

Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway

(higher plants/plant defense/cDNA cloning/deduced amino acid sequence/molecular evolution)

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ABSTRACT Cinnamate 4-hydroxylase [CA4H; *trans*-cinnamate,NADPH:oxygen oxidoreductase (4-hydroxylating), EC 1.14.13.11] is a cytochrome P450 that catalyzes the first oxygenation step of the general phenylpropanoid metabolism in higher plants. The compounds formed are essential for lignification and defense against predators and pathogens. We recently reported the purification of this enzyme from Mn²⁺-induced Jerusalem artichoke (*Helianthus tuberosus* L.) tuber tissues. Highly selective polyclonal antibodies raised against the purified protein were used to screen a λ gt11 cDNA expression library from wound-induced Jerusalem artichoke, allowing isolation of a 1130-base-pair insert. Typical P450 domains were identified in this incomplete sequence, which was used as a probe for the isolation of a 1.7-kilobase clone in a λ gt10 library. A full-length open reading frame of 1515 base pairs, encoding a P450 protein of 505 residues ($M_r = 57,927$), was sequenced. The N terminus, essentially composed of hydrophobic residues, matches perfectly the microsequenced N terminus of the purified protein. The calculated pI is 9.78, in agreement with the chromatographic behavior and two-dimensional electrophoretic analysis of CA4H. Synthesis of the corresponding mRNA is induced in wounded plant tissues, in correlation with CA4H enzymatic activity. This P450 protein exhibits the most similarity (28% amino acid identity) with avocado CYP71, but also good similarity with CYP17 and CYP21, or with CYP1 and CYP2 families. According to current criteria, it qualifies as a member of a new P450 family.

Involvement of cytochrome P450 in the oxidative metabolism of endogenous compounds and of xenobiotics in animals is well documented (1). Over 180 P450 genes, chiefly from animals, fungi, and prokaryotes, have been characterized and classified into 27 gene families (2). It was proposed that the wide expansion of some P450 families which occurred in animals, leading to the diversification of xenobiotic-metabolizing enzymes, resulted from plant–animal coevolution, permitting better exploitation of plant sources (3). However, no information is available to assess the effects of animal–plant and microbe–plant warfare on cytochrome P450 evolution in plants.

In recent years, cytochrome P450 has been shown to catalyze at least 10 oxidative steps involved in the biosynthesis of phenylpropanoid derivatives in plants (4). Cinnamate 4-hydroxylase (CA4H), the second enzyme of the common phenylpropanoid pathway, catalyzes the hydroxylation of *trans*-cinnamic acid into *trans*-*p*-coumaric acid and controls the carbon flux to pigments essential for pollination or UV protection, to numerous phytoalexins synthesized by

plants when challenged by pathogens (5), and to lignins. In terms of biomass processing, CA4H is a major P450 in the biosphere because of the enormous amount of lignin accumulated in both living and fossilized plants. CA4H is highly inducible by light (6) and, like many enzymes involved in the synthesis of plant defense compounds, by wounding (7) and infection (8). This enzyme, essential for plants, has not been found in any invertebrate or vertebrate animal.

We recently purified CA4H from wound- and Mn²⁺-induced Jerusalem artichoke tuber tissues (9). In this paper we report the isolation and characterization of a cDNA encoding this enzyme. The N terminus of the deduced protein is a perfect match to the N-terminal sequence of the purified enzyme. CA4H (CYP73) sequence analysis indicates that it is a member of new P450 gene family.

MATERIALS AND METHODS

Plant Materials. Jerusalem artichoke (*Helianthus tuberosus* L. cv. Blanc Commun) tubers grown in open fields were stored in polyethylene bags at 4°C in the dark. To elicit wound-induced increase of CA4H activity, tubers were sliced (1 mm thick), washed with cold tap water for 30 min, and aged 16 hr in the dark in vigorously aerated distilled water.

Protein Microsequencing. CA4H purified as described (9, 10) was submitted to SDS/PAGE (10% total monomer, 0.3% crosslinker) and transferred onto a ProBlott membrane (Applied Biosystems) in 10 mM Caps, pH 11/10% methanol. The Coomassie blue-stained CA4H band was excised for automated Edman degradation (Applied Biosystems 473A protein sequencer).

Antibody Preparation. Polyclonal antibodies were elicited in two rabbits by subcutaneous injection, repeated at 4-week intervals, of 10–50 μ g of purified CA4H (9) in complete and then incomplete Freund's adjuvant. One rabbit was injected with native enzyme (in the presence of nonionic detergents) concentrated by dialysis against dry CM-cellulose (serum CA4H-pa1). Serum from a second rabbit immunized with enzyme repurified by SDS/PAGE produced poor CA4H inhibition and Western immunoblot response. However, a single additional injection of native enzyme into this rabbit produced a highly crossreactive and specific serum (CA4H-pa2).

Abbreviation: CA4H, cinnamate 4-hydroxylase.

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cDNA Libraries and Colony Screening. A λ gt11 cDNA expression library was constructed (11) from poly(A)⁺ RNA extracted as described (12). First-strand cDNA was synthesized by random priming using tridecamers with avian myeloblastosis virus reverse transcriptase. Filter lifts of the library (9×10^4 plaque-forming units) were screened with CA4H-pa1 antibodies (13) by using alkaline phosphatase detection. The positive plaques were purified by three additional rounds of screening, one of them with CA4H-pa2 serum, and amplified. The inserts were excised and subcloned in the M13 mp18 or mp19 vector for sequencing. One clone, CA4H-8, was used to screen, under stringent conditions, 3×10^5 plaques from a second cDNA library constructed in λ gt10 from oligo(dT)-primed poly(A)⁺-enriched RNA (11). One hundred seventy-six positive clones, purified by three further screening rounds, were subjected to hybridization with a probe corresponding to the 5' region of CA4H-8 insert synthesized as follows: CA4H-8 inserted in M13 mp18 in the appropriate orientation was used as a template for a brief extension reaction of the universal primer with T7 DNA polymerase in the presence of a limiting concentration of [α -³²P]dCTP. Hybridization was performed overnight at 65°C and was followed by four washes in 0.3 M NaCl/0.03 M sodium citrate, pH 7/0.1% SDS at room temperature. The largest inserts selected among the positive λ gt10 clones were excised and subcloned in pBluescript II plasmid (Stratagene) for sequencing.

DNA Sequencing. M13 or double-stranded pBluescript subclones were sequenced by the dideoxynucleotide method (14) with T7 DNA polymerase using synthetic oligonucleotide primers progressively deduced from the DNA sequence.

Northern Blot Analysis. Poly(A)⁺ RNA was prepared (12) from Jerusalem artichoke tuber dormant tissues or slices aged 16 hr in distilled water. Formamide- and formaldehyde-denatured RNA was electrophoresed in a 1.2% agarose/formaldehyde gel and transferred by capillarity onto Hybond N (Amersham). After transfer, RNA was fixed by heating 2 hr at 80°C. CA4H-F5 insert labeled with ³²P by random oligonucleotide-primed synthesis was used as a hybridization probe under high-stringency conditions as described for λ gt10 library screening.

Sequence Alignment, Comparisons, and Phylogenetic Trees. Computer analysis of the protein sequence and local and global alignments were made by using the programs of the Genetics Computer Group package (15) on a VAX. The multiple alignments used to construct phylogenetic trees were made with CLUSTAL (16) and the PHYLIP phylogeny package (17).

RESULTS

N-Terminal Sequence of Purified CA4H. Edman degradation was performed on two CA4H samples, the enzyme purified by conventional chromatography (9) and the immunopurified protein (10). A single amino acid sequence was obtained through the first 19 aa (Table 1). The sequence is essentially hydrophobic but is interrupted by 2 aa of opposite charge (Glu and Lys) in positions 7 and 8.

Isolation of cDNA Clones. In dormant Jerusalem artichoke tubers CA4H activity is practically undetectable. Slicing and aging tuber tissues in aerated water leads to an increase in enzymatic activity, reaching 1–2 nmol/min per mg of microsomal protein after 16 hr (7). This increase is paralleled by an increase in immunodetectable CA4H. Two cDNA libraries, one in λ gt11 and one in λ gt10, were therefore prepared from mRNA of aged tuber tissue. Screening of the λ gt11 expression library led to the isolation of CA4H-8, containing a 1130-bp open reading frame (Fig. 1) and revealing the presence of P450 consensus domains A–C (19). However, domain D, containing the heme-binding cysteine, and the N

Table 1. N-terminal sequencing of purified CA4H

Cycle	CA4H purified as in ref. 1		Immunopurified CA4H	
	Residue	pmol	Residue	pmol
1	Met	7.57	Met	39.02
2	Asp	3.20	Asp	16.08
3	Leu	11.00	Leu	42.73
4	Leu	11.16	Leu	35.30
5	Leu	14.48	Leu	41.34
6	Ile	9.37	Ile	27.59
7	Glu	5.49	Glu	18.97
8	Xaa	—	Lys	19.55
9	Xaa	—	Thr	15.97
10	Leu	5.51	Leu	34.39
11	Val	2.03	Val	21.72
12	Ala	5.10	Ala	27.73
13	Leu	6.25	Leu	28.02
14	Phe	4.06	Phe	23.13
15	Ala	10.14	Ala	25.92
16	Ala	10.64	Ala	30.94
17	Ile	3.95	Ile	25.96
18	Xaa	—	Ile	25.77
19	Gly	6.47	Gly	20.77
20	—	—	Ala	29.68
21	—	—	Ile	26.17

terminus were missing. CA4H-8 thus appeared to encode the central part of a P450 sequence.

Use of CA4H-8, amplified by polymerase chain reaction, as a probe to screen the λ gt10 library permitted isolation of a complete coding sequence, CA4H-F5. It contains an open reading frame of 1515 bp encoding a sequence of 505 aa, from which a protein of 57,927 Da is predicted. This agrees with the molecular mass of 57 kDa estimated for purified CA4H by SDS/PAGE (9). The N-terminal residues of the deduced polypeptide are identical to the 21 aa determined by Edman degradation of purified CA4H. The pI of this polypeptide is 9.78, in good agreement with chromatographic and electric focusing behavior of the enzyme (9). It contains a single potential glycosylation site, Asn⁵⁷.

Sequence comparison of CA4H-8 and CA4H-F5 (Fig. 1) shows that CA4H-8 overlaps the central part of CA4H-F5, with 17 mismatches (1.75%) occurring exclusively at the third position of codons, and thus is encoding the same protein. This heterogeneity may result from the existence of multiple genes coding for CA4H or of allelic variants ($n = 6$ for *H. tuberosus*). Hybridization of CA4H-F5 to multiple fragments on a Southern blot did not permit us to choose between these hypotheses.

Comparison with Other P450 Sequences. The CA4H protein sequence was compared with the other available P450 sequences (Swiss-Prot Release 21) by using both global and local comparison methods. Global comparisons made by pairwise alignment of the complete sequences showed that CA4H resembles most CYP71 from avocado (28.7% identity), CYP17 from chicken (27.7% identity), and human CYP2D6 (28.4% identity). Least identity is shown to CYP6 from housefly (18%), bovine CYP11A (17.9%), and CYP51 from yeast (17.2%). CA4H thus shares <40% positional identity with any characterized member of the cytochrome P450 superfamily and therefore qualifies as the first member of a new family (2). It has been termed CYP73 by the cytochrome P450 Nomenclature Committee.

Although global sequence identity of CA4H with other P450 proteins never exceeds 30%, much higher homology over short sequence segments can be detected by using local alignment methods. Fig. 2 shows a comparison of CA4H with CYP71 from avocado, CYP101 from *Pseudomonas putida*, and six animal P450 sequences from different families that

Table with 3 columns: CA4H.SEQ, CA4H.PEP, and position numbers. It shows the nucleotide and deduced amino acid sequences for CA4H cDNAs, with CA4H-8 sequences in bold. Conserved residues are marked with vertical bars (|), and differences from CA4H-F5 are marked with asterisks (*). Substrate-binding regions are indicated by << and >> symbols.

Fig. 1. Nucleotide (CA4H-F5) and deduced amino acid (CA4H.PEP) sequences of CA4H cDNAs. The beginning and end of the CA4H-8 sequence are marked (<< and >>). Only positions where CA4H-8 differs from CA4H-F5 are indicated.

show remarkable local similarity to CYP73. There is 85% identity in a 20-aa overlap from Lys³¹ to Gly⁵⁰ with the corresponding sequence in rat P450Md (CYP2C22), and 75% identity from Phe¹⁹⁰ to Asp¹⁹⁹ of CA4H to rat CYP2D5. The

region Lys³¹ to Gly⁵⁰ contains the proline-rich sequence highly conserved in P450 families I and II, which is supposed to orient the heme plane perpendicular to the cytosolic surface of the ER membrane. Two regions, spanning from

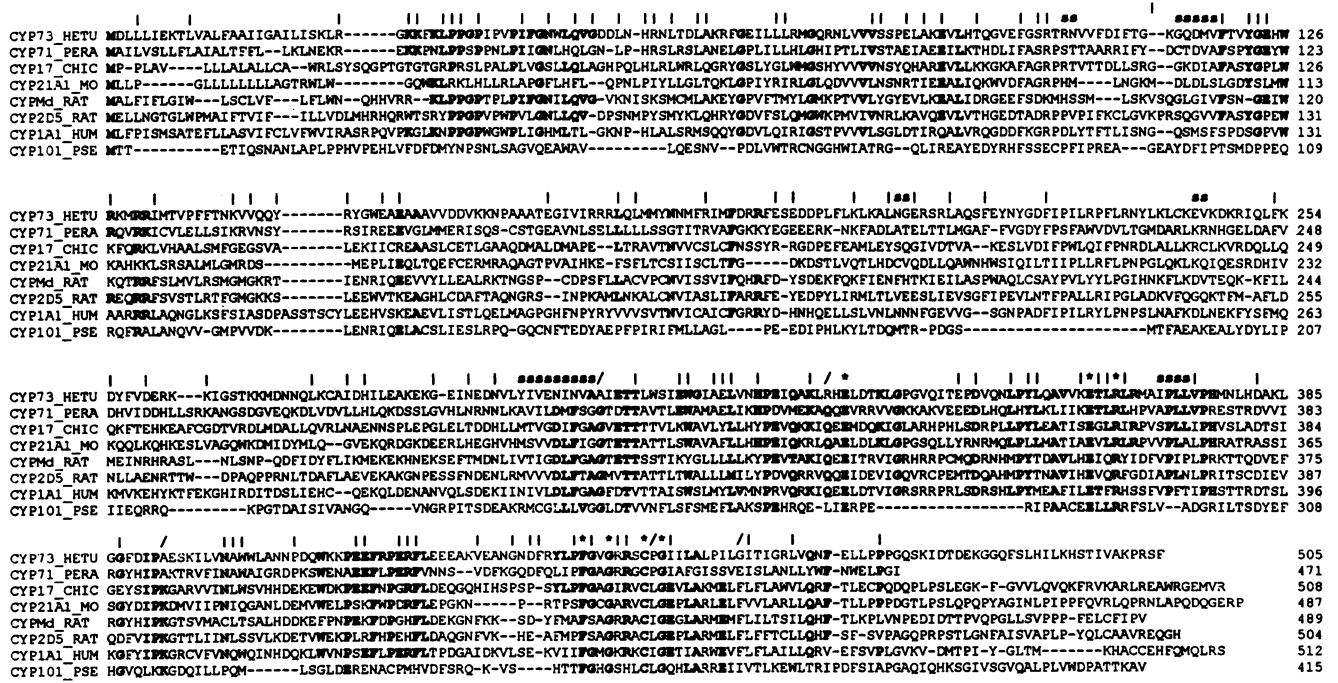


Fig. 2. Alignment of Jerusalem artichoke CYP73 with other known P450 sequences selected from various families: CYP71 from *Persea americana* (N-methyl-p-chloroaniline demethylase, Swiss-Prot P24465), chicken CYP17 (steroid 17-hydroxylase, Swiss-Prot P12394), mouse CYP21A1 (steroid 21-hydroxylase, Swiss-Prot P03940), rat P450Md (= P450C22, male dominant, Swiss-Prot P19225), rat CYP2D5 (Swiss-Prot P12939), human CYP1A1 (aryl hydrocarbon hydroxylase, Swiss-Prot P04798), bacterial CYP101 (camphor-metabolizing, Swiss-Prot P00194). Stars, residues conserved in all P450 sequences; bold lettering, residues conserved in most sequences; |, residues shared between CYP73 and CYP71; /, residues from CYP73 which differ from the consensus; ss (sss, etc.), substrate-binding regions according to ref. 27.

Pro³⁵⁵ to Pro³⁷⁵ and from Trp⁴¹⁰ to Leu⁴²², show considerable (50–75%) conservation with the corresponding regions in chicken CYP17 and CYP71 from avocado. The first region contains the “steroid-binding” consensus domain defined by Picado-Leonard and Miller (20) from Leu³⁵⁴ to Ala³⁷⁰. CA4H differs from this 17-aa consensus at only three positions. The second region is located in the C consensus domain defined by Kalb and Loper (19) and contains highly conserved residues at Trp⁴¹⁰, Pro⁴¹³, Phe⁴¹⁶, Pro⁴¹⁸, and Phe⁴²¹.

Most of the residues conserved in all P450s are present in CYP73. Thr³¹⁰ in particular can be assumed to correspond to Thr²⁵² of CYP101, which has an essential function in the stabilization of the dioxygen-binding groove in helix I and in coupling of the reaction (21). The position just upstream of this Thr³¹⁰ is occupied by a highly conserved acidic residue possibly involved in the electron transfer toward dioxygen. Three residues upstream, all P450 sequences so far determined display a glycine, thought to be located in the dioxygen binding site, that in CYP73 is replaced by Ala³⁰⁷. The heme-binding domain, centered around Cys⁴⁴⁷, shows the remarkable presence of Pro⁴⁴⁸ instead of the usual leucine, isoleucine, valine, alanine, or phenylalanine. Proline is found at this position in only two other P450s: CYP71 from avocado and CYP7. Four lysine residues are believed to be involved in the interaction between microsomal cytochromes P450 and NADPH-cytochrome P450 reductase (22). The corresponding residues, deduced from our alignments, are Lys⁸⁸, Lys²⁵⁴, Lys²⁶², and Lys³⁹⁵. We used the algorithm of Janin *et al.* (23) to confirm that these residues are likely to be exposed at the surface of the protein. Other residues that might be implicated in electron transfer are Trp¹²⁶ and Arg¹³¹ (24). Based on analogy with CYP101, Arg¹³⁰ may interact with one of the heme propionate groups (25).

Extensive P450 sequence comparison (24, 26) have outlined other individual residues that are invariant across all eukaryotic and prokaryotic P450 proteins, likely due to conserved functions. Corresponding positions in CA4H are Glu³³⁵, Glu³⁶³, Arg³⁶⁶, Phe⁴⁴⁰, Gly⁴⁴³, Cys⁴⁴⁷, and Gly⁴⁴⁹.

Phylogenetic Studies. We analyzed the phylogenetic relatedness of CA4H with 120 P450 sequences available in the Swiss-Prot databank (release 21). A subset of 23 sequences representative of the different P450 families was chosen and aligned with increased gap-weight and gap-length penalty to minimize gaps. The alignment was further refined manually by using the alignments of Nelson and Strobel (24) and Gotoh (27) as guides and analyzed by a neighbor-joining method (28) (Fig. 3).

The branching pattern of this tree confirms the hypothesis of Nelson and Strobel (30) that the evolution of a cholesterol side-chain-cleaving P450 (P450_{sc}) preceded the plant-animal divergence. At least two gene duplications occurred between the evolution of P450_{sc} and of CA4H. It is interesting that P450_{sc} bears very little sequence identity with CA4H. The divergence of CA4H from the avocado CYP71 apparently occurred later than the divergence of the *Catharantus roseus* CYP72 from the group of families III, IV, and VI and after the gene duplication that gave rise to the drug-metabolizing P450s, about 900 million years ago. However, it preceded the major diversification of mammalian P450s, some 400 million years ago.

Induction of CA4H Activity and mRNA Accumulation. To correlate wound-induced CA4H activity with expression of CA4H-F5, poly(A)⁺ mRNA was extracted from dormant or wound-induced Jerusalem artichoke tuber tissues for Northern analysis using CA4H-F5 as a probe (Fig. 4). CA4H mRNA was practically undetectable in dormant tissues. Accumulation of a single RNA size class, of about 1700 bases, was promoted by wounding. The size of this mRNA agrees with the 1515-bp size of CA4H-F5 when noncoding sequences and a poly(A) tail are included.

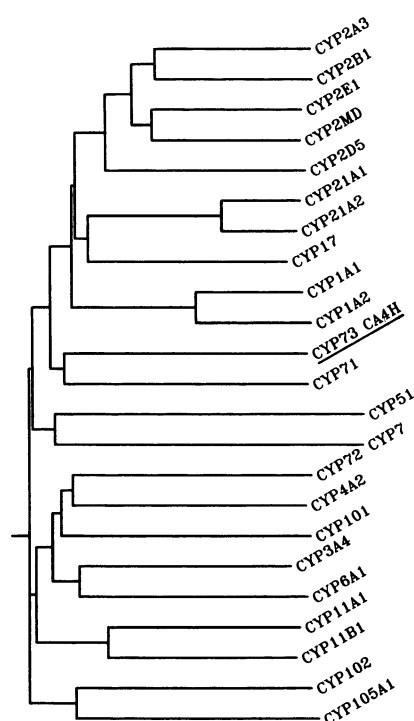


FIG. 3. Phylogenetic tree of 23 cytochrome P450 proteins, calculated by the neighbor-joining method. The number of plant P450 sequences so far available is insufficient to allow a proper calibration of phylogenetic distances. The tree is therefore shown without a time scale. The difference in length of some branches is due to a shortcoming of the method we used, which treats all residues and positions identically, not taking into account the different rates of substitution in different positions of different codons. Swiss-Prot accession numbers: CYP2A3, mammalian, P20812; CYP2B1, mammalian, P00176; CYP2E1, mammalian, P05181; CYP2MD, mammalian, P19225; CYP2D5, mammalian, P12939; CYP21A1, mammalian, P03940; CYP21A2, mammalian, P04033; CYP17, chicken, P12394; CYP1A1, mammalian, P04798; CYP1A2, mammalian, P05177; CYP73 (CA4H), plant; CYP71, plant, P24465; CYP51, fungal, P10614; CYP7, mammalian, P18125; CYP72, plant (29); CYP4A2, mammalian, P20816; CYP101, bacterial, P00183; CYP3A4, mammalian, P08684; CYP6A1, insect, P13527; CYP11A1, mammalian, P05108; CYP11B1, mammalian, P15538; CYP102, bacterial, P14779; CYP105A1, bacterial, P18326.

DISCUSSION

A complete cDNA sequence encoding a P450 protein (CYP73) has been isolated by using antibodies that produce a specific inhibition of CA4H activity (9). That the CYP73 cDNA encodes CA4H is demonstrated by the exact match of the deduced amino acid sequence with the microsequenced N terminus of the purified enzyme. It was recently further confirmed by expressing CA4H-F5 in yeast, resulting in very high turnover number for conversion of cinnamate to coumarate (H.G.T., D.W.-R., M. Kazmaier, C. Mignotte-Vieux, and D. Pompon, unpublished data).

CYP73 provides a sequence of a P450 involved in a plant defense mechanism—i.e., in plant-animal and plant-microorganism warfare. Alignment of its sequence with representative members of the cytochrome P450 superfamily is therefore particularly interesting. Its overall positional identity with other plant, fungal, bacterial, insect, or vertebrate P450 sequences does not exceed 28%. From phylogenetic studies and pairwise alignments it appears that the mammalian steroid-metabolizing CYP17 and CYP21 are the non-plant P450s most closely related to CA4H, followed by members of the CYP1 and CYP2 families. CYP73 shows much less relatedness with CYP3, CYP4, CYP6, and bacte-

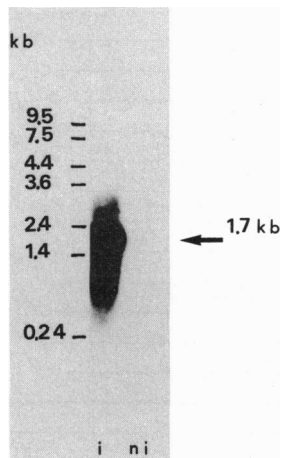


FIG. 4. Northern blot analysis of poly(A)⁺ mRNA isolated from Jerusalem artichoke tuber that was dormant (ni, noninduced) or sliced and aged 16 hr in aerated water (i, induced). CA4H-F5 was used as probe.

rial or fungal P450 sequences. In this respect, the presence in CA4H of a sequence closely matching the steroid-binding consensus sequence (20) is interesting, in view of the well-established binding of phenylpropanoid derivatives on steroid receptors. This similarity may, however, be coincidental since this segment corresponds to one of the most conserved domains in all P450 sequences.

Highest similarity is observed with the ripening-induced CYP71 from avocado. Capacity of the CYP71 protein to catalyze the N-demethylation of N-methyl-p-chloroaniline has been reported (18), but its physiological function is unknown. Since ripe avocado fruit contains high amounts of phenolic compounds, Bozac *et al.* (31) proposed hydroxylation of cinnamic acid as one of the possible physiological functions of CYP71. The failure of yeast cells transfected with CYP71 to catalyze cinnamate hydroxylation (32) cast doubt on this hypothesis, which is now definitely invalidated by the finding that CA4H is a distinct P450. Use of CA4H-pal antibodies has recently shown that CYP73 is expressed in ripening avocado fruit and shows structural relatedness with the orthologous enzyme from Jerusalem artichoke, as evidenced by inhibition of activity, immunoblot analysis of avocado microsomes, and immunopurification of this protein (D.W.-R., unpublished data). In this context, it was interesting to compare CYP73 and CYP71 sequences. Fig. 2 shows the positional identities between these P450s and the location of the putative substrate-recognition sites (SRS) deduced from the alignment of Gotoh (27). It is striking that no, or very little, identity is found at SRS-1 (positions 103–112), SRS-2 (210–211), SRS-3 (244–245), or SRS-4 (298–306), but there is high identity at SRS-5 (371–375). The presence of an unusual proline residue next to the heme-binding cysteine in both CYP71 and CYP73 suggests that they share a common ancestor. From the phylogenetic tree in Fig. 3, it appears that the gene duplication resulting in the separation of CYP71 and CYP73 families is not a recent evolutionary process and has preceded the gene explosion in the CYP2 family. The divergence between the CYP71 and CYP73 genes is, however, impossible to date, due to the paucity of P450 structures available outside the class Mammalia.

Accumulation of CYP73 transcripts after wounding of Jerusalem artichoke tubers correlates with induction of CA4H activity. The cloned cDNA is thus a useful tool to investigate the regulation of the enzyme in response to environmental stress or during plant development. Like many of the enzymes involved in the phenylpropanoid path-

way, CA4H is induced by wounding (7), light (6), phytopathogens (8), and elicitor treatment (33). Like many P450 species it is also induced following exposure to various xenobiotics (34, 35). Whether these various signals trigger a unique or several induction mechanisms is an interesting question. Analysis of the gene(s) coding for CA4H should help to answer this question.

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