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Isolation and Functional Characterization of *CYP71AJ4* Encoding for the First P450 Monooxygenase of Angular Furanocoumarin Biosynthesis*

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The biosynthesis of linear and angular furanocoumarins is still poorly understood at the molecular level, with only psoralen synthase (CYP71AJ1) identified from *Ammi majus*. Using cDNA probes inferred from *CYP71AJ1*, three orthologs were isolated from *Apium graveolens* (*CYP71AJ2*) and *Pastinaca sativa* (*CYP71AJ3* and *-4*) and functionally expressed in yeast cells. CYP71AJ2 and CYP71AJ3 displayed psoralen synthase activity, whereas CYP71AJ4 only catalyzed the conversion of (+)-columbianetin to angelicin and negligible amounts of a hydroxylated columbianetin by-product. CYP71AJ4 thus constitutes the first fully characterized P450 monooxygenase specific for the angular furanocoumarin pathway. The angelicin synthase exhibited an apparent K_m of 2.1 ± 0.4 μM for (+)-columbianetin and a k_{cat} of 112 ± 14 min^{-1}. Moreover, the use of 3′-deuterated (+)-columbianetin as substrate led to an almost complete "metabolic switch," resulting in the synthesis of *anti*-3′-hydroxy-3′-deuterated(+)-columbianetin. This confirms that angelicin synthase attacks columbianetin by *syn*-elimination of hydrogen from C-3′. Sequence comparison between psoralen synthase (CYP71AJ3) and angelicin synthase (CYP71AJ4) showed 70% identity, whereas the identity dropped to 40% in those regions thought to provide the substrate recognition sites. Accordingly, CYP71AJ3 and CYP71AJ4 might be derived from a common ancestor of unknown functionality by gene duplication and subsequent molecular evolution.

Furanocoumarins are natural plant metabolites characterized by a furane moiety fused to benzopyran-2-one. The position of the furane substitution distinguishes two large groups of compounds, the linear (psoralens) and the angular furanocoumarins (angelicin and derivatives) (Fig. 1) (1, 2). The psoralens in particular are known for their photosensitizing and phototoxic effects and have been used in photochemotherapy of skin disorders (psoriasis, vitiligo, and mycosis). In addition, numerous other bioactivities have been attributed to furanocoumarins, which are the focus of many pharmacological screenings, although their mutagenic potential was recognized many years ago (3, 4). Furanocoumarins are formed by only four plant families (*i.e.* Apiaceae, Rutaceae, Fabaceae, and Moraceae) (5, 6). They are allelochemicals that have been assigned ecological functions, such as the defense against phytopathogens, germination inhibition of competitor plants, or herbivore deterrence (7–10). Their toxicity is based on their ability to create covalent adducts of double-stranded DNA, impeding DNA replication (9, 11), as well as to inhibit cytochrome P450 enzymes (12–14). It is noteworthy that plants synthesize either linear furanocoumarins (psoralens) only or both linear and angular furanocoumarins, which are less widely distributed and mainly confined to some Fabaceae and Apiaceae. The association of both forms was demonstrated to be more toxic for insect larvae than the presence of linear furanocoumarins alone, which could reflect the evolutionary adaptation of plants to new herbivores that have acquired the capacity to detoxify psoralens (15–17).

Due to the complex bioactivity of furanocoumarins, its biosynthesis has received continuous attention. The basis of linear furanocoumarin formation was mostly established by the end of the 1980s by a combination of precursor feeding experiments and the biochemical characterization of major enzymes of the pathway. Nevertheless, until recently, the pathway was thought to depend on the *ortho*-hydroxylation of *trans*- or *cis*-4-coumaric acid and cyclization of the product to umbelliferone (see Ref. 6 for a review). However, a dioxygenase has now been cloned from *Arabidopsis* and expressed; this enzyme converts feruloyl-CoA to 2,4-dihydroxy-5-methoxycinnamoyl-CoA and scopoletin (18). To a lesser extent, it also accepts 4-coumaroyl-CoA, which yields umbelliferone (Fig. 1). The data prove for the first time the essential role of CoA-esters. These serve as an active substrate in coumarin biosynthesis, whereas unconjugated cinnamic acids are inactive. This pivotal role of CoA-esters in coumarin synthesis is consistent with the report of a unique 4-coumaroyl-CoA ligase that was specifically assigned to coumarin synthesis in *Ruta graveolens* (19). Umbelliferone is subsequently prenylated to demethylsuberosin by a 6-prenyltransferase also assigned to plastids in *R. graveolens* (20). The conversion of demethylsuberosin to marmesin and further to psoralen is then catalyzed by two distinct P450 enzymes (*i.e.*

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marmesin synthase and psoralen synthase) in *Ammi majus* and *Petroselinum crispum* (21–23). Psoralen is next hydroxylated at C-5 by yet another P450 monooxygenase-generating bergaptol, which is subsequently *O*-methylated to bergapten (5-methoxypsoralen) (24, 25). Alternatively, the formation of xanthotoxin (8-methoxypsoralen) from psoralen is supposed to proceed via analogous reactions (*i.e.* successive hydroxylation at C-8 and *O*-methylation), but this remains to be established. The pathway to angular furanocoumarins has not been as well documented, especially at the level of enzyme activity. This pathway branches off from that for psoralens by divergent prenylation of umbelliferone (Fig. 1) to form osthenol. The two subsequent steps leading to angelicin were assumed to be very similar to the steps leading to the conversion of demethylsuberosin to (+)-marmesin and psoralen. This assumption is particularly convincing when the catalytic mechanisms of psoralen synthase and angelicin synthase are compared. Previous studies using stereospecifically deuterated (+)-marmesin confirmed that psoralen synthase activity catalyzes a single concerted reaction, initiated by the abstraction of the C-3′-hydrogen *syn*-configured to the isopropyloxy group and release of acetone as a by-product (Fig. 1) (26, 27). An equivalent study demonstrated by feeding leaves of *Heracleum mantegazzianum* with stereospecifically 3′-deuterated (+)-columbianetin that the conversion of (+)-columbianetin to angelicin (*i.e.* angelicin synthase activity) also proceeded by a *syn*-elimination at C-3′ (28), although this was inferred by mass spectral analysis of metabolites rather than by *in vitro* enzyme assays. The mechanistic and stereochemical similarities of the two pathways and the limited distribution of angular furanocoumarins to a few plant species that already synthesize linear structures support the assumption that the two pathways are driven by structurally related enzymes.

The molecular genetics of furanocoumarin formation has remained elusive, due to the high lability of the specific activities in crude plant microsomal preparations (21), until the very recent isolation of the first furanocoumarin-committed P450 gene, *CYP71AJ1*, which encodes psoralen synthase. This gene was cloned from *A. majus* (Apiaceae), which produces only linear furanocoumarins (23). Notably, the recombinant enzyme efficiently catalyzes the conversion of (+)-marmesin to psoralen but is unable to metabolize the angular substrate (+)-columbianetin, although the angular compound acts as a competitive inhibitor of psoralen synthase activity. Modeling studies and docking calculations suggested that (+)-columbianetin fitted properly into the catalytic pocket, as required for competitive inhibition, but the distance of C-3′ to the active site porphyrin-iron was too large to initiate the process of abstracting hydrogen (23). The *in silico* results raised the possibility that in plants producing both linear and angular furanocoumarins, a rather limited number of mutations in CYP71AJ1 paralogs could have generated angelicin synthase; this would have been as a pivotal step in the evolution of the angular furanocoumarin pathway, provided that umbelliferone 8-prenyltransferase for the synthesis of osthenol was also present.

In the present work, we describe the cloning and functional expression of two new orthologs of *A. majus CYP71AJ1*, encoding psoralen synthase, from *Apium graveolens* (celery *CYP71AJ2*) and *Pastinaca sativa* (parsnip *CYP71AJ3*). Additionally, the closely related *CYP71AJ4* was isolated from parsnip and annotated as angelicin synthase, the first P450 gene committed to angular furanocoumarin biosynthesis. Alignments of the paralogs (CYP71AJ3 and CYP71AJ4) revealed that the sequence identity dropped to only 40% in substrate recognition sites (SRSs)[3] at an overall identity of 70%. Assays conducted with the recombinant enzyme and *syn*-[3′-D]columbianetin demonstrated a reaction mechanism analogous to that of psoralen synthase initiated by the abstraction of *syn*-C-3′-hydrogen. These results demonstrate that the CYP71AJ subfamily is involved in the synthesis of both linear and angular furanocoumarins and suggest that *CYP71AJ3* and *CYP71AJ4* derived from a common ancestor gene.

EXPERIMENTAL PROCEDURES

Plant Material—Parsnip (*Pastinaca sativa*) plants var. "Demi-Long de Guernesey" (Caillard, Avignon, France) were grown in the university greenhouse for 5 weeks. Celery (*A. graveolens*) plants were bought at a local market.

Chemicals—Umbelliferone, psoralen, xanthotoxin, and cinnamic acids were purchased from Sigma. Xantothoxol was from Roth (Karlsruhe, Germany). Angelicin, bergapten, and bergaptol were from Extrasynthese (Genay, France). Demethylsuberosin was synthesized as described previously (21). (+)-Marmesin and 5-hydroxymarmesin were kindly provided by R. Caniato (University of Padua, Italy). A small sample of *syn*-[3′-D]columbianetin and columbianadin were gifts from W. Boland (Max Planck Institute for Chemical Ecology, Jena, Germany) and J. Hohmann (University of Szeged, Hungary), respectively.

(+)-Columbianetin was preparatively isolated from dried roots of *Angelica pubescens*. The roots (Huisong Pharmaceuticals, Hangzhou, China) obtained from Chongde Sun (Zheijang University, Hangzhou, China) were coarsely chopped, redried in an oven at 80 °C for ~5 h, and then ground to a fine powder by mill and mortar. The powder (30-g aliquot) was stirred for 60 min in acetone (100 ml) at room temperature, the acetone extract was evaporated to dryness, and the oily residue was partitioned in a mixture of *n*-hexane/methanol/water 10:1:9 to remove lipids. The polar phase was reduced by evaporation to remove methanol, and the remaining aqueous phase was extracted with ethyl acetate, yielding the organic crude extract. This extract was applied to a silica gel 60 (Merck) column (3 × 35 cm), and the elution was performed in two steps, with chloroform (250 ml) followed by chloroform/acetone 9:1 (500 ml). After the first 200 ml, fractions of 10 ml each were collected and examined by silica F_{254} thin layer chromatography (Merck) using toluene/acetic acid 4:1 as the mobile phase. Fractions containing blue-fluorescing compounds under UV_{254} irradiation and co-migrating on TLC with authentic columbianetin (*Rf* ~0.3–0.4) were pooled, evaporated to dryness, and subjected to high speed countercurrent chromatography on an HSCCC instrument (P.C. Inc.) equipped with a 400-ml Teflon

[3] The abbreviations used are: SRS, substrate recognition site; HPLC, high pressure liquid chromatography; SPE, solid phase extraction; *syn*-[3′-D]columbianetin, 3′-deuterated (+)-columbianetin.

coil and 10-ml sample loop switch. Solvents were delivered to the coil, the flow was maintained by an L6000 pump (Merck-Hitachi), and fractions were collected manually. The partition coefficients and purity of fractions were determined on an HPLC system (Merck-Hitachi) consisting of a D6000 interface, L6200A gradient pump, AS4000 autosampler, and L4500 diode array detector. Samples were analyzed on an EC 125/4 Nucleodur Sphinx RP column (Machery-Nagel, Düren, Germany) operated at a flow rate of 1 ml/min. The two-solvent system employed for separation was composed of 1.5% (v/v) aqueous phosphoric acid (solvent A) and 80% (v/v) acetonitrile (MeCN) in water (solvent B) and using linear gradients of 80 to 50% A in B within 20 min, followed by 50 to 20% A in B over 20–25 min before a final re-equilibration to the starting conditions. The elution was monitored at 330 nm. Solvents chosen for HSCCC separation were mixed at appropriate ratios in a separation funnel, shaken vigorously, and allowed to stand overnight for complete phase separation. The HSCCC coil was then equilibrated with the stationary phase, and the sample was dissolved in 10 ml of the mobile phase and injected into the coil for separation at 890–900 rpm at room temperature and a flow rate of 1 ml/min. Fractions of 2–10 ml were collected, fluorescent substances were detected in small aliquots spotted on filter paper under UV irradiation, and fractions of interest were examined further by TLC and HPLC analyses. The substance identity was finally verified by negative mode mass spectral fragmentation on a VG Micromass 7070 mass spectrometer at 70 eV ionization energy and comparison with authentic (+)-[3′-D]columbianetin (G. Laufenberg and N. Zitzer, Institute of Pharmaceutical Chemistry, Philipps-University Marburg). Columbianetin-containing fractions were pooled and concentrated to remove methanol; the remaining aqueous phase was extracted with ethyl acetate and dried.

cDNA Cloning—Total RNA was extracted from leaves of 6-h mechanically wounded celery and parsnip with the RNeasy plant extraction kit (Qiagen). The cDNA was generated using SuperScript™ III RT (Invitrogen) and random primers. A full-length cDNA sequence from celery and two full-length cDNA sequences from parsnip were PCR-amplified using *CYP71AJ1* (accession number AY532373.1) specific primers (forward, 5′-CGGTACCATGAAGATGCTGGAACAGAAT; reverse, 5′-GGAATTCTCAAACATGTGGTCTGGCAACCACCAAGAGAGG) and proofreading polymerase FideliTaq (GE Healthcare). The PCR amplification included a touch-down (A) and a classical (B) PCR as follows: 5 min at 94 °C, followed by 10 cycles (30 s at 94 °C, 45 s at 60 °C −1 °C/cycle, and 90 s at 72 °C), 20 cycles (30 s at 94 °C, 45 s at 50 °C, and 90 s at 72 °C), and a final 10-min extension step at 72 °C. The *CYP71AJ2* PCR product from celery (1498 bp) and those from parsnip (*CYP71AJ3*, 1485 bp; *CYP71AJ4*, 1503 bp) were purified and cloned in the pCR8-TOPO vector (Invitrogen) prior to sequencing. In order to identify the specific 5′- and 3′-ends of *CYP71AJ3* and *CYP71AJ4* orthologs, an inverse PCR strategy adapted from Stemmer and Morris (29) was used. The genomic DNA from parsnip leaves was isolated using the DNeasy plant extraction kit (Qiagen). One μg of genomic DNA from each plant was digested during 2 h at 37 °C by 6 units of the restriction enzyme DraI. After heat inactivation of DraI during 20 min at 65 °C, 0.1 μg of digested gDNA was self-ligated overnight at 16 °C using 4 units of T4 DNA ligase (Invitrogen) in a final volume of 50 μl. This ligated gDNA was used as a matrix for PCR amplification using specific primers. The 5′- and 3′-ends of the celery *CYP71AJ2* were amplified using forward primer 5′-GCAATGGCTGTTAATGACCTTGTAGTTGCAAATGTC and reverse primer 5′-ATCATGAGTTTTCAAAACCTCACGAGCCGC. The 5′- and 3′-borders of parsnip *CYP71AJ3* and *CYP71AJ4* were amplified with the forward primers 5′-GCTATGGCTGTTAATGAGCTTGCTGTAGCAAATGTT and 5′-GCAATCTCTGTTAATGAGCTTGTAGTAGCAAAT and the reverse primers 5′-GACTACATTTCTGGTTAGTTGGATTAATTGCTCGCC and 5′-AGAATTTTCAATATTATCGAGTAACAGAGCAAC, respectively. In each case, the temperature cycles consisted of 5 min at 94 °C, 30 cycles (30 s at 94 °C, 45 s at 55 °C, 150 s at 72 °C). PCR products were purified and then cloned into pCR8 TOPO TA GW vector prior to sequencing.

The two parsnip full-length cDNA sequences were PCR-amplified using the 5′-end-specific forward primer 5′-ATGAAGATGCTGGAACAGAATCCCCTGTACCTGTATTTC and the *CYP71AJ3* and *CYP71AJ4* 3′-end-specific primers 5′-TCAAACATGTGGTGTGGCAACCACAAAGAAAGG and 5′-TCAAACATGCGGTGAGGCAACAACCATGAGAGG, respectively. PCR conditions were as follows: 5 min at 94 °C, 30 cycles (30 s at 94 °C, 45 s at 55 °C, 100 s at 72 °C), 10 min at 72 °C using a proofreading polymerase (FideliTaq; GE Healthcare). PCR products were purified, cloned in pCR8 TOPO vector, and sequenced.

Yeast Expression and Enzyme Assays—The celery and parsnip CYP71AJ1 orthologs corresponding ORF were ligated in the yeast expression vector pYeDP60 (30) between KpnI and EcoRI restriction sites. Yeast transformation was performed as described elsewhere (31). Propagation of yeast cells and preparation of microsomes were conducted as described previously (23). Protein amounts were determined according to Bradford (32), and the microsomal P450 contents were determined as described by Omura and Sato (33). Enzymatic screening was done with microsomes incubated in 0.1 M sodium phosphate buffer containing 100 μM substrate and 1 mM NADPH. The incubation was performed for 1 h at 27 °C and 800 rpm agitation (Eppendorf Thermomixer compact). The reaction was stopped by adding 75 μl of MeCN/HCl (99:1). Product formation and substrate consumption were analyzed by reverse phase HPLC on a Purospher 5-μm, 4 × 250-mm end-capped column (Merck). The column was equilibrated in water/acetic acid (99.9/0.1) at a flow rate of 0.9 ml min^{-1}, and the elution was performed under diode array detection (220–400 nm) using a linear gradient of MeCN/methanol (1:1) from 0 to 60% for 25.0 min, followed by 100% MeCN/methanol (1:1) for an additional 10 min.

Kinetic Analyses—Kinetic parameters for psoralen synthase activity were determined as previously described (23). The activity of angelicin synthase was measured for 10.0 min in 0.1 M sodium phosphate buffer, pH 7.0 (150.0 μl total), containing 0.5 mM NADPH, 0.5 pmol of the recombinant CYP71AJ4 and varying the concentration of (+)-columbianetin or *syn*-[3′-D]columbianetin. The apparent K_m values were determined by

Lineweaver-Burk extrapolations. Mechanism-based inactivation assays were performed as described previously (23).

Gas Chromatography-Mass Spectrometry Analyses—The product eluting from HPLC at a retention time corresponding to angelicin was collected from 10 incubations, the solvent was evaporated in an air stream, and the pellet was resuspended in chloroform/MeCN (1:1) for gas chromatography-mass spectrometry analysis. The sample (1.0 μl) was injected into a Varian Star 3400 CX spectrometer fitted with a Varian Factor Four 5MS column (15.0 m × 0.25-mm inner diameter, 0.1-μm film) using helium at 5.5 p.s.i. as carrier gas. The column temperature was initially 60 °C for 2 min, ramped to 90 °C at 40 °C min^{-1} and held for 4 min and then to 200 °C at 10 °C min^{-1}, and finally to 300 °C at 10 °C min^{-1} and held for 10.0 min. Mass spectra were recorded at 70 eV, scanning from 60 to 400 atomic mass units.

HPLC-Solid Phase Extraction (SPE)-^{1}H NMR Analyses—The sample was subjected to coupled HPLC-SPE-^{1}H NMR analysis using the following equipment and experimental conditions. HPLC was performed on an Agilent 1100 chromatography system (quaternary pump G1311A; autosampler G1313A; J&M photodiode array detector operating at a wavelength range between 200 and 620 nm, monitoring wavelength 254 and 325 nm). A LiChrospher 100 RP-18 column (250 × 4 mm, particle size 5 μm) with MeCN/water (0.01% trifluoroacetic acid) as a mobile phase (flow rate 1.0 ml min^{-1}) in linear gradient mode from 10 to 90% MeCN in 40 min was applied. After photodiode array detector detection, the eluting solvent was diluted with H_2O by a makeup pump to decrease eluotropic strength. The peak eluting at R_t 10.25 min was trapped on a poly(divinylbenzene) SPE cartridge (HySphere resin GP) using a Prospekt 2 SPE unit (Spark Holland) as an LC-NMR interface. The cartridge containing the trapped peak was dried with a stream of nitrogen gas for 30 min. Then the compound was eluted with MeCN-d_3 and transferred through the connecting capillary from the Prospekt 2 SPE unit to the NMR spectrometer (Bruker AV 500). The NMR spectrometer was equipped with a 5-mm TCI cryoprobe and Cryofit™ flow insert (30-μl active volume). The ^{1}H NMR spectrum of the trace sample amount of the enzyme product was measured in MeCN-d_3 at 300 K, and 2024 scans were added to the FID. The central line of the solvent signal at δ 1.93 was used for referencing. Residual solvent signals were suppressed using presaturation.

^{1}H NMR data of *anti*-3′-hydroxy-*syn*-[3′-D]columbianetin (enzyme product, 500 MHz, MeCN-d_3): δ 7.78 (1H, d, $J_{H\text{-}4-H\text{-}3}$ = 9.5 Hz, H-4), 7.48 (1H, d, $J_{H\text{-}5-H\text{-}6}$ = 8.5 Hz, H-5), 6.80 (1H, d, $J_{H\text{-}6-H\text{-}5}$ = 8.5 Hz, H-6), 6.17 (1H, d, $J_{H\text{-}3-H\text{-}4}$ = 9.5 Hz, H-3), 4.36 (1H, s), 1.22 (3H, s, CH_3), 1.21 (3H, s, CH_3).

^{1}H NMR data of *syn*-[3′-D]columbianetin (substrate, 500 MHz, MeCN-d_3): δ 7.76 (1H, d, $J_{H\text{-}4-H\text{-}3}$ = 9.5 Hz, H-4), 7.36 (1H, d, $J_{H\text{-}5-H\text{-}6}$ = 8.5 Hz, H-5), 6.73 (1H, d, $J_{H\text{-}6-H\text{-}5}$ = 8.5 Hz, H-6), 6.13 (1H, d, $J_{H\text{-}3-H\text{-}4}$ = 9.5 Hz, H-3), 4.77 (1H, d, $J_{H\text{-}2'-H\text{-}3'}$ = 9.8 Hz, H-2′), 3.28 (1H, brd, $J_{H\text{-}3'-H\text{-}2'}$ = 9.8 Hz, H-3′), 1.25 (3H, s, CH_3), 1.17 (3H, s, CH_3).

Bioinformatic Analysis—Multiple sequence alignments were generated with ClustalX.

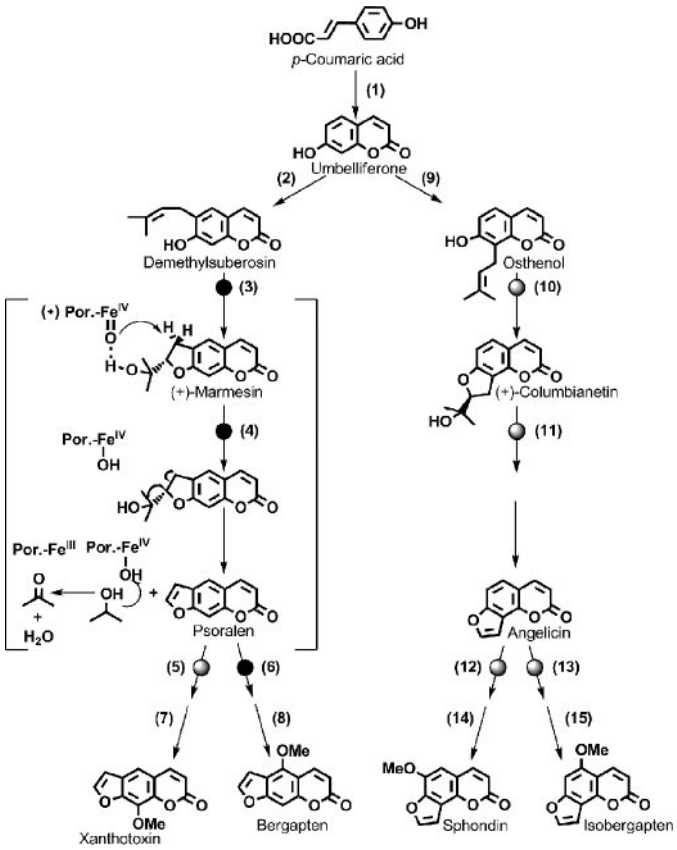

FIGURE 1. **Biosynthetic branch pathways to linear and angular furanocoumarins.** *Black dots* designate steps catalyzed by P450 monooxygenases. *Gray dots* indicate putative P450 steps. Enzymes numbered are 4-coumaric acid 2-hydroxylase (*1*), umbelliferone 6-prenyltransferase (*2*), (+)-marmesin synthase (*3*), psoralen synthase (*4*), psoralen 8-monooxygenase (*5*), psoralen 5-monooxygenase (*6*), xanthotoxol *O*-methyltransferase (*7*), bergaptol *O*-methyltransferase (*8*), umbelliferone 8-prenyltransferase (*9*), (+)-columbianetin synthase (*10*), angelicin synthase (*11*), angelicin 6-hydroxylase (*12*), angelicin 5-hydroxylase (*13*), 6-hydroxyangelicin *O*-methyltransferase (*14*), and 5-hydroxyangelicin *O*-methyltransferase (*15*). Details of the psoralen synthase-catalyzed reaction are in *brackets*.

RESULTS

Isolation of (+)-Columbianetin—The recent characterization of *A. majus* CYP71AJ1 (psoralen synthase) (23) opened the door to further investigations of furanocoumarin biosynthesis in other Apiaceae. The reaction in the angular pathway analogous to psoralen synthase (angelicin synthase), however, depends on (+)-columbianetin as a substrate (Fig. 1), but this was not available commercially or from laboratory collections and therefore had to be isolated from natural sources. Based on the literature (34, 35), several two-phase solvent mixtures were used to examine the separation of columbianetin extracted from *A. pubescens* roots and prepurified by silica column chromatography, and the partition coefficient k = $[columbianetin]_{stat}/[columbianetin]_{mob}$ (36) was analyzed by HPLC. Columbianetin was enriched in the aqueous phase of systems composed of *n*-hexane, ethyl acetate, methanol, water at volume ratios 5:5:5:5 (I) and particularly 5:5:7:4 (II) with k values of 0.40 and 0.11, respectively. The dry prepurified root extract (221 mg from 30 g of dry root) was therefore dissolved in solvent system II (10 ml) and loaded onto a HSCCC coil for separation at 890 rpm, and the

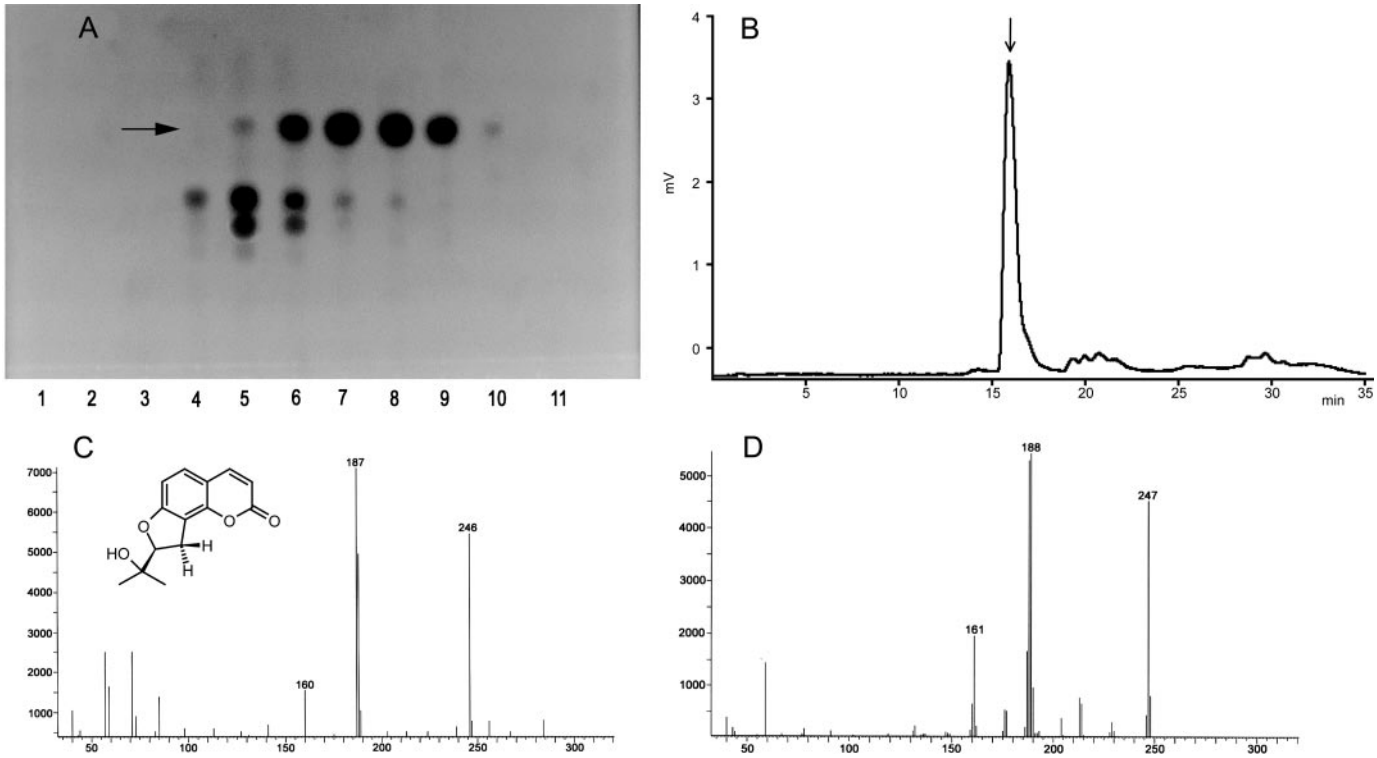

FIGURE 2. **Isolation of (+)-columbianetin from *Angelica pubescens* roots.** Crude extracts were separated by HSCCC in solvent systems I and II, and fractions were analyzed for fluorescent compounds by TLC separation on a transilluminator irradiated at 312 nm (*A*; fractions eluted from HSCCC in solvent II) or HPLC (*B*). The *arrows* indicate the relative mobility of authentic [3′-D]columbianetin. The purified compound was subjected to mass spectroscopy (*C*), and the pattern of fragments was compared with that of reference [3′-D]columbianetin (*D*). Major fragments of columbianetin $[M]^+$ (*m/z* 246) correspond to $[M\text{-}H_2O\text{-}CH_3]^+$ (*m/z* 213), $[M\text{-}C_3H_6O]^+$ (*m/z* 188), and $[M\text{-}C_3H_7O]^+$ (*m/z* 187, base peak) (37).

eluate (20-ml fractions) was analyzed by TLC (Fig. 2*A*). Fractions 6–9 (~85 ml) were combined; columbianetin was extracted from the aqueous phase after the removal of methanol, evaporated to dryness, and subjected to another round of HSCCC separation in solvent system I at 900 rpm. Fractions containing columbianetin only (>95% purity) as judged by TLC were pooled and examined by HPLC (Fig. 2*B*). Unequivocal identification of columbianetin was achieved by mass spectral fragmentation and comparison with authentic [3′-D]columbianetin (Fig. 2, *C* and *D*), revealing the $[M^+]$ at *m/z* 246 with major fragments at *m/z* 187 (base peak) and 160. A yield of 9.48 mg (38.53 μmol) columbianetin was recovered and assigned the (+)-configuration, because only (+)-columbianetin derivatives have been reported from *A. pubescens* (38). The preparative purification also revealed significant amounts of conjugated columbianetin (*i.e.* the angeloylester columbianadin identified by co-chromatography with an authentic sample); although unconjugated (+)-columbianetin had never been described from *A. pubescens* roots, our yield corresponds to 0.03% of the dry herbal drug.

Isolation and Sequence Analysis of CYP71AJ1 Orthologs—Psoralen synthase (CYP71AJ1) possesses a narrow substrate specificity and binds to (+)-columbianetin as a competitive inhibitor of (+)-marmesin only (23). Three-dimensional docking simulations indicated a nearly perfect fitting of the (+)-columbianetin core into the active site pocket (23) and suggested that a limited number of evolutionary mutations might have conferred some angelicin synthase activity on the CYP71AJ1 sequence. Such mutations must have developed to encompass the isopropyloxy side chain and C-2′/3′ of (+)-columbianetin, which are oriented far from the iron-porphyrin active center in the CYP71AJ1-columbianetin model. Under these conditions, angelicin synthase could be spotted beside further psoralen synthases by cloning CYP71AJ1 orthologs. *A. graveolens* (celery) and *P. sativa* (parsnip) belong to the Apiaceae, just like *A. majus*, but are capable of producing linear and angular furanocoumarins when their tissue is wounded (1, 2). Furthermore, their common codon usage (available on the Kazusa World Wide Web server) is an essential prerequisite for a successful nondegenerated PCR approach. The amplification of *CYP71AJ1* orthologs was therefore accomplished using RNA from wounded celery and parsnip leaves as a template for reverse transcription-PCR, followed by a two-step strategy combining the PCR with *CYP71AJ1*-specific primers and inverse PCR techniques. Three full-size cDNA sequences, one from celery and two from parsnip, were isolated; these ranged from 1482 to 1503 bp in length (Fig. 3). Comparison of the translated polypeptides revealed more than 65% identity with CYP71AJ1, which groups the enzymes into the same P450 subfamily. Classification by the international P450 nomenclature committee (available on the cytochrome P450 home page World Wide Web server) assigned the sequences as CYP71AJ2 (celery; GenBank™ accession number EF191022), CYP71AJ3, and CYP71AJ4 (parsnip; GenBank™ accession numbers EF191020 and EF191021). The CYP71AJ2 and CYP71AJ3 polypeptides are closely related to CYP71AJ1 (Fig. 3), with an identity of 89 and 82%, respectively, but contain a four-amino acid insertion at position

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71AJ1    1  MKMLEQNPQYLYFFSLFLVTIFLYKWLTLKKTPLKNLPPSPPQYPIIGNLHQIGPDPQASLRDLAQKYGPLMFLKFGTVPVLVVSSADAA
71AJ2    1  .....................V.V..L.......S........R..............H......L......S.................
71AJ3    1  ..............P.............-V....S........RL.............I......R....V.H....S.........G.
71AJ4    1  ............................-A....S........RL............LHI......R......Q.QL.RI........E.T
                          SRS 1
71AJ1   91  REALKTHDLVFADRPYSSVANKIFYNGKDMVFARYTEYWRQVKSICVTQLLSNKRVNSFHYVREEEVDLLVQNLENSHSKVANLTELLIE
71AJ2   91  ..V......I............V.....................................QN............I...C...I........
71AJ3   90  ..IF.....A...........R.....R................T.......V...Q...N......A..LD.I...K...I..S.M...
71AJ4   90  ..V.....V..SQ..IT.AID.LC.K.R.VA.S..S......R.T........S..H...NI.....A..I..I...A.E.I..G.Q..Q
                                  SRS 2                    SRS 3
71AJ1  181  VTGNVVCRVSVG----SGDKVDSYKILILEIMDMLGYSRSIEDFFPLLGWVDWLTGLRGKVAEAAKGVDTFLEGVLKEHLS----TTGSK
71AJ2  181  .......K....----.................E.............MF..............K......D..........TARASNNA.A
71AJ3  180  L.......AAL.----..YN.....S.L.Q.................S......I...K...EK..N...A.......N.TNP---S.S.A
71AJ4  180  L.R.........SEYL..H.GKL.QK.LA.VTE..A.TY..G..I.........S.SKA..EKT..E..A....A.RD.IKTMASNK..A
                                        SRS 4
71AJ1  263  YNDFVSILLEIQEADAGSSMDNECIKSLIWDMLGAGTETISTALEWTLAALIKNPDAMFKLQNEVREIGKGKSKISEADLVKMNYLQAVM
71AJ2  267  D.....................T.....................................L.....................G..T......
71AJ3  263  NK.....................K.................A......IG....S....S...K.........R.E.G.....D..K...
71AJ4  270  ND..L......R......TL.E....AIV...ILG....T..T...IV..I.....V.....K............E.V........K...
             SRS 5
71AJ1  353  KESMRLYFTAPLLVPREARQDIKFMGYDISSGTQVLINAWAIARDPLLWDKPEEFRPERFLNSPIDYKGFHYEFLPFGAGRRGCPGIQFA
71AJ2  357  .................S...V........A.......V.......S..E.............H......N..Y................
71AJ3  353  .....................V.......K.................SS..N...................N..YI...............
71AJ4  360  .......I..-F.L....K..V.L..............T.ET....S...N...................L...Y....G...........
                                        SRS 6
71AJ1  443  MCINELVVANLVHKFNFELPDGKRLEDLDMTAASGITLRKKSPLLVVARPHV
71AJ2  447  .AV.......VI..........E.........V...................
71AJ3  443  ISV.......V.N.............EM....ST...FH....IF...T...
71AJ4  449  .AV...A...V.Y..D.KM...E.F.....SGVP..S.YR.Y..M...S...
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FIGURE 3. **Amino acid sequence comparison of CYP71AJ1 orthologs.** The translated amino acid sequences of CYP71AJ1 orthologs were compared with CYP71AJ1 polypeptide. *Dots* represent identical amino acid residues. SRSs are assigned in the sequence.

TABLE 1

Identity (percentage) of full CYP71AJ2–4 polypeptide sequences and SRSs with CYP71AJ1

The SRS elements are designated in Fig. 3.

	Full sequence	SRS1	SRS2	SRS3	SRS4	SRS5	SRS6	Outside SRSs
	%	%	%	%	%	%	%	%
CYP71AJ2	89	95	90	92	100	100	93	90
CYP71AJ3	82	89	80	67	92	100	71	85
CYP71AJ4	70	37	40	42	61	67	43	80

TABLE 2

Substrate affinities of CYP71AJ2 and -3 in comparison with affinity of CYP71AJ1

Data were reported by Larbat *et al.* (23).

	(+)-Marmesin K_m	5-Hydroxymarmesin (K_m)
	μM	μM
CYP71AJ1	1.5 ± 0.5	29.3 ± 8
CYP71AJ2	0.54 ± 0.21	54 ± 16
CYP71AJ3	1.3 ± 0.4	19 ± 7

258 (CYP71AJ2) or one deletion at position 28 and a Pro^{257} insertion (CYP71AJ3). In contrast, CYP71AJ4 showed only 70% identity to CYP71AJ1 with two single deletions at positions 28 and 370 and two four-amino acid insertions at positions 192 and 261 (Fig. 3). The divergence in polypeptide sequences becomes even more pronounced when the six substrate recognition sites (SRS1 to -6) (39) previously defined in CYP71AJ1 (23) are compared (Table 1). Most notably, the level of sequence identity with CYP71AJ1 in SRS1, -4, and -5 of CYP71AJ2 and CYP71AJ3 was higher than the level of sequence identity for the full sequence average, whereas the inverse tendency was observed for the sequence identity of CYP71AJ4 dropping to 37, 40, 42, and 43%, respectively, in SRS1, -2, -3, and -6.

Functional Expression of CYP71AJ2, CYP71AJ3, and CYP71AJ4—The genes *CYP71AJ2* to *-4* were individually expressed in yeast strain WAT 11, as described earlier for the functional characterization of *CYP71AJ1* (23). A low level of expression was recorded for each of the transformants, as was true of *CYP71AJ1* (23) and revealed by weak absorbance at 450 nm in the differential CO spectrum (data not shown). Nevertheless, the expression was sufficient for activity screenings of the crude yeast microsomal fractions. In the search for the substrate, enzyme assays were performed with either of two intermediates of the furanocoumarin pathway (demethylsuberosin, (+)-marmesin, 5-hydroxymarmesin, psoralen, bergaptol, xanthotoxol, bergapten, xanthotoxin, isopimpinellin, (+)-columbianetin, or angelicin) as well as with cinnamic acids (cinnamic acid, 4-coumaric acid, 2-coumaric acid, or ferulic acid) and simple coumarins (coumarin, herniarin, scopoletin, or umbelliferone). *CYP71AJ2* and *CYP71AJ3* were shown to encode NADPH-dependent psoralen synthases with maximal activity in sodium phosphate buffer at pH 7.5 and substrate affinities to (+)-marmesin of K_m = 0.54 ± 0.21 μM and 1.3 ± 0.40 μM, respectively (Table 2). Analogous to CYP71AJ1 (23), both recombinant enzymes were capable of converting 5-hydroxymarmesin to bergaptol with much lower affinities of K_m = 54 ± 16 μM and 19 ± 7 μM (Table 2); none of the other compounds included in the assays were used. These data clearly proved that CYP71AJ2 and CYP71AJ3 are psoralen synthases. In contrast, recombinant CYP71AJ4 did not exhibit psoralen synthase activity but catalyzed the NADPH-dependent conversion of (+)-columbianetin (Fig. 1) to a major product and a minor by-product, eluting at 27.5 and 21.3 min, respectively, from HPLC (Fig. 4, *A* and *B*). Neither was detected in control assays employing microsomes from yeast transformed with the empty plasmid vector. Maximal conversion occurred at pH 7.0 in phosphate buffer and a temperature range of 27–30 °C. The major product was unequivocally identified as angelicin due to its ability to absorb λ_{max} 300 nm and electron impact mass spectrum with an $[M^+]$ of *m/z* 186 and fragments at *m/z* 158, 130, 102, and 51, which fully matched the fragmentation of commercial angelicin (Fig. 4, *C–F*). Mass spectral analysis of the minor by-product revealed an $[M^+]$ of *m/z* 263 and fragments corresponding to a hydroxylated columbianetin derivative. The fragmentation pattern (*m/z* fragments at 249, 229, and 187) clearly indicated that hydroxylation of the isopropyloxy side chain had occurred (Fig. 4*G*). Further incubation of the isolated hydroxyl derivative of columbianetin with CYP71AJ4 failed to give angelicin (data not shown), ruling out the possibility that this derivative was an intermediate in angelicin formation. Thus, *CYP71AJ4* is the first gene reported from the angular branch of furanocoumarin biosynthesis and encoding angelicin synthase.

Characterization of Recombinant CYP71AJ4—Microsomes harvested from yeast cells expressing CYP71AJ4 showed narrow substrate specificity for (+)-columbianetin, since none of the furanocoumarins, coumarins, or cinnamic acids listed

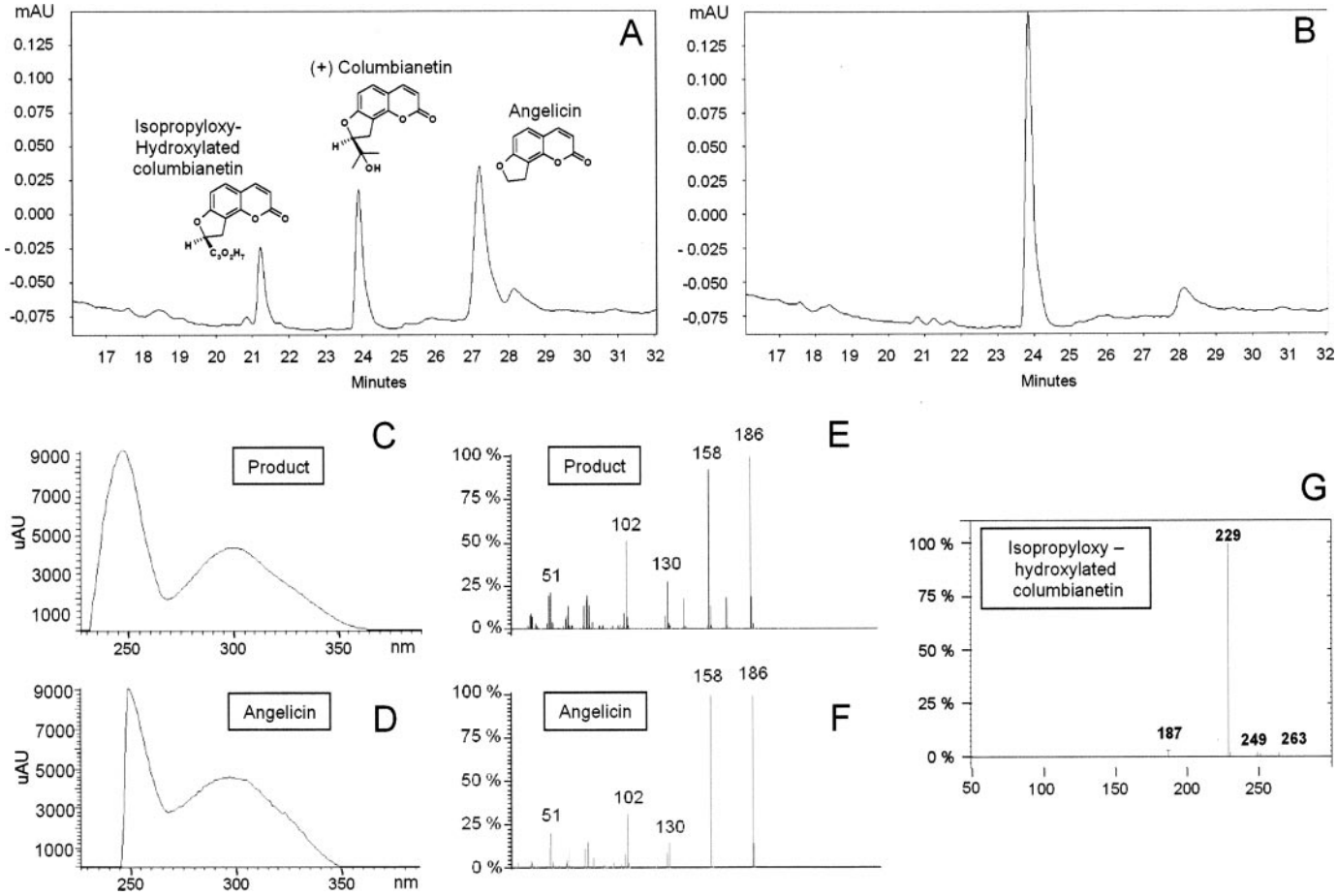

FIGURE 4. **Reaction product profiles obtained from incubations of CYP71AJ4 with (+)-columbianetin.** Incubations were conducted in 0.1 M sodium phosphate buffer, pH 7, with 3 μM (+)-columbianetin, employing microsomes expressing CYP71AJ4, in the presence (*A*) or in the absence (*B*) of 0.5 mM NADPH. The products were extracted and separated by HPLC monitored at 310 nm, which is specific for angelicin. Hydroxycolumbianetin, the minor product, is more readily apparent at 325 nm. The UV spectrum and mass spectrometry fragmentation pattern of the major product eluting at 27.5 min (*C* and *E*) are compared with those of commercial angelicin (*D* and *F*). *G*, mass spectrometry fragmentation pattern of the minor product eluting at 21.3 min showing an [M^+] of *m/z* 263 and major fragments at *m/z* 249, 229, and 187.

TABLE 3

Kinetic parameters and product specificity of CYP71AJ4 with (+)-columbianetin *versus* *syn*-[3′-D]columbianetin as substrate

	Substrates	
	(+)-Columbianetin	*syn*-[3′-D]Columbianetin
Formation of angelicin		
K_m (μM)	2.1 ± 0.4	1.4 ± 0.2
k_{cat} (min^{-1})	112 ± 14	16.1 ± 1.4
k_{cat}/K_m	55.2 ± 6.4	11.3 ± 1.7
Isopropyloxy-hydroxylation		
K_m (μM)	1.4 ± 0.4	2 ± 0.9
k_{cat} (min^{-1})	18.9 ± 5.8	3.4 ± 0.8
k_{cat}/K_m	13.6 ± 0.3	1.8 ± 0.5
Anti-3′-hydroxylation		
K_m (μM)		3.8 ± 1.5
k_{cat} (min^{-1})		234 ± 66
k_{cat}/K_m		58 ± 12

above was accepted. Kinetic assays revealed an apparent K_m of 2.1 ± 0.4 μM and a k_{cat} of 112 ± 14 min^{-1} for the angelicin synthase reaction, which is in line with the values recorded for psoralen synthase (23) and other plant enzymes of the CYP71 family (40, 41). The formation of the hydroxycolumbianetin by-product by angelicin synthase occurred 3–4 times less efficiently than the formation of angelicin, as indicated by the k_{cat}/K_m ratios (Table 3). Angelicin synthase assays were also conducted in the presence of increasing concentrations of (+)-marmesin (30–200 μM). Surprisingly, no inhibition was observed (data not shown). Angelicin synthase assays were also performed in the presence of psoralen, 5- or 8-methoxypsoralen (100 μM), and angelicin (100 μM). All of these compounds failed to inhibit angelicin synthase *in vitro* (data not shown), which is consistent with the proposal that P450s from furanocoumarin-producing plants are resistant to the mechanism-based inhibition by furanocoumarins reported for various other P450s (12, 42, 43).

Mechanistic Considerations—Precursor feeding experiments using leaves of *H. mantegazzianum* had shown that the conversion of (+)-columbianetin to angelicin proceeds by 2′,3′-*syn*-elimination of the isopropyloxy side chain. Although no direct proof was given, this reaction is probably initiated by the abstraction of the respective C-3′-hydrogen (28). The analogous reaction was confirmed *in vitro* for the conversion of (+)-marmesin by psoralen synthase (27). The availability of recombinant angelicin synthase and specifically deuterated (+)-columbianetin offered the opportunity to extend the *in vivo* investigations of angelicin formation by *in vitro* studies. Incubation of *syn*-[3′-D]columbianetin with recombinant angelicin synthase revealed a new product. This compound is synthe-

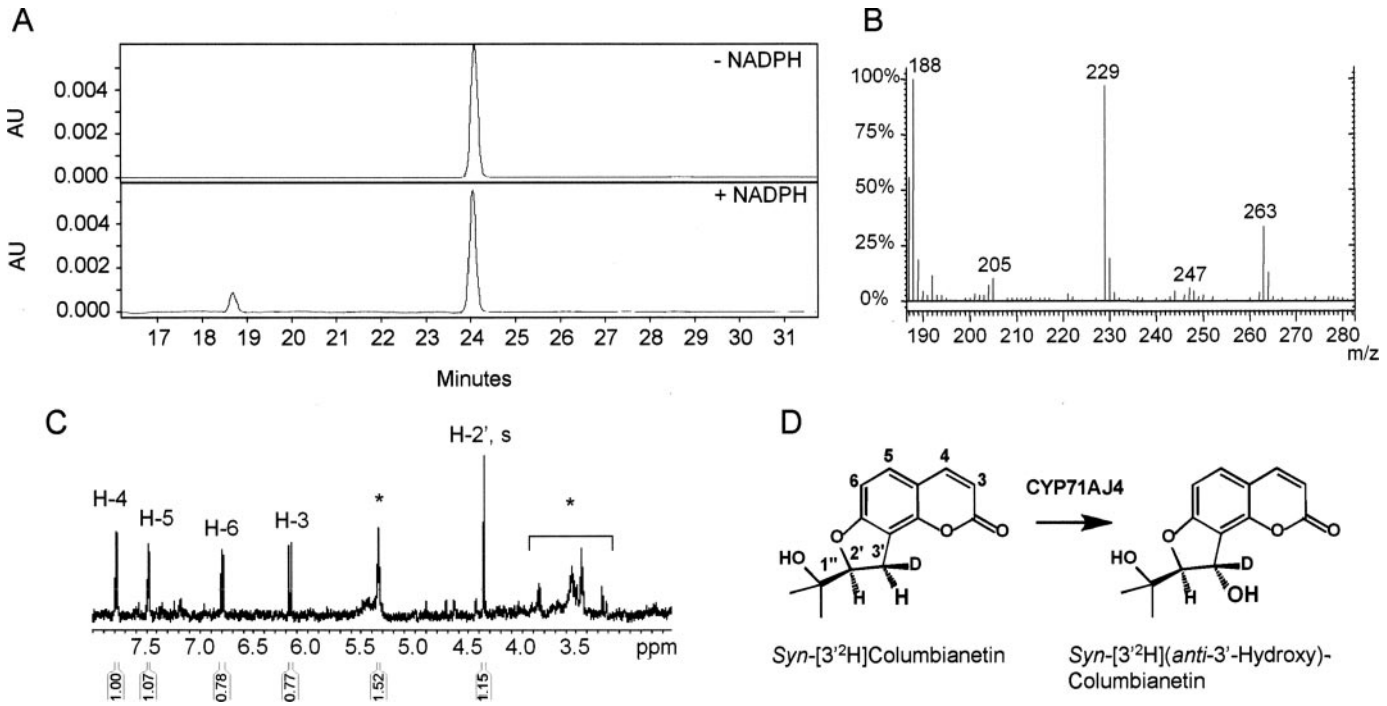

FIGURE 5. **Reaction product profiles obtained from incubations of CYP71AJ4 with *syn*-[3′-D]columbianetin.** *A*, HPLC separation profile of the reaction product generated by CYP71AJ4 with *syn*-[3′-D]columbianetin in the presence (+) or absence (−) of 0.5 mM NADPH. The products were extracted and separated by HPLC monitored at 325 nm, which is specific for hydroxycolumbianetin. Elution of *syn*-[3′-D]columbianetin was observed at 24 min, whereas the major product peak eluting at 18.8 min corresponds to *syn*-[3′-D](anti-3′-hydroxy)-columbianetin. *B*, mass spectrometry fragmentation pattern of the product showing an [M^+] of *m/z* 263 and major fragments at *m/z* 247, 229, and 205. *C*, ^{1}H NMR spectrum of the product. The *asterisks* indicate likely chemical impurities. *D*, regio- and stereospecific hydroxylation of *syn*-[3′-D]columbianetin catalyzed by CYP71AJ4.

sized at the expense of a relatively large decrease in the formation of angelicin and hydroxycolumbianetin by-product, as compared with control assays employing natural (+)-columbianetin as substrate (Fig. 5*A*). HPLC and gas chromatography-mass spectrometry (*n*) analyses unambiguously assigned the new product as another hydroxylated columbianetin derivative retaining the deuterium label (Fig. 5*B*). Roughly 2 μg of the new product was collected from several incubations for NMR analysis. The LC-SPE-^{1}H NMR spectrum displayed two AB spin systems comprising four doublet resonances for H-3 (δ 6.17, $J_{H\text{-}3\text{–}H\text{-}4} = 9.5$ Hz), H-4 (δ 7.78, $J_{H\text{-}4\text{–}H\text{-}3} = 9.5$ Hz), H-5 (δ 7.48, $J_{H\text{-}5\text{–}H\text{-}6} = 8.5$ Hz), and H-6 (δ 6.80, $J_{H\text{-}6\text{–}H\text{-}5} = 8.5$ Hz) (Fig. 5*C*), matching the chemical shifts and coupling constants of corresponding protons in *syn*-[3′-D]columbianetin as well as the two singlets due to two methyl groups at δ 1.22 and 1.21 (not shown). The presence of these proton signals suggests that hydroxylation can only occur at C-2′ or C-3′. Hydroxylation at C-2′ would, in the spectrum of the deuterated substrate, [3′-D]columbianetin, wipe out the doublet for H-2′ at δ 4.77 ($J_{H\text{-}2'\text{–}H\text{-}3'} = 9.8$ Hz), and change the broad doublet at δ 3.28 ($J_{H\text{-}3'\text{–}H\text{-}2'} = 9.8$ Hz) to a singlet. This was apparently not the case. Instead, the signal of H-3′ disappeared from the spectrum of the new product, indicating that the proton had been abstracted. Thus, hydroxylation at C-2′ can be excluded, and the deuterium label at C-3′ must be retained. Moreover, compared with the deuterated substrate (44), which was measured under the same conditions as the product (see "Experimental Procedures"), the signal of H-2′ was shifted upfield to δ 4.36 and collapsed to a singlet, confirming loss of the adjacent proton at C-3′ (Fig. 5*C*). The unresolved signals between δ 3.2 and 3.9 ppm can be ascribed to impurities of the minute product sample. Moreover, hydroxylation at C-2′ is highly improbable in light of the literature (1, 2). Taken together, these results are fully consistent with the new product as *syn*-[3′-D](*anti*-3′-hydroxy)-columbianetin (Fig. 5*D*).

Kinetic studies revealed that the affinities of recombinant angelicin synthase to natural columbianetin or *syn*-[3′-D]columbianetin were almost identical (Table 3). However, the conversion rates k_{cat} to angelicin and its derivative hydroxylated in the isopropyloxy residue observed in assays with natural columbianetin were dramatically affected when the C-3′ deuterium-labeled substrate was used, because the ratio k_{cat}/K_m dropped from 55.2 to 11.3 (Table 3). Furthermore, gas chromatography-mass spectrometry analysis of angelicin isolated from the CYP71AJ4 assays with *syn*-[3′-D]columbianetin revealed the loss of deuterium (data not shown), demonstrating that syn-3′D was the site of attack by the iron-oxo center of angelicin synthase. In contrast, formation of the new product hydroxylated in *anti*-orientation at C-3′ in these assays was considerably favored, as indicated by a k_{cat} of 234 ± 66 min^{-1} (Table 3).

DISCUSSION

Despite the considerable interest in furanocoumarins as therapeutics or plants' herbivore defense factors, the biosynthesis and genetics of these compounds, particularly the angular furanocoumarins, are still poorly understood (45, 46). Evolution of the angular pathway is thought to have developed later from genes of the pathway leading to psoralens and as a consequence of herbivore selection pressures (47); such a later devel-

opment would predict a close relationship between enzymes involved in the two pathways. Following this hypothesis, sequence elements of *CYP71AJ1*, the first coumarin-specific P450 gene isolated recently from *A. majus* and encoding psoralen synthase (23), were used as primers to amplify related genes from plants producing both linear and angular furanocoumarins. Celery (*A. graveolens*) and parsnip (*P. sativa*) fulfill this requirement and belong to the same taxon (Apiaceae), suggesting a common codon usage. Three new members of the CYP71AJ subfamily (CYP71AJ2 to -4) were cloned by this approach and expressed with a relatively low activity in yeast cells. The marginal degree of expression was also encountered with CYP71AJ1 and partially resolved by replacing the N-terminal membrane anchor with that of CYP73A1 (23). This, however, turned out not to be necessary for CYP71AJ2 to -4, since the activity of the transformants was sufficient for kinetic identification. *CYP71AJ2* (celery) and *71AJ3* (parsnip) encode psoralen synthases showing substrate affinities to (+)-marmesin or (+)-5-hydroxymarmesin very similar to those reported for CYP71AJ1 (23). It is noteworthy that in these enzymes, SRS4 and SRS5 were completely conserved, whereas SRS6 showed some sequence divergence.

CYP71AJ4 (parsnip) was shown to encode angelicin synthase, thus representing a P450 dedicated to angular furanocoumarin biosynthesis. The recombinant enzyme exhibited stringent substrate selectivity ($K_m = 2.1 \pm 0.4\ \mu$M) to (+)-columbianetin and yielded a by-product in minor quantity identified as a (+)-columbianetin derivative hydroxylated in the isopropyloxy side chain. *CYP71AJ4* could be cloned from leaves only after wounding to elicit furanocoumarins (data not shown), which is fully compatible with the proposed role of angelicin in plant defense against herbivores. Activity assays conducted with *syn*-[3′-D]columbianetin (Fig. 5) severely affected the kinetics of angelicin formation as well as the product pattern, shifting the reaction toward the formation of *anti*-3′-hydroxy-*syn*-[3′-D]columbianetin. The considerable isotopic effect (*i.e.* the drop in k_{cat}/K_m) (Table 3) clearly indicated that angelicin synthase attacks the substrate by abstracting the *syn*-hydrogen from C-3′ in the context of the 2′,3′-elimination reaction. However, the enzyme is also capable of abstracting the *anti*-hydrogen from C-3′, which is then followed by rebound hydroxylation at C-3′, and this reaction is strongly favored if deuterium replaces the *syn*-3′-hydrogen (Table 3). The data suggest that the initial abstraction and the elimination reaction (angelicin formation) or rebound hydroxylation (formation of *anti*-3′-hydroxycolumbianetin) by angelicin synthase do not occur concomitantly but, rather, sequentially. This might also be the reason for the by-product formation when unlabeled (+)-columbianetin is used as a substrate. Overall, subtle differences become apparent in the catalytic processes catalyzed by angelicin synthase (CYP71AJ4) compared with those catalyzed by psoralen synthase (CYP71AJ1). Psoralen synthase exclusively abstracts the hydrogen isotope *syn* to the C-3 substituent when *syn*-[3′-D]- or *anti*-[3′-D]marmesin is offered as a substrate with a kinetic isotope effect of $k_H/k_D \sim 4$ (27), which is consistent with previous precursor feeding studies (48). Most importantly, psoralen is the only product of the reaction.

Both *CYP71AJ3* (psoralen synthase) and *CYP71AJ4* (angelicin synthase) were isolated from *P. sativa* and constitute the first paralogs *stricto sensu* described for the linear and angular furanocoumarin pathways. The overall sequence identity of 70% suggests that *CYP71AJ3* and *CYP71AJ4* could have arisen from a common ancestor of unknown functionality by a process involving gene duplication followed by mutations. This is consistent with a reduced sequence identity in the SRS elements to 40% that fully matches a divergence of substrate specificity between the two enzymes. The competitive inhibition of *A. majus* psoralen synthase by (+)-columbianetin was demonstrated previously and illustrated by *in silico* docking experiments (23), which supported the idea of psoralen synthase structurally close to angelicin synthase. Alternatively, in this report, (+)-marmesin was not accepted as a substrate or competitive inhibitor by angelicin synthase. Altogether, these differences suggest a mode of action for angelicin synthase that is distinct from that for psoralen synthase, although the reasons underlying the discrepancy remain to be determined.

The expression of *CYP71AJ4*-like genes is necessary but not sufficient for the evolution of the angular furanocoumarin pathway. Indeed, angelicin synthase needs (+)-columbianetin as a substrate and depends on a transferase activity prenylating carbon-8 of umbelliferone (Fig. 1). The complementary characterization of the umbelliferone 8-prenyltransferase will constitute a major step forward to our understanding of the divergence between angular and linear furanocoumarin pathways in higher plants.

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REFERENCES

1. Murray, R. D. H., Mendéz, J., and Brown, S. A. (1982) *The Natural Coumarins*, John Wiley & Sons, Inc., New York
2. Murray, R. D. H. (2002) *Prog. Chem. Org. Nat. Prod.* **83,** 1–619
3. Kawase, M., Sakagami, H., Motohashi, N., Hauer, H., Chatterjee, S. S., Spengler, G., Vigyikanne, A. V., Molnar, A., and Molnar, J. (2005) *In Vivo* **19,** 705–711
4. Pereira, L. E., Villinger, F., Wulff, H., Sankaranarayanan, A., Raman, G., and Ansari, A. A. (2007) *Exp. Biol. Med.* **232,** 1338–1354
5. Bourgaud, F., Allard, N., Guckert, A., and Forlot, P. (1989) *Natural Sources of Fucocoumarins*, pp. 219–230, John Libbey Eurotext, Ltd., Paris
6. Bourgaud, F., Hehn, A., Larbat, R., Doerper, S., Gontier, E., and Kellner, Matern, U. (2006) *Phytochem. Rev.* **5,** 293–308
7. Beier, R. C., and Oertli, E. H. (1983) *Phytochemistry* **22,** 2595–2597
8. Baskin, J. M., Ludlow, C. J., Harris, T. M., and Wolf, F. T. (1967) *Phytochemistry* **6,** 1209–1213
9. Ojala, T., Remes, S., Haansuu, P., Vuorela, H., Hiltunen, R., Haahtela, K., and Vuorela, P. (2000) *J. Ethnopharmacol.* **73,** 299–305
10. Neal, J. J., and Wu, D. (1994) *Pestic. Biochem. Phys.* **50,** 43–50
11. Dall'Acqua, F., Vedaldi, D., and Recher, M. (1978) *Photochem. Photobiol.* **27,** 33–36
12. Letteron, P., Descatoir, D., Larrey, M., Tinel, J., Geneve, J., and Pessayre, D. (1986) *J. Pharmacol. Exp. Ther.* **238,** 685–692
13. Scott, J. G. (1996) *Insect Biochem. Mol. Biol.* **26,** 645–649
14. Zumwalt, J. G., and Neal, J. J. (1993) *Comp. Biochem. Phys.* **106,** 111–118

15. Berenbaum, M. R., and Zangerl, A. R. (1996) *Recent Adv. Phytochem.* **30,** 1–24
16. Ma, R., Cohen, M. B., Berenbaum, M. R., and Schuler, M. A. (1994) *Arch. Biochem. Biophys.* **310,** 332–340
17. Berenbaum, M. R., and Zangerl, A. R. (2006) *Ecology* **87,** 3070–3081
18. Kai, K., Mizutani, M., Kawamura, N., Yamamoto, R., Tamai, M., Yamaguchi, H., Sakata, K., and Shimizu, B. (2008) *Plant J.* **55,** 989–999
19. Endler, A., Martens, S., Wellmann, F., and Matern, U. (2008) *Plant Mol. Biol.* **67,** 335–346
20. Dhillon, D. S., and Brown, S. A. (1976) *Arch. Biochem. Biophys.* **177,** 74–83
21. Hamerski, D., and Matern, U. (1988) *Eur. J. Biochem.* **171,** 369–375
22. Wendorff, H., and Matern, U. (1986) *Eur. J. Biochem.* **161,** 391–398
23. Larbat, R., Kellner, S., Specker, S., Hehn, A., Gontier, E., Hans, J., Bourgaud, F., and Matern, U. (2007) *J. Biol. Chem.* **282,** 542–554
24. Hamerski, D., and Matern, U. (1988) *FEBS Lett.* **239,** 263–265
25. Hehmann, M., Lukacin, R., Ekiert, H., and Matern, U. (2004) *Eur. J. Biochem.* **271,** 932–940
26. Stanjek, V., Piel, J., and Boland, W. (1999) *Phytochemistry* **50,** 1141–1145
27. Stanjek, V., Miksch, M., Lüer, P., Matern, U., and Boland, W. (1999) *Angew. Chemie-Int. Ed.* **38,** 400–402
28. Stanjek, V., and Boland, W. (1998) *Helv. Chim. Acta* **81,** 1596–1607
29. Stemmer, W. P. C., and Morris, S. K. (1992) *BioTechniques* **13,** 214–220
30. Pompon, D., Louerat, B., Bronine, A., and Urban, P. (1996) *Methods Enzymol.* **272,** 51–64
31. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) *Nucleic Acids Res.* **20,** 1425
32. Bradford, M. M. (1976) *Anal. Biochem.* **7,** 248–254
33. Omura, T., and Sato, R. (1964) *J. Biol. Chem.* **239,** 2370–2378
34. Liu, R., Sun, Q., Shi, Y., and Kong, L. (2005) *J. Chromatogr. A* **1076,** 127–132
35. Wei, Y., and Ito, Y. (2006) *J. Chromatogr. A* **1115,** 112–117
36. Ito, Y. (2005) *J. Chromatogr. A* **1065,** 145–168
37. Cappeletti, E. M., Innocenti, G., Caniato, R., Filippini, R., and Piovan, A. (1998) in *Biotechnology in Agriculture and Forestry - 41. Medicinal and Aromatic Plants* (Bajaj, P. S., ed), pp. 238–260, Springer, Heidelberg
38. Zschoke, S., Liu, J. H., Stuppner, H., and Bauer, R. (1998) *Phytochem. Anal.* **9,** 283–290
39. Gotoh, O. (1992) *J. Biol. Chem.* **267,** 83–90
40. Takahashi, S., Zhao, Y., O'Maille, P. E., Greenhagen, B. T., Noel, J. P., Coates, R. M., and Chappell, J. (2005) *J. Biol. Chem.* **280,** 3686–3696
41. Takahashi, S., Yeo, Y. S., Zhao, Y., O'Maille, P. E., Greenhagen, B. T., Noel, J. P., Coates, R. M., and Chappell, J. (2007) *J. Biol. Chem.* **282,** 31744–31754
42. Koenigs, I. L., and Trager, W. F. (1998) *Biochemistry* **37,** 13184–13193
43. Gravot, A., Larbat, R., Hehn, A., Lièvre, K., Gontier, E., Goergen, J.-L., and Bourgaud, F. (2004) *Arch. Biochem. Biophys.* **422,** 71–80
44. Stanjek, V. (1998) *Studien zur Biosynthese der Furanocumarine*, Ph.D. thesis, Universität Bonn, Germany
45. Stern, R. S. (2007) *New Eng. J. Med.* **357,** 682–690
46. Schuler, M. A., and Berenbaum, M. R. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** Suppl. 2, 14593–14598
47. Berenbaum, M., and Feeny, P. (1981) *Science* **212,** 927–929
48. Stanjek, V., Miksch, M., and Boland, W. (1997) *Tetrahedron* **53,** 17699–17710