Divergent evolution of flavonoid 2-oxoglutarate-dependent dioxygenases in parsley[1]

Stefan Martens[a], Gert Forkmann[b], Lothar Britsch[c], Frank Wellmann[a], Ulrich Matern[a,*], Richard Lukačin[a]

[a] *Institut für Pharmazeutische Biologie, Philipps-Universität Marburg, Deutschhausstrasse 17 A, D-35037 Marburg, Germany*
[b] *Technische Universität München, Wissenschaftszentrum für Ernährung, Landnutzung und Umwelt, Department für Pflanzenwissenschaften, Lehrstuhl für Zierpflanzenbau, Am Hochanger 4, D-85350 Freising, Germany*
[c] *Merck KgaA, Frankfurterstrasse 250, D-64271 Darmstadt, Germany*

Received 4 April 2003; accepted 22 April 2003

First published online 12 May 2003

Edited by Ulf-Ingo Flügge

Abstract **Flavone synthases (FNSs) catalyze the oxidation of flavanones to flavones, i.e. the formation of apigenin from (2*S*)-naringenin. While many plants express a microsomal-type FNS II, the soluble FNS I appears to be confined to a few species of the Apiaceae and was cloned recently from parsley plants. FNS I belongs to the Fe^{II}/2-oxoglutarate-dependent dioxygenases characterized by short conserved sequence elements for cofactor binding, and its evolutionary context and mode of action are under investigation. Using a homology-based reverse transcription polymerase chain reaction approach, two additional flavonoid-specific dioxygenases were cloned from immature parsley leaflets, which were identified as flavanone 3β-hydroxylase (FHT) and flavonol synthase (FLS) after expression in yeast cells. Sequence alignments revealed marginal differences among the parsley FNS I and FHT polypeptides of only 6%, while much less identity (about 29%) was observed with the parsley FLS. Analogous to FNS I, FLS oxidizes the flavonoid γ-pyrone by introducing a C2, C3 double bond, and (2*R*,3*S*)-dihydrokaempferol (*cis*-dihydrokaempferol) was proposed recently as the most likely intermediate in both FNS I and FLS catalysis. Incubation of either FNS I or FLS with *cis*-dihydrokaempferol exclusively produced kaempferol and confirmed the assumption that flavonol formation occurs via hydroxylation at C3 followed by dehydratation. However, the lack of apigenin in these incubations ruled out *cis*-dihydrokaempferol as a free intermediate in FNS I catalysis. Furthermore, neither (+)-*trans*-dihydrokaempferol nor unnatural (−)-*trans*-dihydrokaempferol and 2-hydroxynaringenin served as a substrate for FNS I. Overall, the data suggest that FNS I has evolved uniquely in some Apiaceae as a paraphyletic gene from FHT, irrespective of the fact that FNS I and FLS catalyze equivalent desaturation reactions.**

Key words: Apiaceae; Flavonoid biosynthesis; 2-Oxoglutarate-dependent dioxygenase; *Petroselinum crispum*

1. Introduction

A plethora of natural flavonoids have been reported and some have been credited with a function for the integrity and propagation of the producing plant, e.g. due to a scavenging potential or the recruitment of insect pollinators [1,2], as well as for their neutraceutical benefit [3–5]. The respective features depend, in part, on the mesomeric character and the extent of hydroxylation of the flavonoids. Basically, four soluble dioxygenase activities are responsible for the classification of flavanols, flavones, flavonols and anthocyanidins (Fig. 1), which depend on Fe^{II} and 2-oxoglutarate and have conceivably evolved from a common ancestral gene. Flavanone 3β-hydroxylase (FHT) catalyzing the conversion of (2*S*)-flavanones to (2*R*,3*R*)-dihydroflavonols (Fig. 1) was characterized first [6–9], and loss of function mutants were shown to cause a white flower phenotype accumulating exclusively flavanones [10]. FHT cDNA was cloned from *Petunia hybrida* [11] and cDNAs of 70–90% similarity were assigned from 15 additional plants at least. However, only few of the genes have been functionally expressed, which is a constant matter of concern, and the recombinant enzymes have hardly been studied in biochemical terms. The product of the FHT reaction may be reduced to the flavane-3,4-*cis*-diol (leucoanthocyanidin), which paves the road to the anthocyanidins, proanthocyanidins and catechins (Fig. 1). The conversion of leucoanthocyanidins to anthocyanidins is catalyzed by another 2-oxoglutarate-dependent dioxygenase, anthocyanidin synthase (ANS), which was cloned and functionally expressed very recently from *Anthirrinum*, *Petunia*, *Torenia* and *Zea mays* [12]. The ANS polypeptide sequences share 48–87% similarity with each other and less, albeit significant, similarity with FHT sequences (about 30%). Dihydroflavonols such as dihydrokaempferol (DHK) may alternatively be oxidized to the corresponding flavonols by a third 2-oxoglutarate-dependent dioxygenase, flavonol synthase (FLS), which was reported initially from irradiated cultured parsley cells [13]. Seven FLS cDNAs have been cloned so far from different plant species, and the translated polypeptide sequences showed a remarkable degree of similarity (about 85% with 50% identity), while the similarity to other intermolecular dioxygenases ranged in the order of 30–60% [14].

A divergent route is accessed by flavone synthase (FNS) which oxidizes flavanones to flavones (Fig. 1), and notably two types of FNSs have evolved. Most plants express a micro-

*Corresponding author: Fax: (49)-6421-282 6678.
E-mail address: matern@mailer.uni-marburg.de (U. Matern).

[1] Nucleotide and polypeptide sequence data reported here will appear in the EMBL, GenBank and DDBJ databases under the accession numbers AY230247 (FNS I), AY230248 (FHT) and AY230249 (FLS).

Abbreviations: ANS, anthocyanidin synthase; FNS, flavone synthase; FHT, flavanone 3β-hydroxylase; FLS, flavonol synthase; AP, apigenin; DHK, dihydrokaempferol; KM, kaempferol; NAR, naringenin

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00479-4

somal, cytochrome-P450-dependent FNS II activity which was described initially from cell cultures of soybean [15]. Full-size FNS II cDNAs were cloned recently from *Gerbera hybrida*, *Antirrhinum majus* and *Torenia fournieri* [16,17]. In contrast, the soluble 2-oxoglutarate-dependent FNS I was reported in 1981 from irradiated parsley cells [13] and appears to be confined to a few additional species of the Apiaceae, particularly carrot and celery [18]. FNS I was cloned recently from immature parsley leaves [19], allowing a provisional classification on comparison with heterologous dioxygenases. The stringency of FNS I expression among the Apiaceae and the lack of information on homologous 2-oxoglutarate-dependent dioxygenases encouraged us to search for additional flavonoid-specific dioxygenases in parsley. Furthermore, recent studies on ANS from *Arabidopsis* provided evidence for a mechanism requiring the initial hydroxylation of the flavonoid substrate at C3, and this suggested that FLS and FNS I may also catalyze the sequential C3 hydroxylation and dehydratation [20] instead of the vicinal desaturation process proposed previously [21]. Therefore, the modes of action of FNS I and FLS were reexamined with the highly active recombinant enzymes.

2. Materials and methods

2.1. Plant material

Petroselinum crispum cv. Italian Giant plants were grown in the botanical gardens and in the greenhouse (Technical University Munich). Immature leaves were harvested at four stages of maturation (about 1–25 mm in length), frozen in liquid nitrogen and stored at −80°C until use. The leaf tissues were combined for RNA extraction.

2.2. Chemicals

Biochemicals of analytical grade were purchased from Biomol (Hamburg, Germany), Roche Diagnostics (Mannheim, Germany), Merck (Darmstadt, Germany), Amersham-Pharmacia (Freiburg, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) or Sigma (Deisenhofen, Germany). Reference samples of (*R*/*S*)-naringenin (NAR), apigenin (AP) and kaempferol (KM) were bought from Roth (Karlsruhe, Germany), and (+)-*trans*-DHK was from our laboratory collection. The identity of all the samples was confirmed by ultraviolet and mass spectroscopies.

2.3. Labeled substrates

[2-^{14}C]Malonyl-CoA was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). (2*S*)-[4a,6,8-^{14}C]NAR (2.34–4.25 GBq/mmol) was collected from preparative incubations of [2-^{14}C]-malonyl-CoA and 4-coumaroyl-CoA as described previously [6], employing pure recombinant chalcone synthase from *Ruta graveolens* L. [22] and partially purified chalcone isomerase from parsley [10]. (2*S*)-[4a,6,8-^{14}C]DHK was prepared from (2*S*)-[4a,6,8-^{14}C]NAR by incubation with pure recombinant FHT from *P. hybrida* [7]. The purity of the labeled flavonoids was routinely verified by thin-layer radiochromatography, and the pure compounds dissolved in ethyl acetate were stored at −20°C until use.

2.4. RNA isolation

Total RNA was extracted from 200 mg of ground frozen *P. crispum* leaf tissue according to Giuliano et al. [23].

2.5. Sequence analysis

The cDNAs amplified by reverse transcription polymerase chain reaction (RT-PCR) were sequenced by the dideoxy nucleotide chain termination technique [24], using the universal and reverse sequencing primers and additional oligonucleotide primers designed as required for accurate sequencing following the primer walking strategy (Medigenomics, Martinsried, Germany). GenBank accessions of six FHTs (*Callistephus chinensis*, *Daucus carota*, *Dendranthema grandiflorum*, *Ipomoea batatas*, *Matthiola incana* and *P. hybrida*), four FLSs (*C. unshiu*, *Eustoma grandiflorum*, *P. hybrida* and *Solanum tuberosum*) and four ANSs (*C. chinensis*, *D. carota*, *M. incana* and *P. hybrida*) were examined with the MultAlin software [25] for conserved elements enabling the construction of degenerate PCR primers, and BLAST searches (NCBI server) were carried out with the cloned cDNA sequences. More elaborate analysis of DNA and translated polypeptide sequences was carried out with the OMIGA software (Oxford Molecular, Oxford, UK) including Clustal W [26].

2.6. PCR cloning and heterologous expression

Oligo(dT)-primed cDNA was synthesized from 5 μg of total RNA in a 25 μl incubation using Superscript II reverse transcriptase (Life Technologies, Karlsruhe, Germany) and following the supplier's instruction. Three degenerate primers, designated DIOXY1H (5′-GAITGGGGIRTIWTICAIKTIRYIRAYCAYGG-3′), DIOXY2H (5′-CCIMMITGYCCIMRRCCIGAITTRRCITTRGG-3′) and DIOXY1R (5′-TCIGTRTGISYIWYIAMICCYAAITCIGG-3′), were employed for PCR amplification (50 μl per assay) with one nested PCR round using AGS Gold Taq DNA polymerase (Hybaid, Heidelberg, Germany) and the following primer combinations: DIOXY1H/oligo(dT), DIOXY1H/DIOXY1R and DIOXY2H/oligo(dT). 3′- and 5′-rapid amplification of cDNA ends (RACE) was carried out with the Gene Racer Kit (Invitrogen, Groningen, The Netherlands) and employing gene-specific primers deduced from the PCR fragments. Three full-size cDNAs putatively encoding different dioxygenases were amplified using sets of 25 pmol each of gene-specific primers, employing High Fidelity DNA polymerase in 50 μl PCR assay (Roche Diagnostics, Mannheim, Germany).

The three cDNAs were cloned into the T/A cloning yeast expression vector pYES2.1 (Invitrogen), the orientation was examined by restriction analysis, and the yeast strain INV Sc1 (Invitrogen) was transformed with the sense or antisense construct by a modified lithium acetate method [27]. The growing of yeast transformants and protein isolation were performed as described [28].

2.7. Analytical methods

The activities of FNS I, FHT and FLS were determined as reported previously [6–9]. The flavonoids (NAR, AP, DHK and KM) were extracted from the incubations with 150 μl ethyl acetate and analyzed by chromatography on cellulose thin-layer plates (Merck) with the solvents trichloromethane/acetic acid/water 10:9:1 (v/v/v) or 20% aqueous acetic acid. The flavonoids were spotted on the plates by bioimaging or by spraying with 0.1% aqueous fast blue B salt and exposure to ammonia vapors as described elsewhere [6,13]. Labeled products were measured quantitatively by a Fuji BAS 1000 Bio-Imaging Analyzer with TINA software (Raytest, Straubenhardt, Germany). The protein amounts were determined by the method of Bradford [29] using bovine serum albumin as a standard.

3. Results and discussion

3.1. Cloning and relationship of flavonoid-specific dioxygenases

Three oligonucleotide primers were deduced from the consensus sequences of 14 database accessions of 2-oxoglutarate-dependent dioxygenases assigned to flavonoid biosynthesis and used with total RNA from immature parsley leaf tissues for RT-PCR amplifications. Five cDNA fragments were distinguished, and the corresponding full-size clones were generated by 3′- and 5′-RACE [19]. Two of these clones encoded very similar polypeptides of 365 and 368 amino acid residues (Fig. 2), respectively, with a high degree of homology to each other (94%) and to FHTs from various plant sources (70–85%). Both these enzymes were expressed in yeast cells and identified as FNS I and FHT, respectively, by assays oxidizing (2*S*)-NAR to AP [19] or hydroxylating (2*S*)-NAR to DHK (data not shown). A third cDNA clone encoded a polypeptide with pronounced homology to heterologous FLSs (50–76% identity and 63–87% similarity), but low homology of roughly 29% to the parsley FHT and FNS I; this cDNA was corroborated to encode FLS by expression in yeast cells and assays employing (+)-(2*R*/3*R*)-DHK as a substrate (data not shown).

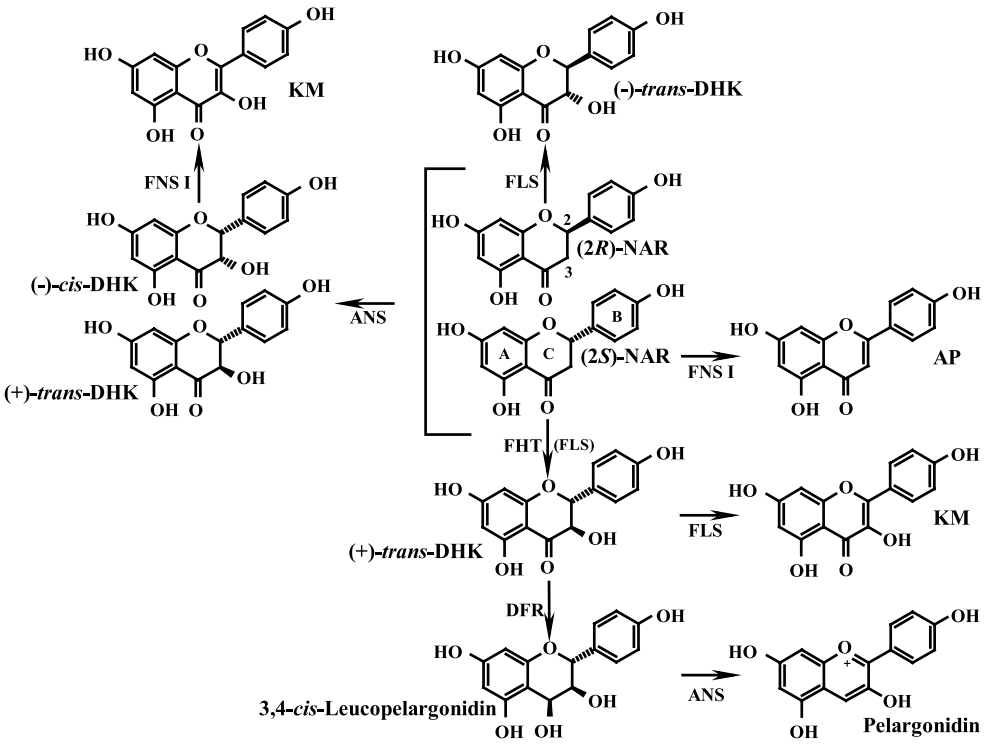

Fig. 1. Reactions catalyzed by 2-oxoglutarate-dependent dioxygenases in flavonoid biosynthesis. FHT converts (2*S*)-NAR to (+)-(2*R*/3*R*)-DHK, while the oxidation by FNS I yields the flavone AP. Analogous oxidation of flavanols, i.e. (+)-*trans*-DHK, by FLS leads to flavonols such as KM. FLS and ANS are capable of catalyzing more than one reaction. In addition to the desaturation of flavanols to flavonols, recombinant FLSs from *C. unshiu* and *P. crispum* have been reported to accept (2*S*)-NAR to form KM most likely via (+)-*trans*-DHK [36], which might be reduced by dihydroflavonol reductase (DFR) to 3,4-*cis*-leucopelargonidin as a substrate for ANS. In contrast, FLS assays performed with unnatural (2*R*)-NAR produced (−)-*trans*-DHK [36]. ANS from *Arabidopsis* [20] or *Gerbera* hybrids (Martens et al., unpublished) convert (±)-NAR to DHK isomers, and the resulting *cis*-form only was converted efficiently by FNS I to KM.

```
                10        20        30        40        50        60        70        80
                 |         |         |         |         |         |         |         |
PcFHT     MAPSTLTALAQEK----TLNSKFVRDEDERPKIAYNK-FSDEIPVISLAGIGDDSVDKRSQICRK-VEACEDWGIFQVVD
PcFNS     MAPTTITALAKEK----TLNLDFVRDEDERPKVAYNQ-FSNEIPIISLAGLDDDSDGRRPEICRKIVKACEDWGIFQVVD
PcFLS     MEVERVQAISKMSRCMDTIPSEYIRSESEQPAVTTMQGVVLQVPVIDLG---S-SNNTEENLVELIAEASREWGIFQVVN
          *    . *... .    *.  ...*.*.*.* ..  . .  ..*.*.*.   . * . . .. .  ..*..*******.

                90       100       110       120       130       140       150       160
                 |         |         |         |         |         |         |         |
PcFHT     HGIDIDLISEMTRLARQFFALPAEEKLRFDMTGGKKGGFIVSSHLQGEA--VQDWREIVAYFSYPIQARDYSRWPDKPEG
PcFNS     HGIDSGLISEMTRLSREFFALPAEEKLEYDTTGGKRGGFTISTVLQGDD--AMDWREFVTYFSYPINARDYSRWPKKPEG
PcFLS     HGIPDDAIAKLQKVGKEFFELPQQEKEVIAKPEGYQGVEGYGTKLQKELGGKKGWVDHLFHIIWPKSAVNYNFWPNNPPL
          ***  . *... .....** ** .**     . * .*    .. ** .     .* . . .. .* .* .*. **..*

               170       180       190       200       210       220       230       240
                 |         |         |         |         |         |         |         |
PcFHT     WRSITEMYSDKLMALACKLLEVLSEAMGLEKEGLTKAC--VDMDQKVIVNYYPKCPQPDLTLGLKRHTDPGTITLLLQDQ
PcFNS     WRSTTEVYSEKLMVLGAKLLEVLSEAMGLEKGDLTKAC--VDMEQKVLINYYPTCPQPDLTLGVRRHTDPGTITILLQDM
PcFLS     YREANEEYAVALRGVVDKLFEALSLGIGLEKHELKKASGGDDLIYMLKINYYPPCPRPDLALGVVAHTDMSAITILVPNE
          .*. .* *.  *  .  **.*.** ..****  *.**.   *.   . .**** **.***.**.  *** ..**.*. .

               250       260       270       280       290       300       310       320
                 |         |         |         |         |         |         |         |
PcFHT     VGGLQATRDGGKTWITVQPVEGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNSSRMSIATFQNPAPNATVYPLKIREGEK
PcFNS     VGGLQATRDGGKTWITVQPVEGAFVVNLGDHGHYLSNGRFRNADHQAVVNSTSSRLSIATFQNPAQNAIVYPLKIREGEK
PcFLS     VQGLQVHKD--DHWYDVKYIPNALIIHIGDQIEIMSNGKYKSVYHRTTVNKDKTRMSWPVFLEPPPELLTGPISKLITD-
          * ***. .*  . *  *. . .*.....**. . .***..... *...**. ..*.* ..* .*. .  . *..    .

               330       340       350       360       370
                 |         |         |         |         |
PcFHT     AVMEEPITFAEMYKRKMSRDIEMATLKKLAKEKVLQDQEVEKAKLQMTPKSADEIFA
PcFNS     AILDEAITYAEMYKKCMTKHIEVATRKKLAKEKRLQD---EKAKLEMKSKSADENLA
PcFLS     ------E-NPAKFKTKKYKDYVYCKLNKLPQ--------------------------
                . .*   ..  .. .**..
```

Fig. 2. Alignment of the translated polypeptides of FHT (PcFHT), FNS I (PcFNS) and FLS (PcFLS) from parsley. Conserved amino acids are marked by asterisks and conservative exchanges are indicated by dots. Two regions highly conserved in flavonoid-specific dioxygenase polypeptides are underlined. Amino acids ligating ferrous iron and residues participating in 2-oxoglutarate binding (RXS motif) are printed in bold.

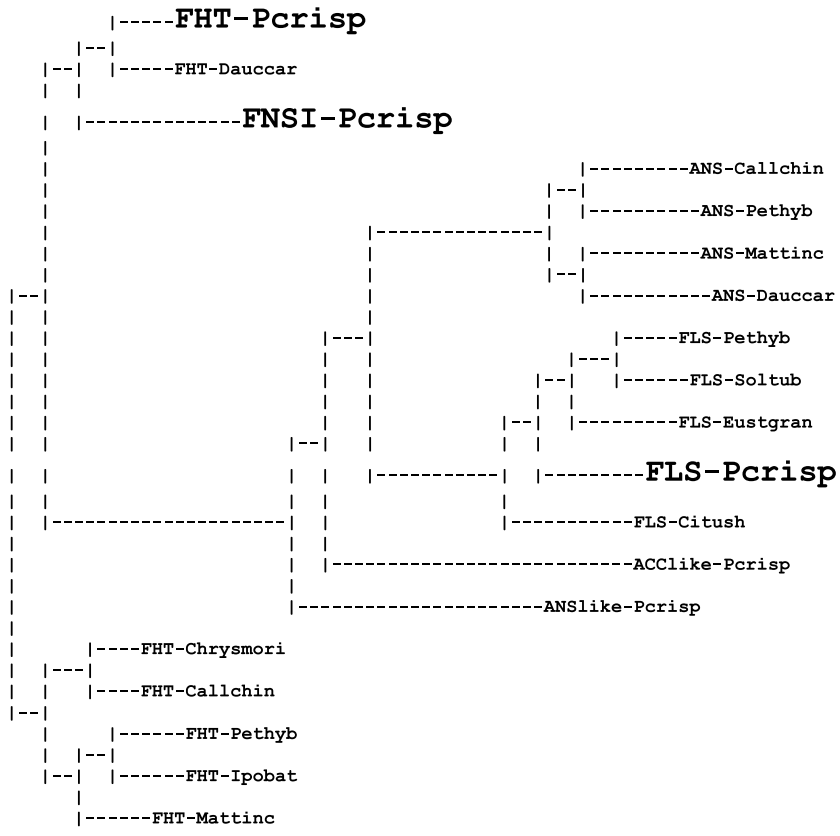

Fig. 3. Phylogenetic analysis of 19 flavonoid-specific 2-oxoglutarate-dependent dioxygenases, including FHT, FLS, FNS I (highlighted), an aminocyclopropane-1-carboxylate oxidase (ACC) and an ANS-like enzyme from parsley (Pcrisp). Heterologous FHTs and ANSs were from carrot *D. carota* (Dauccar), *Chrysanthemum moriflorium* (Chrysmori), *C. chinensis* (Callchin), *P. hybrida* (Pethyb), *I. batatas* (Ipobat) and *M. incana* (Mattinc); FLS sequences were from *P. hybrida* (Pethyb), *S. tuberosum* (Soltub), *E. grandiflorum* (Eustgran) and *C. unshiu* (Citush).

The conserved motifs required for ferrous iron (H-X-D) and 2-oxoglutarate binding (R-X-S) (Fig. 2) classified the amplified sequences as 2-oxoglutarate-dependent dioxygenases [8]. Base alignments, however, are insufficient to prove the functionality of the encoded polypeptides, and, as a striking example, the marginal differences in the DNA sequences of the parsley FHT and FNS I of only 10% emphasize the need to express and characterize the recombinant enzymes. Similar conclusions have been reached with unrelated plant enzymes, where a few point mutations dramatically changed the substrate specificity [30,31]. The cladistic analysis (Fig. 3) suggested that FNS I is much more closely related to the Apiaceae FHTs than to any other homologous or heterologous 2-oxoglutarate-dependent dioxygenase. For comparison, the parsley FLS sequence revealed a different tendency with a close relationship to heterologous FLSs rather than to parsley dioxygenases. Overall, these data (Fig. 3) point to a paraphyletic evolution of FNS I from FHT, and the confinement of FNS I to a few species of the Apiaceae nourishes the assumption that this process happened not too long ago in evolutionary terms.

3.2. Differential FNS I and FLS catalysis

Recent investigations of the in vitro mode of action of *Arabidopsis* ANS [20,32,33] revealed overlapping selectivities for flavonoid dioxygenases, because the ANS also oxidized *trans*-dihydroquercetin to quercetin in an FLS-type reaction [32]. Furthermore, the ANS produced DHK as a major product from of (2*R*/2*S*)-NAR (Fig. 1) with a *cis*/*trans* ratio of ca. 4:1 [20]. Extrapolating these results, the authors suggested a common type of mechanism for ANS, FLS and FNS I catalysis via 'α-face' hydroxylation [20] at C3 (3-pro*S* in 2*S*-NAR) followed by dehydratation. This would imply a 2,3-*cis*-dihydroflavonol (*cis*-DHK) as an intermediate in the formation of AP from (2*S*)-NAR by FNS I (Fig. 1) or the formation of KM from (2*S*)-NAR by FLS. In order to gain experimental proof, (−)-*cis*- and (+)-*trans*-DHK were generated from NAR through ANS incubations as suggested by Welford et al. [20]. An ANS cDNA was isolated from *Gerbera* hybrids and functionally verified after expression in yeast cells by assays converting (2*R*,3*S*,4*R*)-*cis*-[4a,6,8-^{14}C]leucopelargonidin to pelargonidin (Martens et al., unpublished). In the presence of Fe^{II}/2-oxoglutarate and ascorbate, the partially purified recombinant *Gerbera* ANS converted (2*R*/*S*)-NAR to *cis*- and *trans*-DHK with a strong bias towards *cis*-DHK (Fig. 4A), as had been reported for the *Arabidopsis* ANS [20]. Control experiments with the enantiomeric substrates revealed that the *cis*-configured product was generated exclusively from (2*S*)-NAR, thus representing (2*R*,3*S*)-DHK (Fig. 1), and the 2,3-*cis*-configuration was confirmed by small coupling constants ($J_{2,3} \approx 2.5$ Hz) on ^{1}H nuclear magnetic resonance spectroscopy (Lukačin et al., unpublished). When these products were separately incubated further with highly active recombinant FNS I, a considerable extent of *cis*-DHK was converted to KM (Fig. 4B), whereas (+)-*trans*-DHK remained unchanged (Fig. 4A) and no other product was observed. This definitely

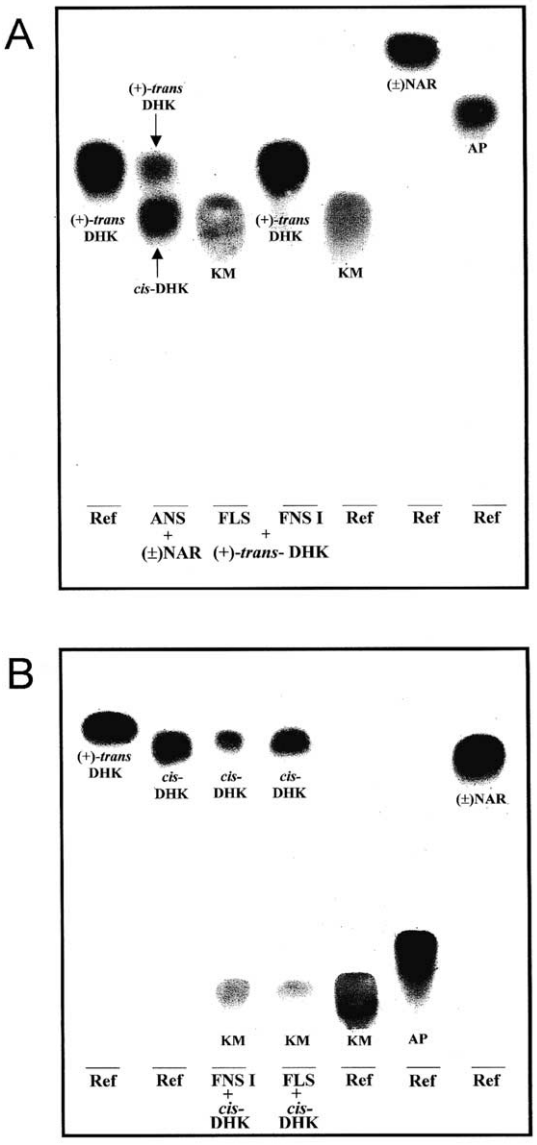

Fig. 4. Substrate specificities of ANS, FLS and FNS I. Flavonoids were extracted from enzyme assays with ethyl acetate, subjected to cellulose thin-layer chromatography in (A) trichloromethane/acetic acid/water 10:9:1 (v/v/v) or (B) 20% acetic acid and stained by spraying the plates with 0.1% aqueous fast blue salt and exposure to ammonia vapors. Authentic (+)-*trans*-DHK and commercially available KM, (±)-NAR, AP as well as *cis*-DHK, isolated from ANS incubations, served as references (Ref). Incubations of (±)-NAR with *Gerbera* ANS (ANS+(±)NAR in A) generated (−)-*cis*- and (+)-*trans*-DHK. Recombinant FLS from *C. unshiu* oxidized (+)-*trans*-DHK exclusively to KM (FLS+(+)-*trans*-DHK in A), whereas no product was formed from (+)-*trans*-DHK in assays employing recombinant parsley FNS I (FNS I+(+)-*trans*-DHK in A). FNS I oxidized isolated *cis*-DHK to a considerable extent to KM (FNS I+*cis*-DHK) while the FLS produced only traces of KM under these conditions (FLS+*cis*-DHK).

excludes *cis*- or *trans*-DHK as an intermediate in the FNS I-catalyzed oxidation of NAR to AP (Fig. 1), irrespective of the FLS-like activity observed with *cis*-DHK.

Since 2-oxoglutarate-dependent dioxygenases predominantly catalyze hydroxylation reactions, 2-hydroxyflavanone has also been considered a potential intermediate in flavone biosynthesis [13]. This compound is known as a natural metabolite and can be converted to AP by chemical dehydration under mild conditions [21]. Nevertheless, incubations employing FNS I of low specific activity partially purified from parsley cells had already indicated that 2-hydroxyflavanone is unlikely to occur as a dissociable intermediate in the biosynthesis of flavones and is incapable of competing with the flavanone substrate [21]. These findings were reexamined with the highly active recombinant FNS I (>1 mkat/kg), which also completely failed to yield AP from 2-hydroxyflavanone on repeated incubations (data not shown). Thus, 2-hydroxyflavanone can likely be excluded as a pseudosubstrate for FNS I. Taken together, the data appear to rule out a sequential hydroxylation/dehydratation mechanism for FNS I and support the direct 2,3-desaturation of flavanones as postulated earlier [21]. Precedents for this kind of desaturation can be found in the literature [34,35].

In contrast to FNS I, FLS produced only traces of the flavonol from *cis*-DHK (Fig. 4B), in addition to efficiently converting (+)-*trans*-DHK to KM (Fig. 4A). Furthermore, FLS from *C. unshiu* accepted (2*S*)- or unnatural (2*R*)-NAR to form KM and (−)-*trans*-DHK [36]. The same specificity was recorded for the recombinant FLS from parsley, although the conversion rates were relatively low (data not shown). Nevertheless, both FLSs totally lacked FNS I activity. Thus, FLS and ANS belong to a group of dioxygenases with broad substrate specificities, while FNS I and FHT are far more selective and appear to form a separate group of dioxygenases in accordance with their high degree of sequence homology.

Acknowledgements: Financial support for the work described in this report by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie is gratefully acknowledged. We thank S. Schreiner for skilled technical assistance, M. Schwarzkopf-Hofmann (Merck KgaA, Darmstadt) for ^{1}H NMR measurements and W. Heller (GSF Forschungszentrum für Umwelt und Gesundheit, Neuherberg) for critical reading of the manuscript.

References

[1] Koes, R.E., Quattrocchio, F. and Mol, J.N.M. (1994) BioEssays 16, 123–132.
[2] Harborne, J.B. and Grayer, R.J. (1994) in: The Flavonoids (Harborne, J.B., Ed.), pp. 589–618, Chapman and Hall, London.
[3] Hollman, P.C.H. and Katan, M.B. (1999) Food Chem. Toxicol. 37, 937–942.
[4] Huang, Y.T., Hwang, J.J., Le, P.P., Ke, F.C., Huang, J.H., Huang, C.J., Kandaswami, C., Middleton, E. and Lee, M.T. (1999) Br. J. Pharmacol. 128, 999–1010.
[5] Harborne, J.B. and Williams, J.B. (2000) Phytochemistry 55, 481–504.
[6] Britsch, L. and Grisebach, H. (1986) Eur. J. Biochem. 156, 569–577.
[7] Lukačin, R., Gröning, I., Schiltz, E., Britsch, L. and Matern, U. (2000) Arch. Biochem. Biophys. 375, 364–370.
[8] Lukačin, R., Gröning, I., Pieper, U. and Matern, U. (2000) Eur. J. Biochem. 267, 853–860.
[9] Lukačin, R., Urbanke, C., Gröning, I. and Matern, U. (2000) FEBS Lett. 467, 353–358.
[10] Forkmann, G., Heller, W. and Grisebach, H. (1980) Z. Naturforsch. 35c, 691–695.
[11] Britsch, L., Ruhnau-Brich, B. and Forkmann, G. (1992) J. Biol. Chem. 267, 5380–5387.
[12] Nakajima, J., Tanaka, Y., Yamazaki, M. and Saito, K. (2001) J. Biol. Chem. 276, 25797–25803.
[13] Britsch, L., Heller, W. and Grisebach, H. (1981) Z. Naturforsch. 36c, 742–750.
[14] Wellmann, F., Lukačin, R., Moriguchi, T., Britsch, L., Schiltz, E. and Matern, U. (2002) Eur. J. Biochem. 269, 4134–4142.

[15] Kochs, G. and Grisebach, H. (1987) Z. Naturforsch. 42c, 343–348.
[16] Martens, S. and Forkmann, G. (1999) Plant J. 20, 611–618.
[17] Akashi, T., Fukuchi-Mizutani, M., Aoki, T., Ueyama, Y., Yonekura-Sakakibara, K., Tanaka, Y., Kusumi, T. and Ayabe, S. (1999) Plant Cell Physiol. 40, 1182–1186.
[18] Lukačin, R., Matern, U., Junghanns, K.T., Heskamp, M.-L., Britsch, L., Forkmann, G. and Martens, S. (2001) Arch. Biochem. Biophys. 393, 177–183.
[19] Martens, S., Forkmann, G., Matern, U. and Lukačin, R. (2001) Phytochemistry 58, 43–46.
[20] Welford, R.W.D., Turnbull, J.J., Claridge, D.W., Prescott, A.G. and Schofield, C.J. (2001) Chem. Commun. 1828–1829.
[21] Britsch, L. (1990) Arch. Biochem. Biophys. 282, 152–160.
[22] Springob, K., Lukačin, R., Ernwein, C., Gröning, I. and Matern, U. (2000) Eur. J. Biochem. 267, 6552–6559.
[23] Giuliano, G., Bartley, G.E. and Scolnik, P.A. (1993) Plant Cell 5, 379–387.
[24] Sanger, F., Nicklen, S. and Coulsen, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[25] Corpet, F. (1988) Nucleic Acids Res. 16, 10881–10890.
[26] Thompson, J.D., Higgins, D.J. and Gibson, T.J. (1994) Nucleic Acids Res. 2, 4673–4680.
[27] Gietz, D., St. Jean, A., Woods, R.A. and Schiestl, R.H. (1992) Nucleic Acids Res. 20, 1425.
[28] Urban, P., Mignotte, C., Kazmeier, M., Delorme, F. and Pompon, D. (1997) J. Biol. Chem. 272, 19176–19186.
[29] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[30] Frick, S., Ounaroon, A. and Kutchan, T.M. (2001) Phytochemistry 56, 1–4.
[31] Lukačin, R., Schreiner, S. and Matern, U. (2001) FEBS Lett. 508, 413–417.
[32] Turnbull, J.J., Sobey, W.J., Aplin, R.T., Hassan, A., Firmin, J.L., Schofield, C.J. and Prescott, A.G. (2000) Chem. Commun. 2473–2474.
[33] Wilmouth, R.C., Turnbull, J.J., Welford, R.W.D., Clifton, I.J., Prescott, A.G. and Schofield, C.J. (2002) Structure 10, 93–103.
[34] Albone, K., Gaskin, P., MacMillan, J., Smith, V.A. and Weir, J. (1989) Planta 177, 108–115.
[35] Mansuy, D. (1998) Comp. Biochem. Physiol. C 121, 5–14.
[36] Lukačin, R., Wellmann, F., Martens, S., Britsch, L. and Matern, U. (2003) Phytochemistry 62, 287–292.