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Molecular evolution of flavonoid dioxygenases in the family Apiaceae

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Abstract

Plant species of the family Apiaceae are known to accumulate flavonoids mainly in the form of flavones and flavonols. Three 2-oxoglutarate-dependent dioxygenases, flavone synthase or flavanone 3 β-hydroxylase and flavonol synthase are involved in the biosynthesis of these secondary metabolites. The corresponding genes were cloned recently from parsley (*Petroselinum crispum*) leaves. Flavone synthase I appears to be confined to the Apiaceae, and the unique occurrence as well as its high sequence similarity to flavanone 3β-hydroxylase laid the basis for evolutionary studies. In order to examine the relationship of these two enzymes throughout the Apiaceae, RT-PCR based cloning and functional identification of flavone synthases I or flavanone 3β-hydroxylases were accomplished from *Ammi majus*, *Anethum graveolens*, *Apium graveolens*, *Pimpinella anisum*, *Conium maculatum* and *Daucus carota*, yielding three additional synthase and three additional hydroxylase cDNAs. Molecular and phylogenetic analyses of these sequences were compatible with the phylogeny based on morphological characteristics and suggested that flavone synthase I most likely resulted from gene duplication of flavanone 3β-hydroxylase, and functional diversification at some point during the development of the apiaceae subfamilies. Furthermore, the genomic sequences from *Petroselinum crispum* and *Daucus carota* revealed two introns in each of the synthases and a lack of introns in the hydroxylases. These results might be explained by intron losses from the hydroxylases occurring at a later stage of evolution.

Keywords: Apiaceae; Flavonoid 2-oxoglutarate dependent dioxygenases; Evolution; Flavone synthase I; Flavanone 3β-hydroxylase

1. Introduction

Among the low molecular weight polyphenols flavonoids comprise a large and widespread group of secondary metabolites, which has evolved as a consequence of the plants continuous interactions with the environment (Oksman-Caldentey and Inze, 2004), and may be traced back along the molecular relationship of relevant enzymes. Flavonoids are structurally composed of two aromatic rings (ring A and B) joined by a heterocycle (ring C). Modifications of the central C-ring differentiate the flavonoid subtypes as flavanones, flavones, isoflavones, dihydroflavonols, flavonols, flavan-3-ols and anthocyanins (Forkmann and Heller, 1999). Flavonoids serve the plants in a wide range of functions, for example as pigments in color signatures and UV-protection of tissues, or as chemical signal compounds in plant–microbe and plant–insect interactions (Schijlen et al., 2004; Harborne and Williams, 2000). Moreover, some flavonoids

Abbreviations: 2-ODD, 2-oxoglutarate-dependent dioxygenases; FHT, flavanone 3β-hydoxylase; DHF, dihydroflavonol; ANS, anthocyanidin synthase; FNS I, flavone synthase I; FLS, flavonol synthase; FNS II, flavone synthase II.

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have received considerable attention because of their beneficial effects on human health. Although flavonoids are not considered to be essential nutrients, anticarcinogenic, antiinflammatory, antihistaminic, antioxidant or antiviral activities have been reported (Hempel et al., 1999; Nielsen et al., 1999; Fejes et al., 2000; Middleton et al., 2000; Akihisa et al., 2003; Parejo et al., 2004).

The common flavonoid pathway has been well documented at the biochemical and molecular level, but little is known about the evolution of the respective enzymes. Several of these enzymes were assigned to the abundant group of 2-oxoglutarate-dependent dioxygenases (2-ODDs) which use molecular oxygen as the co-substrate and are characterized by their co-factor requirements of ferrous iron, 2-oxoglutarate and/or ascorbate (Forkmann and Heller, 1999; Springob et al., 2003). In general, these dioxygenases group into a large, functionally heterogeneous class of enzymes with widely divergent substrate specificities, ranging from flavonoids, gibberellins and alkaloids to penicillins and cephalosporins (Prescott, 1993; De Carolis and De Luca, 1994; Prescott and John, 1996; Prescott and Lloyd, 2000).

Flavanone 3β-hydoxylase (FHT) is a 2-ODD acting early in the flavonoid pathway by hydroxylating (2*S*)-flavanones stereospecifically to (2*R*,3*R*)-dihydroflavonols (DHFs) (Fig. 1). DHFs may be reduced to the corresponding flavan-*cis*-3,4-diols (leucoanthocyanidins) which are then converted further by another 2-ODD, anthocyanidin synthase (ANS), to anthocyanidins. Anthocyanidin conjugates, stabilized by glycosylation, are responsible for the coloration of many plant tissues. Alternative to the anthocyanidin branch pathway, (2*S*)-

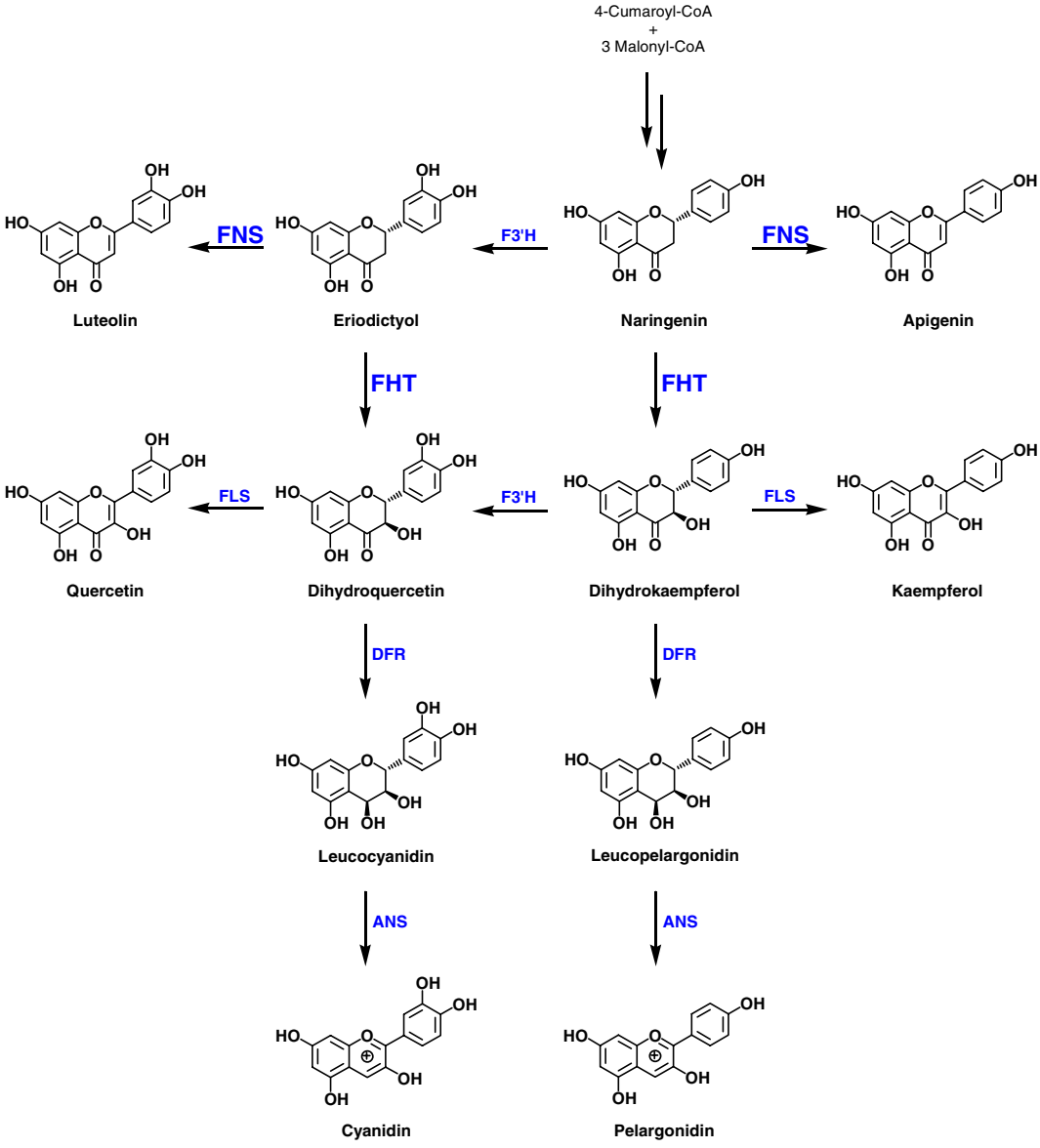

Fig. 1. Schematic flavonoid pathway. 2-ODDs: FNS I: flavone synthase I (Apiaceae), FHT: flavanone 3β-hydroxylase, FLS: flavonol synthase, ANS: anthocyanidin synthase. Others: DFR: dihydroflavonol 4-reductase, F3′H: flavonoid 3′-hydroxylase, FNS II: flavone synthase II.

flavanones and DHFs can be converted to flavones and flavonols, respectively, by the action of flavone synthase I (FNS I) or flavonol synthase (FLS). These two 2-ODDs are capable of oxidizing flavanones and DHFs by introducing a C2/C3 double bond (Fig. 1). Recombinant FLS appears to catalyze both the 3β-hydoxylation of flavanones and the C2/C3 desaturation, at least in one case (Lukačin et al., 2003). The expression of FNS I is a peculiar feature of the Apiaceae, since all other plants investigated, which accumulate flavones rely on the activity of flavone synthase II (FNS II) which is a membrane-bound, cytochrome P450-dependent monooxygenase (Forkmann and Heller, 1999; Springob et al., 2003).

The unique set of flavonoid 2-ODDs in plant species of the Apiaceae makes this family particularly attractive for molecular evolutionary studies. Recently, we cloned a FNS I from immature parsley leaves which shares around 90% similarity (81% identity) with the FHT polypeptide from the same tissue (Martens et al., 2001, 2003). Detailed sequence alignments of these enzymes which yield different products from the same substrate revealed only a small number of amino acid substitutions differentiating FNS I from FHT (Martens et al., 2003). This led to the speculation that FNS I has evolved from FHT by gene duplication and subsequent functional diversification.

To date, only three sequences from the Apiaceae encoding FNS I (*Petroselinum crispum* AY230247) or FHTs (*P. crispum* AY230248 and *Daucus carota* AF184270) have been annotated in GenBank (Hirner et al., 2001; Martens et al., 2003). Therefore, a homology-based RT-PCR approach was used in this report to collect more data on FNS I and FHT from additional species of Apiaceae for comparison. All plants investigated in this report belong to the largest subfamily of the Apiaceae, the Apioideae, which is considered more advanced than the Saniculoideae and Hydrocotyloideae, because of the accumulation of flavones, methylated flavonoids and phenylpropanes (Harborne, 1971). Six cDNAs coding for novel 2-ODDs were cloned, functionally verified, and their evolutionary origin evaluated.

2. Results

2.1. Flavonoid patterns and FNS I/FHT activities

The pattern of flavonoids, predominantly kaempferol, quercetin, and luteolin, found in the various Apiaceae subfamilies was proposed to be a taxonomic marker. The Apioideae produce flavone glycosides which are absent from the Hydrocotyloideae and Saniculoideae. Furthermore, in the majority of cases flavones were assigned to species considered to be more highly advanced (e.g., *Daucus*), whereas flavonols are a trait of less specialised taxa (e.g., *Hydrocotyle* (Harborne, 1971)). The quality of flavonoids depends on the activities of either FNS I or FHT, and was thus an important lead to the choice of plants to be investigated. Both flavonols and flavones were reported from *Ammi majus*, *Conium maculatum*, *Daucus carota*, *Petroselinum crispum*, and *Pimpinella anisum*, suggesting that these plants express FNS I and FHT. *Apium graveolens* accumulates predominantly flavones, while *Anethum graveolens* was reported to produce mostly flavonols (Table 1). The majority of the flavonoids appears to be glycosylated for vacuolar storage.

Extracts from parsley plants were analysed by thin-layer chromatography for their flavonoid contents, and flavones and flavonols were detected in all tissues (stem, leaves, flowers and fruits; data not shown). Furthermore, the activities of FNS I and FHT were measured in these tissues and at various developmental stages (Fig. 2). Both enzyme activities were present in crude extracts from stems, young flower buds, open flowers, unripe fruits and different leaf stages, but were lacking in extracts from mature fruits. The examination of leaf tissues of four developmental stages revealed an early expression of FNS I activity with a maximum in

Table 1
Flavonoid pattern in the Apiaceae species under investigation. The flavonoids were classified as flavones (e.g., apigenin, luteolin, chrysoeriol), flavonols (e.g., kaempferol, quercetin, isorhamnetin), and anthocyanidins (e.g., cyanidin)

Type of flavonoid	*Ammi majus*[e,f,g,h]	*Anethum graveolens*[b,e,f,h]	*Apium graveolens*[f,e,h]	*Conium maculatum*[f,g,c,h]	*Daucus carota*[a,f,g,h]	*Petroselinum crispum*[f,a,h]	*Pimpinella anisum*[d,h]
Flavones	+	–	+	+	+	+	+
Flavonols	+	+	–	+	+	+	+
Anthocyanidins	–	–	–	+	+	–	–

[a] Harborne and Baxter (1999).
[b] Teubert et al. (1977).
[c] Teubert and Herrmann (1978).
[d] Kunzemann and Herrmann (1977).
[e] Hegnauer (1973).
[f] Harborne and Williams (1972).
[g] Crowden et al. (1969).
[h] Gebhardt et al. (unpublished).

(A)

Tissue	FNS I	FHT
Stem	18.2%	27.7%
Young flower buds	61.1%	28.2%
Open flowers	70.9%	18.6%
Unripe fruits	26.9%	30.8%
Mature fruits	7.5%	4.9%
Leafs stage 4	28.5%	10.7%

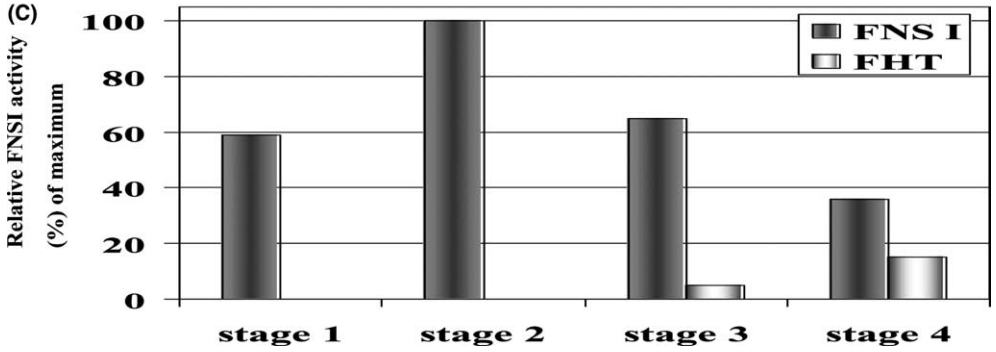

Fig. 2. (A) FNS I and FHT activity in crude extracts prepared from 200 mg fresh tissue of different plant parts. Assays were carried out with 5000 dpm C^{14} Naringenin (approximately 45 pmol) as substrate. Activities are indicated as % conversion. (B) Developmental stages of parsley leaves. (C) FNS I and FHT activities in different developmental stages of parsley leaves. Maximum conversion was set as 100%.

stage 2, whereas significant FHT activity developed with stage 3 (Fig. 2).

2.2. cDNA cloning and functional characterisation of FNS I and FHTs

The amplification of cDNA fragments by RT-PCR with degenerate primers (Martens et al., 2003) resulted in two putative 2-ODD fragments from *Daucus carota* and *Apium graveolens*. The full-size cDNAs were generated by 3′- and 5′-RACE, and specific end to end primers were inferred, which enabled the amplification of the final cDNA clones via proof-reading RT-PCR. Alignments of the translated polypeptides identified both clones as being closely related to parsley FNS I with 88% (*Daucus*) and 91% (*Apium*) identity. Due to the previously reported close relationship of FNS I and FHT (Martens et al., 2003) the cloned cDNAs were functionally expressed for unequivocal identification. The expression in yeast cells furnished active enzyme extracts which efficiently converted (2*S*)-naringenin (NAR) to apigenin (Ap). Thus, both cDNAs encode FNS I (GenBank accession numbers: AY817675 *Daucus* FNS I and AY817676 *Apium* FNS I).

Alignment of the five cDNA sequences so far available encoding Apiaceae FNS I/FHT revealed two highly conserved regions, one spanning the N-terminus including the start codon and the other far downstream in the open reading frame close to the stop codon (Fig. 3). These elements allowed the design of another set of degenerate oligonucleotide primers (DIOXY3H and DIOXY2R) which were used to amplify additional 2-ODDs. The combination of these primers in RT-PCR amplified putative FNS I or FHT fragments beginning at the 5′-border of the open reading frame (start ATG) and extending over nearly 900 bp downstream. Such cDNA fragments were thus amplified from *Ammi majus*, *Anethum graveolens*, *Conium maculatum* and *Pimpinella anisum*, and the corresponding full-size cDNAs were generated by 3′-RACE followed by full length proof-reading PCR. The cDNA products were ligated into the pYES2 vector, expressed in yeast cells, and the extracts were assayed for FNS I or FHT activity with (2*S*)-[4a,6,8-^{14}C] NAR (Martens et al., 2003) as substrate (data not shown) with extracts of untransformed host cells or of transformants with empty vector serving as controls. Overall, these experiments resulted in the identification of one additional FNS I (AY817677, *Conium*, FNS I) and three FHTs (AY817678, *Ammi*, FHT, AY817679, *Anethum*, FHT, AY817674, *Pimpinella*, FHT).

2.3. FNS I and FHT genes

Combinations of gene specific primers spanning the N-terminal coding region and a sequence element of the downstream untranslated region, respectively, were employed to amplify the genomic sequences of *FNS I*

```
Api_graFNSI          -------AAAAATGGCTCCTACAACTATAACTGCACTGTCTCAAGAGAAGACACTGAACT 53
Pet_criFNSI          CTCGCCCTTCAATGGCTCCTACAACAATAACCGCATTAGCCAAGGAGAAAACACTAAACT 60
Dau_carFNSI          ----------AATGGCTCCAACAACTATTACTGCATTGGCCAAGGAAAAGACACTTAACT 50
Pet_criFHT           CTCGCCCTTCAATGGCTCCTTCAACTCTCACTGCCTTGGCCCAAGAGAAAACTCTAAATT 60
Dau_carFHT           ---------AAATGGCCCCTTCAACTCTAACTGCTCTAGCCCAAGAGAAAACCCTCAATT 51
                         ****** **  ****  * ** **  *  *  * ** ** ** ** ** * *

Dioxy3H:                     5'AATGGCYCCWWCAACWMTWACYGC3'

Api_graFNSI          AAGCAGTAGTGAATTCAACTTCCACCAGATTGTCAATTGCAACTTTCCAGAACCCGGCTC 893
Pet_criFNSI          AAGCAGTAGTGAATTCAACCTCTAGCAGATTGTCAATTGCAACTTTCCAGAACCCGGCTC 900
Dau_carFNSI          AAGCAGTAGTGAATTCAACYTCTAGCAGATTGTCCATCGCAACTTTCCAGAACCCGGCTC 890
Pet_criFHT           AAGCAGTTGTGAATTCCAATTCTAGCAGAATGTCAATTGCAACTTTCCAGAACCCGGCTC 900
Dau_carFHT           AAGCAGTCGTGAATTCCAACTACAGCAGATTGTCAATTGCAACTTTCCAGAACCCGGCTC 885
                     ******* ******** *  *  * **** **** ** **********************

Dioxy2R:                                                  3'CGTTGAAAGGTCTTGGGCC 5'
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Fig. 3. Oligonucleotide primers designed to amplify putative *FNS I* and *FHT* fragments from members of the Apiaceae. Wobble symbols were used according to IUPAC-IUB.

or *FHT* by PCR. For the *FHT*s from *Petroselinum* and *Daucus*, single bands were generated and visualized upon agarose gel electrophoresis (Fig. 4(A)), corresponding in size to the respective cDNAs. Therefore, introns are lacking from these FHT genes or they are small enough (<50 nt) to escape detection. In the cases of *Petroselinum* and *Daucus FNS* I genes; however, the amplicons appeared in bands approximately 300–500 bp larger than observed for the respective cDNAs (Fig. 4(A)). The data suggest that the coding regions of these genes contain at least one intron each. The four genomic amplicons were ligated into pTZ57R for DNA sequencing which confirmed the lack of introns for both *FHT*s, but documented two introns in each of the *FNS* I genes. The introns differed in size being 169 and 192 bp for *Daucus* FNS I, and, in case of *Petroselinum* FNS I, 244 and 247 bp (Fig. 4(B)). Furthermore, the intron sequences showed no similarity except for a small region of 37 bp at the end of intron 2 (92% similarity). Nevertheless, alignments of the translated FNS I polypeptides revealed an identical positioning of the introns in both sequences, separating Gln121 from Gly122 and His264 from Tyr265, respectively.

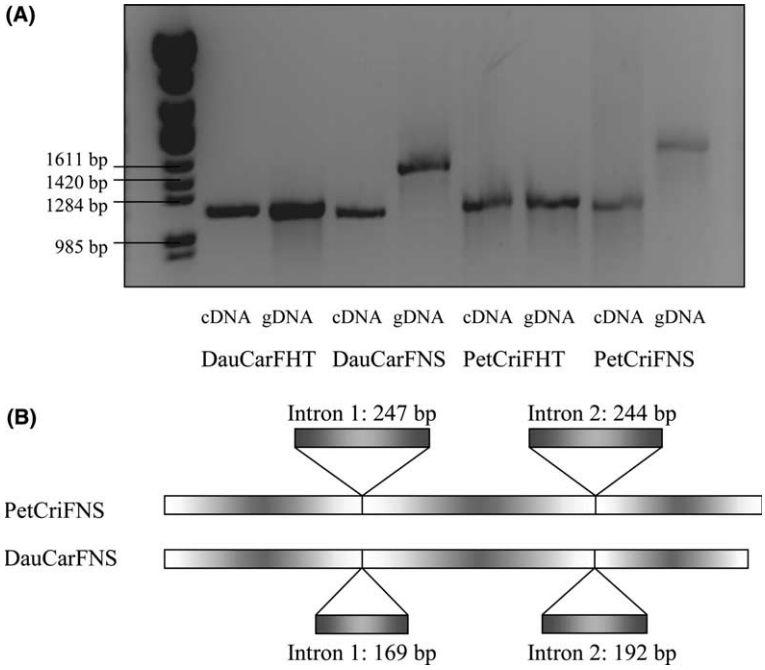

Fig. 4. Structure of FNS I and FHT genes from *Daucus carota* and *Petroselinum crispum*. (A) Electrophoretic separation of PCR products from genomic and cDNA templates with primers specific for either FHT or FNS I. The FHT amplicons from genomic or cDNA templates are of equivalent length for both plants, whereas genomic FNS I is ca. 300–500 bp longer as compared to the amplicon from cDNA template. Sequencing confirmed the abscence of introns in both FHT genes. (B) Organisation of PetCriFNS and DauCarFNS genes. Both genes contain two introns located at the same positions. Intron sizes and sequences do not match, except for a 37 bp region at the 3′ end of Intron 2.

2.4. Sequence analysis

Pairwise alignments of the translated polypeptides of the new and three previously cloned Apiaceae *FNS* I and *FHT* cDNAs documented the relationship of the various FHT and FNS I enzymes, respectively, as well as the relationship of FNS I to FHT. The identity ranged from 87% for *Daucus* FHT with FHTs from *Ammi*, *Anethum* or *Petroselinum* to 97% for *Ammi* and *Pimpinella* FHT (Table 2). Concerning FNS I, the closest relationship was observed between *Apium* and *Petroselinum* (91% identity), whereas *Daucus* FNS I appeared more remote (87% identity with *Apium* FNS I). The FNS I polypeptides are more closely related to each other than to the Apiaceae FHTs, albeit still at 80–83% identity, which is in good agreement with our previous findings for the enzymes from *Petroselinum* (Martens et al., 2003). The high sequence homology among FNS I and FHTs is particularly noteworthy on comparison with other flavonoid 2-ODDs, e. g. 23–31% identity with FLS or ANS and with functionally unrelated 2-ODDs from other pathways, e.g., <32% identity with 1-aminocyclopropan-4-carboxylate oxidase (ACCO). Anyhow, 2-ODDs of rather diverse functions and origins share a number of conserved elements for functional reasons (Prescott and John, 1996; Prescott and Lloyd, 2000). These include the motifs responsible for cofactor binding (Lukačin and Britsch, 1997), which are present in the sequences from Apiaceae as well (Fig. 5).

Minor differences in the polypeptide sequences of FNS I and FHTs obviously resulted from small deletions accounting for size differences from 355 (*Apium* FNS I) to 368 (*Ammi* FHT or *Petroselinum* FHT) residues. The other three FHTs (*Anethum*, *Daucus*, *Pimpinella*) lack two or three residues at position 48 or 49 from the N-terminus, which is a less conserved region. Although the C-terminus also appears to be less important for substrate specificity (Wellmann et al., 2004), most FHTs end in conserved triplets (Ile, Phe, Ala or Ile, Leu, Ala or Asn, Leu, Ala). In most Apiaceae sequences these triplets are preserved as well or vary only marginally (Ile, Ser, Ala). However, the FNS I from *Daucus* differs significantly in this respect due to a deletion (19 nucleotides between nt 1070 and 1071, resulting in a frame shift) truncating the C-terminus by 8 residues (Fig. 5). Similarly, a point mutation in *Apium* FNS I (at nt 1068) introduced a premature stop codon and the loss of 10 C-terminal residues (Fig. 5). Both FNS I sequences were verified by repeated end to end PCR with primers binding to the 3′-untranslated region and sequencing. Nevertheless, these mutations appear to be irrelevant for the FNS I specificity, because a common C-terminus was preserved in the FNS I from *Conium*, and *Petroselinum*. Thus, only two sequence differences of FHT vs.

Table 2
Relationships of the FNS I and FHT polypeptides cloned from Apiaceae (% identity)

Gene	Amm_maj FHT	Api_gra FNS	Con_mac FNS	Dau_car FHT	Dau_car FNS	Ane_gra FHT	Pet_cri FHT	Pet_cri FNS	Pim_ani FHT
Amm_majFHT		81	82	87	82	93	94	82	97
Api_graFNS			91	80	87	80	82	91	80
Con_macFNS				80	88	82	83	92	81
Dau_carFHT					81	87	87	80	88
Dau_carFNS						81	82	88	81
Ane_graFHT							93	81	93
Pet_criFHT								81	93
Pet_criFNS									81
Pim_aniFHT									

Fig. 5. Schematic map of FHT and FNS I sequences under investigation: Shown are the aminoacids conserved in either FNS I or FHT which may be responsible for the conversion of FHT into FNS I. Bars indicate unimportant regions for the functional change of FHT into FNS I containing either nonconserved aminoacids or those identical in FHTs and FNS I. Regions responsible for cofactor binding (two His and one Asp residues for Fe (II) binding, and the R–X–F motif as part of the 2-oxoglutarate binding site) are indicated as white marks on the bars (Lukačin and Britsch, 1997).

FNS I deserve further attention: the deletion of an amino acid triplet (Gln, Glu/Asp, Ala/Val/Trp) at position 344/345 was observed in all FNS I sequences, and several amino acid residues are conserved exclusively in either FNS I or FHT (Fig. 5). These features might be considered as driving factors for the change in functionality of a proposed ancestor FHT during evolution, and will be investigated in future mutagenesis studies.

A cladogram was constructed for flavonoid 2-ODDs including FHTs, FLSs and ANSs from plant sources other than the Apiaceae (Fig. 6). FLSs, ANSs, and FHTs each form a separate clade, and within the FHT clade the Apiaceae FNS I and FHT sequences clearly group apart from the FHTs of other plant families and further split into a FHT and FNS I subclade. The relation of FNS I and FHT sequences in the Apiaceen branch of the tree mostly agrees with the systematic classification of the plant species. According to Downie et al. (2000) *Ammi majus*, *Anethum graveolens*, *Apium graveolens* and *Petroselinum crispum* belong to the

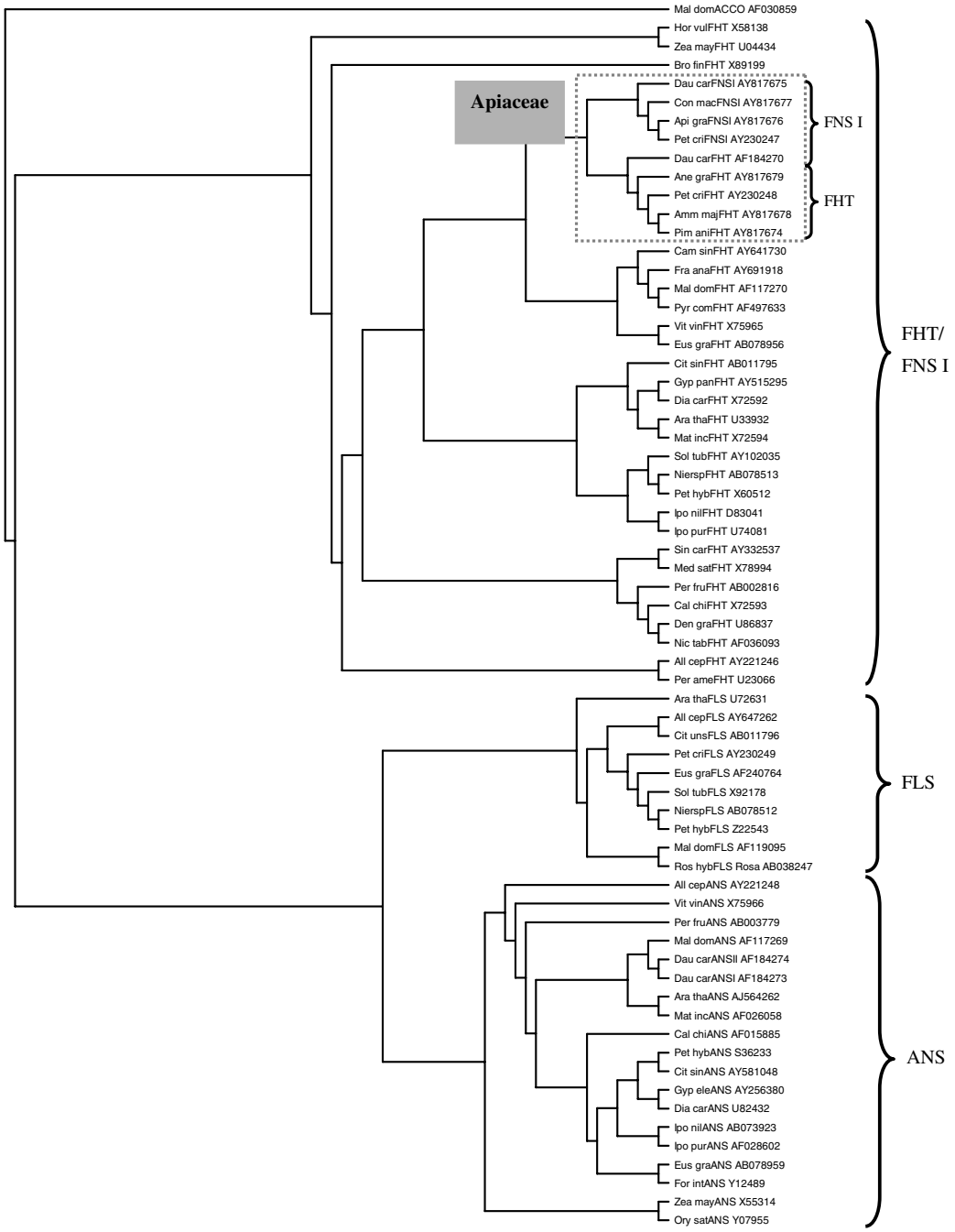

Fig. 6. Cladogram of the deduced amino acid sequences of flavonoid 2-ODDs. Apiaceae sequences are boxed.

Apium clade within the apioid superclade, which is mirrored by the closeness of their FHT and FNS sequences (Fig. 6). In the same superclade but a different clade *Conium maculatum* (apioid superclade incertae sedis) is found. Accordingly, *Conium* FNS I is closer to *Apium* FNS I and *Petroselinum* FNS I. *Daucus carota*, whose FNS I and FHT show the most differences to the other sequences, groups also more distantly to the other species examined in belonging to a different tribe altogether (Scandiceae, subtribe Daucinae). The only exception from this scheme is *Pimpinella anisum* which, although further apart systematically (Pimpinella clade within Apioid superclade), has an FHT that shares higher identity with *Ammi* FHT than either *Anethum* FHT, or *Petroselinum* FHT.

3. Discussion

Although the biosynthesis of flavonoids has been extensively studied, particularly in species of the Apiaceae such as parsley which accumulate a large diversity of flavones and flavonols in green tissues, little is known about the evolution of flavonoid-specific enzymes. The evolutionary process which leads to enzymes of divergent specificities may have followed one or other of two routes: in one scenario, a single enzyme might develop multifunctionality and produce various products from one or more substrates, and this has been ascribed to FLS, ANS, and other enzymes in vitro (Steele et al., 1998; Prescott et al., 2002; Lukačin et al., 2003; Martens et al., 2003). The second scenario requires gene duplication, and mutational evolution of the copy to gain a new functionality while the ancestral gene maintains the primary function (Lynch and Force, 2000). In both instances, the advantage provided to the plant is the driving force of evolution. Gene duplications were proposed for several genes of the flavonoid pathway, e.g., chalcone synthase in *Gerbera* and *Ipomoea* species, chalcone isomerase in *Lotus* or dihydroflavonol-4-reductase in *Ipomoea* species (Helariutta et al., 1996; Hoshino et al., 2001; Shimada et al., 2003), and other pathways, e.g., homospermidine synthase catalyzing the first committed step of pyrrolizidine alkaloid biosynthesis in angiosperms (Ober and Hartmann, 1999), 2-ODDs in glucoseinolate biosynthesis of *Arabidopsis thaliana* (Kliebenstein et al., 2001).

FHT, *FLS* and *FNS* I were previously cloned from parsley (Martens et al., 2003), and the high degree of similarity (90% at the polypeptide level) of FNS I to Apiaceae FHT suggested that *FNS* I had evolved from an ancestral *FHT* by gene duplication. These two enzymes use the same substrates with narrow specificity (Forkmann and Heller, 1999; Martens et al., 2003), in contrast to *FLS* or *ANS* (Prescott et al., 2002; Lukačin et al., 2003; Martens et al., 2003), but produce different products. Nevertheless, the evolutionary context remained to be established, because *FNS* I is exclusively expressed in species of the Apiaceae, but was cloned from one species only. Therefore, three additional *FNS* I (*Apium*, *Conium*; and *Daucus*) and three *FHT* (*Ammi*, *Anethum*, and *Pimpinella*) cDNAs were cloned in this study. All attempts to clone *FNS* I from *Ammi*, and *Pimpinella* or *FHT* from *Conium* failed, unfortunately, although the flavonoid pattern suggested the expression of these enzymes. However, the abundance of the specific transcripts may vary considerably in plant tissues with the developmental stage (Fig. 2), and this parameter will be considered in future studies. The remote possibility of FNS II expression in *Ammi* or *Pimpinella* will also be considered, but this activity has not been detected in preliminary assays. The lack of FHT in *Apium* and the lack of FNS I in *Anethum* fit the respective flavonoid pattern (Table 1) and might suggest that the evolutionary pressure was insufficient to maintain *FHT* or gain *FNS* I function, respectively. Nevertheless, a silent *FHT* gene might exist in *Apium*. Since *FNS* I is predicted to evolve from *FHT* by gene duplication this might apply also to *FNS* I in *Anethum*, in particular because both FHT and FNS I are expressed in closely related parsley and in the systematically more distant carrot. Parsley and *Ammi majus* plants had been taxonomically classified as being closely related, and this is supported by very similar sequences of their FHTs (94% identity) and of other enzymes (Hübner et al., 2003). Therefore, the lack of *FNS* I from *Ammi* appears unlikely, and the genomic DNAs of *Apium*, *Anethum*, and *Ammi* need to be investigated to search for genomic FHT and FNS I genes, respectively, and to determine the number of gene copies.

The divergent intron–exon composition of *FNS* I and *FHT* genes in the Apiaceae seems to be inconsistent with the hypothesis of *FNS* I evolution by *FHT* gene duplication. The two *FNS* I genes examined so far both contain two introns downstream from the Fe(II) and 2-oxoglutarate binding sites in contrast to the *FHT* genes examined in this report. However, *FHT* genes containing two, albeit much longer, introns at equivalent positions as found in the *FNS* I genes have been reported from *Dianthus caryophyllus* (X70378; Dedio et al., unpublished) and *Medicago sativa* (X78994 and X81812; Charrier et al., 1995 and 1996). Accordingly, the lack of introns in Apiaceae FHT genes might be explained by intron losses which have been described for other plant genes as well, e.g., class III peroxidases from *Arabidopsis thaliana* (Tognolli et al., 2002), catalases in Angiosperms (Frugoli et al., 1998). Considering the proposed mechanism of intron loss, reverse transcription of spliced mRNA followed by gene conversion (Roy and Gilbert, 2004; Roy et al., 2003; Baltimore, 1985), the loss of both FHT introns is coherent.

Examination of the evolutionary relationship of flavonoid 2-ODDs (Fig. 6) revealed that the phylogeny of Apiaceae 2-ODD sequences is in good agreement with the organismal phylogeny reconstructed mainly on morphological characteristics, except for *Pimpinella* FHT. However, this is not the case for the phylogenetic relation of the whole tree, but incongruence of organismal and gene phylogenies can occur, when the number of sampled organisms is too small and orthologues genes are missing (Doyle and Gaut, 2000). Nevertheless, the *FNS* I sequences clearly separated within the *FHT* cluster, indicating that this group of genes shares a specific ancestral gene which presumably originated by gene duplication of *FHT*. Since *FNS* I evolved in more highly advanced members of the Apiaceae only, the duplication event likely occurred after the speciation of plant families and during the development of the apiaceae subfamilies. Considering the lack of flavones in the Apiaceae subfamilies Saniculoideae and Hydrocotyloideae (Harborne, 1971), these plants do not require functional *FNS* I. However, gene duplication might have occurred at some point during the separation of the three subfamilies, and scrutinuous studies on FHTs and possibly silent genomic gene duplicates in these subfamilies are necessary to pinpoint the time of gene duplication.

The fact that paralogous *FNS* I genes have not been reported from plants beyond the Apiaceae appears to exclude a horizontal gene transfer of the *FNS* I gene into a common precursor species in Apiaceae as well as another round of gene duplication events in evolutionary history until now. On the other hand, the capability of other species to express *FNS* I might have been lost once the physiological function of synthesizing flavonols and/or flavones as UV-protecting pigments (Middleton and Teramura, 1993; Solovchenko and Schmitz-Eiberger, 2003; Logemann et al., 2000) could be fulfilled by FNS II. The production of flavones seems to confer selective advantages to the Apiaceae, and therefore, the function gained by *FHT* duplication was preserved during evolution. This is also supported by the early expression of *FNS* I during parsley growth, and the fact that in some species of the Apiaceae flavonols were replaced by flavones (Harborne, 1971). It is thus conceivable that flavones contribute significantly to UV-protection within the Apiaceae, which could have been the driving force for the evolution of *FNS* I.

Flavones occur abundantly in plants including primitive plant species, such as the liverworts, division Bryophyta, class Jungermanniopsida, e.g., *Tylimanthus renifolius* (Acrobolbaceae; Feld et al., 2003), and the horse tails, division Pteridophyta, class Equisetopsida, e.g., *Equisetum arvense* (Equisetaceae; Oh et al., 2004). This underlines the early evolution of biosynthetic pathways and their importance for plant persistence. In addition, flavanones, flavonols, dihydroflavonols and anthocyanins were reported from plants of the devision Bryophyta (Zinsmeister et al., 1991, 1994), but it is still unknown whether FNS I or FNS II is responsible for the formation of flavones in these primitive plants. Even in the genus *Bupleurum*, assigned to the monogeneric tribe Bupleureae, and considered as an ancestral group in Apiaceae mainly because it includes woody species and some of the earliest pollen fossil records known for the family, flavones were described (Downie et al., 2000; Chang et al., 2003). Phylogenetic analysis suggested that the genus *Bupleurum* diverged early in the history of Apioideae, but its relationships to other genera has remained uncertain (Neves and Watson, 2004). Therefore, the occurrence of flavones together with flavonols is not in conflict with the assumptions of Harborne (1971) which exclude the occurrence of flavones in primitive genera.

4. Conclusions

The analysis of flavonoid 2-ODD sequences in context with the pertaining literature on the Apiaceae suggests that *FNS* I very likely evolved by a gene duplication of *FHT* followed by functional diversification. Further screening of Apiaceae plants for their flavonoid pattern and functional characterization of *FNS* I and *FHT*s are necessary to identify the most proximal ancestor *FHT* gene.

5. Experimental

5.1. Plant material

All plant materials were derived from isogenic lines and grown under greenhouse conditions in the experimental garden of the Institute (Marburg/Lahn, Germany). The following species were subjected to investigation: *Ammi majus*, *Anethum graveolens* cultivar "Tetra", *Apium graveolens* cultivar "Monarch", *Conium maculatum*, *Daucus carota* ssp. *sativa* cultivar "Nantaise", *Petroselinum crispum* cultivar "Gigante d'Italia" and *Pimpinella anisum*.

Nucleic acids were extracted from young leaves. Developmental stages of parsley leaves were defined as follows (see also Fig. 2): stage 1: leaves < 0.5 cm, completely folded; stage 2: leaves between 0.5 and 1 cm in length, folded, beginning of stem extension; stage 3: leaves > 1 cm, nearly unfolded and clear stem extension; stage 4: leaves unfolded > 1.5 cm.

5.2. Chemicals, buffer and media

Flavonoid standards naringenin, apigenin, kaempferol were purchased from Sigma (Deisenhofen, Germany). Dihydrokaempferol was from our laboratory

collection. All other chemicals were purchased from Roth (Karlsruhe, Germany) or Sigma (Deisenhofen, Germany) and biochemicals from MBI Fermentas (St. Leon-Rot, Germany). [2-^{14}C]Malonyl-CoA was bought from Hartmann Analytik (Braunschweig, Germany). Labeled substrates were synthesised as described in Martens et al. (2003).

5.3. Flavonoid and protein extraction of plant tissue

For the extraction of flavonoids, fresh plant material (1 g) was extracted with 40 ml of ethylacetate at 2 °C in the dark for 48 h. The solvent was vacuum-evaporated to dryness, and the resulting residue was dissolved in 1 ml of methanol. This extract was mixed with equal amounts of 4 N HCl and hydrolysed for 30 min at 95 °C. Flavonoid aglycones were extracted from this solution with ethyl acetate and analysed by thin-layer chromatography on cellulose plates (Merck, Darmstadt, Germany) in different solvent systems as described (Martens et al., 2003). Preparation of crude enzyme extracts from plants and enzyme assays were performed as described previously (Martens et al., 2003).

5.4. Homology based RT-PCR

Isolation of total RNA, reverse transcription, and PCR amplification of sequences with 2-ODD specific, degenerate primers (DIOXY1H, or DIOXY 2H and DIOXY1R) were carried out as described in Martens et al. (2003). Another set of PCR primers was designed from conserved regions of the new Apiaceae dioxygenases obtained during this study. The forward primer, DIOXY3H (5′-AATGGCYCCWWCAACWMTWACYGC-3′), covered the transcription start site. The reverse primer, DIOXY2R (5′-CCGGGTTCTGGAAAGTTG-C-3′), was chosen to bind around 100 bp 5′ from the end of the coding region.

The first strand cDNA products were PCR-amplified in a reaction mix containing 2.5 U Taq DNA polymerase (MBI Fermentas), 25 pmol each of the degenerate primers DIOXY1H, or DIOXY 2H and DIOXY1R or DIOXY3H and DIOXY2R. Fourty cycles of PCR amplification were carried out, each cycle consisting of denaturation at 95 °C for 1 min, 50–60 °C annealing for 1 min and 72 °C extension for 2 min using Robocycler Gradient 96 (Stratagene, Amsterdam, The Netherlands). The PCR amplification was completed with final extension at 72 °C for 10 min. PCR products were electrophoresed on 1.5% agarose-TAE gels at 100 V for 45 min, stained with ethidium bromide and visualized under UV-illumination (ltf-Labortechnik, Wasserburg, Germany).

The PCR products were subcloned into the TA cloning system pTZ57R (InsT/AClone™ PCR Cloning Kit, MBI Fermentas) and were custom sequenced in both directions at MWG Biotech (Ebersberg, Germany). Overlapping clones for the complete coding regions were obtained by 5′- or 3′-RACE with gene specific primers deduced from the known sequences. All full length cDNAs were sequenced in both directions. End-to-end PCR amplification was performed with gene-specific primers for each clone and High Fidelity Polymerase mix (MBI Fermentas) using the amplification protocol described above.

5.5. Functional expression of full length cDNAs in Saccharomyces cerevisiae

In order to confirm whether the isolated cDNAs encoded FNS I, FHT or another 2-ODD, each full-length cDNA (in pTZ57) was ligated into pYES2.1 TOPO TA or pYES2 yeast expression vector (Invitrogen) using either A-overhangs generated by Taq polymerase or using unique restriction sites (5′*Hin*dIII and 3′*Xba*I) that were introduced via the end to end primers outside the coding region of these cDNA sequences. Competent yeast cells and transformation of strain INV *Sc*1 (Invitrogen) was performed according to Dohmen et al. (1991). Expression, cell disruption and protein isolation was done as described in Martens et al. (2003).

5.6. Enzyme assay of 2-ODDs

For identification and analysis of the putative 2-ODDs activities in vitro enzyme assays with recombinant and native protein extracts were performed using ^{14}C-labeled flavonoids as substrates together with the known cofactors as described in Martens et al. (2003). The reaction products were identified by co-chromatography on cellulose thin-layer plates (Merck, Darmstadt, Germany) in solvent system CAW (chloroform acetic acid:water; 10:9:1) or 15% aqueous acetic acid with authentic standards.

5.7. PCR analysis of genomic DNA

Genomic DNA was isolated from leaves of each species according to Dellaporta et al. (1983). PCR amplification of the different genomic DNAs with gene specific primers was performed using 0.5, 1.0, 1.5 and 2.0 μg of genomic DNA in the PCR reaction mix described above. To obtain the maximum yield for these PCR products, 40 cycles of PCR amplification were performed with each cycle consisting of a 95 °C denaturation for 1 min, 57 °C annealing for 1 min, and 72 °C extension for 2 min, and the reaction was completed by a final 72 °C extension for 10 min. PCR products were electrophoresed on 1.5% agarose–TAE gels at 80 V for 2 h, stained with ethidium bromide and visualized under UV-illumination (ltf-Labortechnik,

Wasserburg, Germany). For sequencing, PCR products were ligated into pTZ57R.

5.8. Cladogram

The protein sequences of *Ammi*, *Anethum*, *Apium*, *Conium*, *Daucus*, and *Pimpinella* FNS I/FHTs were aligned with those known from *Petroselinum* and *Daucus*, and 53 other 2-ODD sequences derived from GenBank, with 1-aminocyclopropane-1-carboxylate oxidase (ACCO) from *Malus* × *domestica* serving as the outgroup. The GenBank accession numbers of genes are listed in Fig. 6. Multiple alignments of predicted protein coding sequences were performed using Clustal W (Thompson et al., 1997; http://clustalw.genome.jp/). The phylogenetic tree was displayed by TreeView software (R.D.M. Page, University of Glasgow, UK).

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