Biochemical and Biophysical Research Communications **261,** 156–162 (1999)
Article ID bbrc.1999.0999, available online at http://www.idealibrary.com on IDEAL®

Cloning and Functional Characterization of CYP94A2, a Medium Chain Fatty Acid Hydroxylase from *Vicia sativa*

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Received May 28, 1999

A full length cDNA encoding a new cytochrome P450-dependent fatty acid hydroxylase (CYP94A2) was isolated from a *Vicia sativa* library. CYP94A2 displays 58% sequence identity with CYP94A1, a fatty acid ω-hydroxylase isolated from the same material. Heterologous expression of CYP94A2 in *Saccharomyces cerevisiae* yeast strain WAT11 shows that it catalyses the hydroxylation of myristic (C14) acid with a $K_{m(app)}$ of 4.0 μM and a turnover rate number of 80 min^{-1}. In addition, lauric (C12) and palmitic (C16) acids were hydroxylated at a ten-fold lower rate, while C18 fatty acids were not oxidized. Remarkably, the regiospecificity of hydroxylation is different for the C12, C14, and C16 fatty acids and appears to be correlated with the length of the carbon chain. Northern blot analysis showed a low level of constitutive expression of *CYP94A2* in *V. sativa* seedlings. In contrast to *CYP94A1*, transcript accumulation of *CYP94A2* was only weakly enhanced in seedlings treated with clofibrate or methyl jasmonate, indicating that both substrate range and gene regulation of the two fatty acid hydroxylases are different.

Besides the major fatty acids (FAs) from C16 and C18 families found in plants, there is a large number of quantitatively minor FAs (1), which often are oxygenated, and whose functions remain largely unknown. Among the oxygenated FAs, ω-hydroxy FAs are major constituents of cutin, the biopolymeric layer which forms the outermost protective barrier of aerial plant organs (2). Furthermore, short- and mid-chain length ω-hydroxy FAs appear to be involved in suberisation in elicitor-treated French bean cells (3). Besides this role as structural constituents of the cuticle and suberin, mono- or poly-hydroxylated FAs have been described as elicitors of defense reactions in pathogen-challenged plants (4, 5), indicating that hydroxy FAs may play an important role in plant defense mechanisms. In view of the remarkable similarity between the eicosanoid signalling pathway found in animals and the octadecanoid signalling pathway in plants (6), it is expected that hydroxylated FAs are part of this array of signalling molecules. The enzymology of FA oxygenation is complex and only partly resolved at the molecular level. In plants, in-chain hydroxy groups may be introduced either by enzymes of the lipoxygenase pathway (7), or by the desaturase-like diiron 12-hydroxylase recently isolated (8), or by cytochrome P450-dependent hydroxylases (9, 10). In contrast, only cytochrome P450 enzymes have been demonstrated to catalyze hydroxylation at the end of the aliphatic chain, i.e. at the ω-, (ω-1)- or (ω-2)-positions of saturated and unsaturated FAs of various chain length.

The cytochrome P450-dependent hydroxylation of FAs has been amply documented at the biochemical level. The studies by Kolattukudy (2) offered the first evidence that the formation of C16 and C18 hydroxy FAs which constitute the main cutin monomers was catalyzed by cytochrome P450 enzymes. More detailed studies from our laboratory have demonstrated that at least three distinct P450 systems were present in microsomes from higher plants, which were able to hydroxylate free FAs either in the chain (9, 10) or at the (ω-1)- or the ω-position (11). These enzymes proved also to be active epoxygenases when presented with unsaturated substrates and were selectively induced by chemicals like phenobarbital and clofibrate which also induce FA hydroxylases in animal and plant tissues (11). Taken together with results from inhibition studies using selective and tailored mechanism-based inhibitors, these findings suggested that hydroxylation of FAs was performed by an array of P450 isoforms with defined regioselectivity of oxidation and chain-length preference (11, 12). Recently, we have cloned the two first plant P450-dependent FA ω-hydroxylases, CYP86A1 from *Arabidopsis thaliana* which ω-hydroxylates mainly

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Abbreviations: *CYP94A1* and *CYP94A2*, the genes coding for the hydroxylases; CYP94A1 and CYP94A2, the hydroxylase proteins; PCR, polymerase chain reaction; FA(s), fatty acid(s).

0006-291X/99 $30.00

palmitic acid (13), and CYP94A1 from *V. sativa* which is able to ω-hydroxylate FAs from 10 to 18 carbon atoms (14).

In view of the physiological importance of oxygenated FAs and of the diversity of their roles, and based also on our biochemical studies suggesting the involvement of distinct isoforms, it was expected that hydroxylation of FAs was catalyzed by several fine regulated, and probably tissue-specific, enzymes. Here, we report the cloning and characterization of *CYP94A2*, a cDNA coding for a second P450-dependent FA hydroxylase from *Vicia sativa*. Using heterologous expression in an *ad hoc* yeast expression system, we demonstrate that this hydroxylase has a different substrate specificity and a different regioselectivity of oxidative attack than CYP94A1. Furthermore, induction studies show that the regulation of CYP94A2 is clearly distinct and independent from that of CYP94A1.

MATERIALS AND METHODS

Chemicals. Radiolabeled [1-^{14}C]capric acid (12.2 Ci/mol) was from Sigma Chimie (La Verpillière, France). [1-^{14}C]lauric acid (45 Ci/mol) was from CEA (Gif sur Yvette, France). [1-^{14}C]myristic acid (55 Ci/mol), [1-^{14}C]palmitic acid (54 Ci/mol), [1-^{14}C]stearic acid (57 Ci/mol), [1-^{14}C]oleic acid (50 Ci/mol), [1-^{14}C]linoleic acid (58 Ci/mol), [1-^{14}C]linolenic acid (52 Ci/mol) were from NEN-Dupont (England). Dichlorodi[U-^{14}C]phenyltrichloroethane (DDT; 104 Ci/mol), [4-^{14}C]testosterone (54.5 Ci/mol) and [1-^{14}C]hexadecane (52 Ci/mol) were from Amersham (Les Ulis, France). Methyl jasmonate was from Sigma-Aldrich. Clofibrate and NADPH were purchased from Sigma Chimie (La Verpillière, France). N-methyl-N(trimethyl)-trifluoro-acetamide (MSTFA) was from Pierce. Thin-layer plates (Silica gel G60F254) were from Merck (Darmstadt, Germany).

Screening of the cDNA library. The λ-ZAP cDNA library, prepared from poly(A^+) RNAs from 48 h-clofibrate-treated *Vicia sativa* seedlings (14), was screened using different probes (see Results) following the manufacturer's instructions (Stratagene). Positive plaques were screened under the same conditions, and pBluescript phagemids were rescued from the λ-ZAP vector as described by the Stratagene protocols. Sequencing reactions of cDNA clones of interest were performed with a dye terminator cycle sequencing kit (Applied Biosystems Inc.) and standard T3 and T7 primers in conjunction with sequence-specific primers. Reactions were resolved on an automated ABI 377 instrument (Applied Biosystems Inc.).

5′-RACE. RNA was isolated from clofibrate-treated *Vicia sativa* seedlings *via* the procedure described by Lesot *et al.* (15) and mRNA purified on an oligo(dT) column (16). First-strand cDNA was synthesized by use of SuperScript II reverse transcriptase from 500 ng of poly(A^+) mRNA as specified by the manufacturer (Gibco Life Technologies). 5′ Rapid amplification of cDNA ends (17) was performed with the 5′ RACE System for Rapid Amplification of cDNA Ends kit (Gibco Life Technologies 18374-025) according to the manufacturer's instructions, with the specific primer 5′-atttccggagggatctagt-3′. After purification and tailing, an aliquot of the dC-tailed cDNA was amplified by PCR using 30 thermal cycles (1 min 93°C, 2 min 52°C, 3 min 72°C) with the anchor primer supplied by BRL and the specific primer (5′-gggatctagtgttgaattcg-3′). A 517 bp amplification product was cloned using SpeI and EcoRI restriction sites in the pBluescript vector and sequenced using vector specific primers.

Heterologous expression of CYP94A2 in yeast. For functional expression of the full-length *CYP94A2* clone, we used a yeast expression system specifically developed for the expression of P450 enzymes (18) consisting of plasmid pYeDP60 and *Saccharomyces cerevisiae* strains W(R) and WAT11. Reformatting and cloning of *CYP94A2* into pYeDP60 was performed by PCR amplification using two specific primers to delete its 5′- and 3′-non coding regions and to introduce a SmaI restriction site upstream of the initiation codon and a SacI site immediately downstream of the stop codon. Forward primer: 5′-ggat**cccggg**gaATGGAACTCGAAACATTG-3′, reverse primer: 5′-atccgctc**gagctc**TCATACAAGTGGGCTTC-3′.

The coding region was amplified using 25 thermal cycles (1 min 93°C, 2 min 56°C, 3 min 72°C) with HiFi polymerase (Boehringer) and directly inserted in the pYeDP60 vector using the SmaI and SacI restriction sites. The construction was sequenced as described previously. Sequence data were aligned and evaluated using ClustalX (19) and Genedoc (http://www.cris.com/~ketchup/genedoc.html). The P450 Nomenclature Committee (http://drnelson.utmem.edu/nelsonhomepage.html) assigned the new gene as the second member of the 94A subfamily.

Yeast transformation was performed as described by Schiestl and Gietz (20). Yeast cultures were grown and induced according to Pompon *et al.*, (21) from one isolated transformed colony. After growth, cells were collected and microsomes were prepared by differential centrifugation. Microsomal membranes were resuspended in 50 mM Tris-HCl, pH 7.4, EDTA 1 mM, 30% glycerol (v/v), and stored at −30°C. Microsomal proteins were quantified by a microassay procedure from BioRad using bovine serum albumin as a standard. Cytochrome P450 was measured by the method of Omura and Sato (22).

Enzyme activity. FA hydroxylase activities were measured at 27°C in the presence of 1 mM NADPH plus a regenerating system consisting of a final concentration of 6.7 mM glucose-6-phosphate and 0.4 U of glucose-6-phosphate dehydrogenase as previously described (23). The standard assay in a final volume of 0.2 ml contained 0.5 mg of microsomal protein from transformed yeast, 20 mM sodium phosphate buffer, pH 7.4, and 100 μM radiolabeled substrate. Reactions were initiated with NADPH and stopped with 0.1 ml acetonitrile/acetic acid (99.8/0.2, v/v), mixed and directly spotted onto silica plates. Kinetic parameters were determined using 60 μg of microsomal protein corresponding to 0.66 pmol of P450 and seven data prints ranging from 0.1 to 100 μM radiolabeled substrate.

Chromatographic analysis. Metabolites were resolved by reverse phase-HPLC using a mixture of acetonitrile/water/acetic acid (25/75/0.2, v/v/v) for C12, (37/63/0.2) for C14 and (47/53/0.2) for C16 as described previously in (24, 25), or by silica thin-layer chromatography with a mixture of diethylether/light petroleum (bp 40-60°C)/formic acid (70/30/1, v/v/v) for C10 to C16 FAs and a 50/50/1 mixture of the same solvents for long-chain FAs. The plates were scanned with a thin layer scanner (LB 2723, Berthold Analytical; EG & G Wallac, Gaithersburg, MD). The areas corresponding to polar metabolites generated from each FA were scraped directly into counting vials and quantified by liquid scintillation.

Gas chromatography-mass spectrometry analysis. Reactions were stopped with acetonitrile/acetic acid (99.8/0.2), incubation media were centrifuged and supernatants were loaded on sep-pak C18 reverse phase cartridges (Waters, division of Millipore). Cartridges were washed with acetonitrile/water/acetic acid (10/90/0.2, v/v/v). The retained compounds were eluted with acetonitrile/acetic acid (99.8/0.2), evaporated to dryness and silylated with MSTFA overnight at room temperature, before gas chromatography and electron impact (70 eV) ionization mass spectrometry. The analysis was performed on a DB-1 capillary column (30 m, 0.25 μm) programmed to rise from 120 to 280°C at 4°C/min, coupled to LKB 9000 S mass spectrometer with a LKB 2130 computer on line.

Northern blot analysis. Northern blot analysis were performed as previously described by Tijet *et al.* (14).

RESULTS

Isolation of CYP94A2. A first FA ω-hydroxylase, *CYP94A1*, had been isolated from a cDNA library from clofibrate-treated *V. sativa* seedlings using a gene specific probe derived from the amino acid sequence (14). Using the same probe at lower stringency (hybridization at 55°C; washing at 45°C in 0.2 × SSC, 0.1% SDS), three additional clones were isolated. Upon sequencing, a 1423 bp clone, clone 811, was found to encode a new cytochrome P450, missing about 100–200 bp at the 5′ end. The protein encoded by clone 811 appeared 60% identical to CYP94A1, suggesting that this new P450 also belonged to the CYP94 family and could therefore display FA hydroxylase activity.

A high stringency screen of circa 4.10^6 pfu from the *V. sativa* clofibrate-treated seedling cDNA library with clone 811 led to the isolation of a longer (1602 bp) but still incomplete cDNA (clone H22). The 5′ missing sequence was obtained via 5′-RACE using an EcoRI restriction site in clone 811 (Fig. 1). The full length cDNA (GenBank accession number AF092917) is 1681 bp long with 69 bp 5′- and 70 bp 3′-untranslated regions. It encodes a protein of 513 amino acids with a calculated mass of 58,406 Da and a basic isoelectric point of 8.49. The protein shares 58% identity and 76% similarity with CYP94A1. It was therefore assigned as CYP94A2.

Substrate specificity of CYP94A2. To determine the catalytic activity of CYP94A2, the corresponding cDNA, inserted into the galactose-inducible multicopy expression vector pYeDP60, was expressed in W(R) and WAT11 yeast strains (21). The typical P450 CO-difference spectrum (22) was readily measurable in microsomes from transformed WAT11 yeast, but not in microsomes prepared from W(R) or from WAT11 cells transformed with the void pYeDP60 (Fig. 2). Microsomes from WAT11 transformed with *CYP94A2* were used to investigate the hydroxylation of five saturated (C10 to C18) and three unsaturated (C18:1, C18:2 and C18:3) FAs (Table I). Myristic acid was hydroxylated most efficiently, whereas oxidation of laurate and palmitate was 8- to 10-fold lower respectively. Metabolism, if any, of capric, stearic, oleic, linoleic and linolenic acids was below the limits of detection by our techniques (0.1 mol/min/mol of P450). Myristic acid, which is by far the best substrate for this hydroxylase, was oxidized following classical Michaelis-Menten kinetics, with a $K_{m(app)}$ of 4.0 μM and a V_{max} of 80 mol/min/mol P450.

A related compound such as hexadecane was not metabolized by CYP94A2 (result not shown) indicating that the carboxyl group of FAs is essential for binding of substrate to the enzyme. Although other P450s with known physiological functions are able to metabolize foreign compounds (26), DDT, testosterone, 7-ethoxycoumarin and 7-ethoxyresorufin were not metabolized by CYP94A2 (results not shown).

Regiospecificity of hydroxylation. As shown in Fig. 3, CYP94A2 catalyzed the formation of two products from each of the three FA substrates. The metabolites were identified as monohydroxy FA and the position of hydroxylation was determined by GC/MS analysis. Laurate and myristate were hydroxylated on the terminal (ω) and sub-terminal carbon (ω-1) in respective ratio of 95/5 for C12 and 25/75 for C14. The metabolism of C16 lead to the formation of (ω-1)- and (ω-2)-hydroxy palmitate in proportion a of 80/20.

Regulation. Preliminary studies of the expression of *CYP94A2* by Northern analysis of mRNA from control *V. sativa* seedlings, or seedlings treated with 0.5 mM clofibrate or 1 mM methyl jasmonate showed that the low constitutive level of transcription of *CYP94A2* is barely enhanced by these treatments, in sharp contrast to the strong induction of *CYP94A1* observed in the same tissues (14, 27).

DISCUSSION

Our previous biochemical studies provided evidence that at least two P450 isoforms were involved in the hydroxylation of FAs in microsomes from *V. sativa*, and that one of these forms preferentially oxidizes short and medium chain FAs (28). Recently, we purified the major FA hydroxylase from *V. sativa* microsomes and used PCR primers deduced from an internal peptide of the protein to isolate the corresponding cDNA (14). This hydroxylase, CYP94A1, exhibits a broad substrate range and catalyzes the ω-hydroxylation of all FAs from C10 through C18:3, apart from stearic acid (C18:0) (14). During this cloning work, several other clones were isolated in addition to *CYP94A1*. Upon sequencing, one clone, lacking about 100-200 nucleotides at its 5′ terminus, appeared to encode a new P450 with high identity to *CYP94A1*. Here, we report the cloning of the corresponding full length cDNA, coding for CYP94A2, a new plant FA hydroxylase which in contrast to the previously characterized CYP94A1, is clearly a medium chain FA hydroxylase. Furthermore, this enzyme shows a very distinct and unusual regiospecitivity of hydroxylation which greatly varies according to the carbon chain length of the substrate.

The full-length *CYP94A2* clone could not be isolated by screening the *V. sativa* cDNA library. It was obtained by performing 5′-RACE on purified mRNA from clofibrate-treated *Vicia* seedlings. The encoded protein shows 58% positional amino acid identity with CYP94A1 and less than 35% with CYP86A1, a broad substrate range FA hydroxylase that we have previously isolated and characterized in *A. thaliana* (13).

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cDNA     ccaagaataatccaagtgtaattacttttttagctctcactactcaatcataactatcaa 60
cDNA     acttgaaaaATGGAACTCGAAACATTGATTTCTTGGTTACTTTTCTCTACAAGTTTATTT 120
Protein           M  E  L  E  T  L  I  S  W  L  L  F  S  T  S  L  F  17
cDNA     TGGTTCTTATTCTTAGCCACAAAAACAAAATCCAAACCCCCAAAAACACCTTCCTCTACC 180
Protein   W  F  L  F  L  A  T  K  T  K  S  K  P  P  K  T  P  S  S  T  37
cDNA     ACCAACACCCCAATTCCTAAATCTTACCCCATTTTCGGTTCTGCCTTCTCTCTGCTAGCC 240
Protein   T  N  T  P  I  P  K  S  Y  P  I  F  G  S  A  F  S  L  L  A  57
cDNA     AACTTCCACCGACGCATACAATGGACCTCCGACATTCTCCAAACCATCCCTTCCTCCACC 300
Protein   N  F  H  R  R  I  Q  W  T  S  D  I  L  Q  T  I  P  S  S  T  77
cDNA     TTCGTCCTCCACCGCCCTTTCGGCGCTCGCCAAGTCTTCACGGCTCAACCCGCCGTGGTG 360
Protein   F  V  L  H  R  P  F  G  A  R  Q  V  F  T  A  Q  P  A  V  V  97
cDNA     CAACACATTCTCAGAACCAATTTCACTTGCTACGGCAAAGGTCTCACGTTTTACCAATCT 420
Protein   Q  H  I  L  R  T  N  F  T  C  Y  G  K  G  L  T  F  Y  Q  S  117
cDNA     ATCAATGATTTTCTCGGCGACGGAATCTTCAATGCCGACGGTGAATCTTGGAAGTTCCAA 480
Protein   I  N  D  F  L  G  D  G  I  F  N  A  D  G  E  S  W  K  F  Q  137
cDNA     CGACAAATCTCCAGCCACGAATTCAACACTAGATCCCTCCGGAAATTCGTTGAAACCGTA 540
Protein   R  Q  I  S  S  H  E  F  N  T  R  S  L  R  K  F  V  E  T  V  157
cDNA     GTTGACGTTGAACTCTCCGATCGCCTAGTTCCTGTTCTCTCCCAAGCTTCTAACAGCCAA 600
Protein   V  D  V  E  L  S  D  R  L  V  P  V  L  S  Q  A  S  N  S  Q  177
cDNA     ACCACTCTTGATTTCCAAGACATCCTCCAACGTTTAACTTTTGACAACATTTGCATGATT 660
Protein   T  T  L  D  F  Q  D  I  L  Q  R  L  T  F  D  N  I  C  M  I  197
cDNA     GCGTTTGGATACGATCCAGAGTACCTCCTTCCTTCCCTTCCTGAAATACCATTTGCAAAA 720
Protein   A  F  G  Y  D  P  E  Y  L  L  P  S  L  P  E  I  P  F  A  K  217
cDNA     GCCTTCGACGAAAGCTCGCAACTCAGTATCGAGAGGCTAAACGCGTTGATTCCATTACTA 780
Protein   A  F  D  E  S  S  Q  L  S  I  E  R  L  N  A  L  I  P  L  L  237
cDNA     TGGAAAGTGAAAAGATTCCTGAACATCGGAGTGGAGCGACAGCTGAAAGAAGCGGTTGCT 840
Protein   W  K  V  K  R  F  L  N  I  G  V  E  R  Q  L  K  E  A  V  A  257
cDNA     GAAGTAAGAGGACTCGCCACTAAAATCGTTAAGAATAAGAAAAAAGAGCTTAAAGAAAAA 900
Protein   E  V  R  G  L  A  T  K  I  V  K  N  K  K  K  E  L  K  E  K  277
cDNA     GCACTACAGTCGGAATCCGAATCTGTTGATCTTTTATCGCGATTTTTAAGTTCTGGACAT 960
Protein   A  L  Q  S  E  S  E  S  V  D  L  L  S  R  F  L  S  S  G  H  297
cDNA     TCAGATGAATCTTTTGTTACTGATATGGTAATAAGTATTATTCTTGCTGGGAGAGATACG 1020
Protein   S  D  E  S  F  V  T  D  M  V  I  S  I  I  L  A  G  R  D  T  317
cDNA     ACTTCAGCTGCACTCACGTGGTTCTTTTGGTTACTCTCGAAGCATAGTCATGTGGAGAAT 1080
Protein   T  S  A  A  L  T  W  F  F  W  L  L  S  K  H  S  H  V  E  N  337
cDNA     GAGATTCTCAAAGAGATAACTGGAAAATCGGAAACTGTTGGATACGATGAGGTGAAGGAT 1140
Protein   E  I  L  K  E  I  T  G  K  S  E  T  V  G  Y  D  E  V  K  D  357
cDNA     ATGGTTTACACTCACGCGGCGCTTTGCGAGAGTATGAGGCTATATCCTCCGCTTCCGGTG 1200
Protein   M  V  Y  T  H  A  A  L  C  E  S  M  R  L  Y  P  P  L  P  V  377
cDNA     GATACTAAAGTAGCCGTGCACGACGATGTTTTGCCGGATGGGACTTTAGTGAAGAAAGGA 1260
Protein   D  T  K  V  A  V  H  D  D  V  L  P  D  G  T  L  V  K  K  G  397
cDNA     TGGAGAGTGACGTATCATATATATGCTATGGGAAGATCTGAGAAGATATGGGGACCGGAT 1320
Protein   W  R  V  T  Y  H  I  Y  A  M  G  R  S  E  K  I  W  G  P  D  417
cDNA     TGGGCTGAATTTCGACCCGAGAGGTGGTTGAGTCGGGATGAGGTTGGGAAGTGGAGCTTT 1380
Protein   W  A  E  F  R  P  E  R  W  L  S  R  D  E  V  G  K  W  S  F  437
cDNA     GTTGGGATTGATTATTATAGTTATCCGGTTTTCCAGGCTGGACCGAGGGTGTGTATAGGG 1440
Protein   V  G  I  D  Y  Y  S  Y  P  V  F  Q  A  G  P  R  V  C  I  G  457
cDNA     AAGGAGATGGCATTTTTGCAGATGAAGAGGGTGGTTGCCGGGATTATGGGGCGGTTTAGG 1500
Protein   K  E  M  A  F  L  Q  M  K  R  V  V  A  G  I  M  G  R  F  R  477
cDNA     GTGGTTCCGGCTATGGTTGAAGGGATTGAGCCGGAGTACACTGCCCACTTTACCTCAGTA 1560
Protein   V  V  P  A  M  V  E  G  I  E  P  E  Y  T  A  H  F  T  S  V  497
cDNA     ATGAAAGGTGGCTTCCCCGTGAAGATCGAAAAGAGAAGCCCACTTGTATGAataaaaagg 1620
Protein   M  K  G  G  F  P  V  K  I  E  K  R  S  P  L  V  *           514
cDNA     aaataatacaccatcaatttgaaatataaattacttttcttttaaaaaaaaaaaaaaaaa 1680
cDNA     a                                                            1681
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FIG. 1. Nucleotide sequence and the derived amino acid sequence of the CYP94A2 cDNA. The coding regions are in uppercase and the non-coding in lowercase. The EcoRI restriction site is in bold and the position of primer hybridization used for 5′-RACE is underlined.

The catalytic activity of the new P450 was assessed by functional expression in yeast strains W(R) and WAT11 overexpressing respectively the native yeast P450 reductase or a P450 reductase from *A. thaliana*. Yeast is particularly well suited for expressing FA hydroxylases since sequencing of the complete genome of *S. cerevisiae* confirmed that only three P450s were present in this organism, none of which is able to oxidize FA at any measurable rate. Moreover, under the growth conditions used, expression of these endogenous P450s is very low and their contribution to spectrally measurable P450 is negligible (21). When expressed in W(R), most of CYP94A2 detectable by CO difference spectroscopy was found as the inactive P420 form (Fig. 2), which correlates well with the low hydroxylase activity found in microsomes from transformed W(R) cells. In contrast, a high level of expression was achieved in WAT11. Similar results were obtained for the expression of CYP81B1 and CYP94A1 in W(R) and WAT11 yeast strains (10, 14). It is tempt-

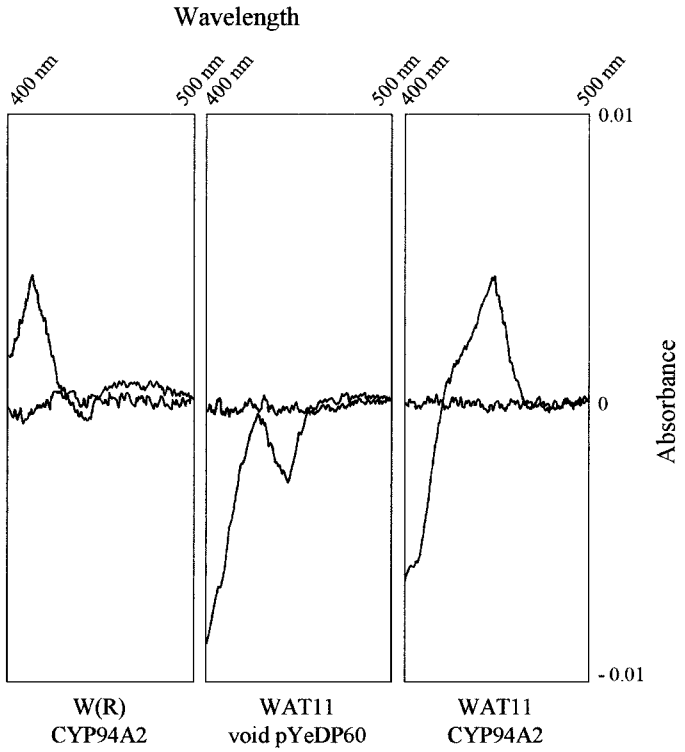

FIG. 2. CO difference spectra of microsomes from W(R) yeast strain transformed with the construction pYeDP60/CYP94A2, WAT11 yeast strain transformed with the void plasmid pYeDP60 or with the construction pYeDP60/CYP94A2. Spectra were recorded with 2 mg of microsomal protein in Tris/HCl (50 mM, pH 7.4) containing 1 mM EDTA and 30% (v/v) glycerol. A base line was recorded after addition of an excess of sodium dithionite to both cuvettes. The difference spectrum was obtained after bubbling CO for 30 seconds in the sample cuvette.

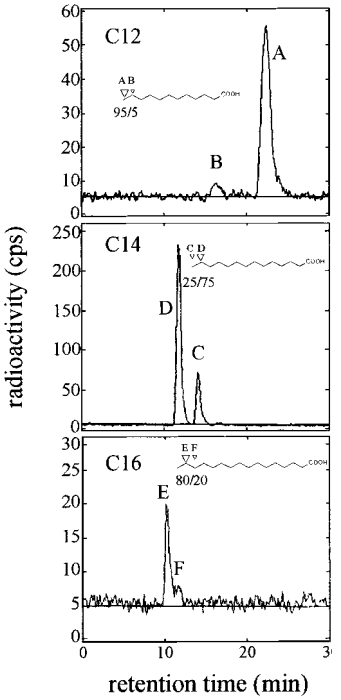

FIG. 3. RP-HPLC elution profiles of radiolabeled metabolites from incubation of yeast microsomes, transformed with CYP94A2, with [1-^{14}C]laurate (C12), [1-^{14}C]myristate (C14) and [1-^{14}C]palmitate (C16) using a mixture of acetonitrile/water/acetic acid (25/75/0.2, v/v/v), (37/63/0.2) and (47/53/0.2) respectively. The metabolites formed from C12 and C14 were identified by GC-MS as the corresponding ω- (A, C) and (ω-1)- (B, D) hydroxy FAs and from C16 the corresponding (ω-1)- (E) and (ω-2)- (F) hydroxy FAs. The molecular formulas show the position of hydroxylation on the substrate and the ratio of formed metabolites.

ing to hypothesize that interaction of plant P450s with the plant reductase not only favors electron transfer but also stabilizes the protein.

TABLE I

Metabolism of FAs by CYP94A2 Expressed in WAT11 Yeast Strain

Fatty acid	CYP94A2
C10:0	n.d.
C12:0	3.8 ± 0.2
C14:0	30.4 ± 0.2
C16:0	4.0 ± 0.3
C18:0	n.d.
C18:1	n.d.
C18:2	n.d
C18:3	n.d

Note. Values are expressed in mole product formed per minute per mole of P450 enzyme. Incubations have been performed with 5.5 pmol of P450 (0.5 mg of microsomal protein) and 100 μM of each FA for 10 min at 27°C in presence of NADPH. Results are the mean of duplicate assays. n.d.: no detectable metabolites are formed during incubation.

The substrate specificity of CYP94A2 was examined with FAs ranging from C10 through C18:3. Compared to the three plant FA hydroxylases described previously (10, 13, 14) and also to the hydroxylases from animal CYP4 (29) and CYP2 family, CYP94A2 is unique by its high affinity and narrow range of substrates and regiospecificity of hydroxylation. The enzyme oxidized exclusively lauric, myristic and palmitic acids, with substrate preference of C14 > C16 = C12. For myristic acid, a turnover of 80 mol/min/mol of P450 and an apparent K_m of 4.0 μM were determined. The enzyme also showed a surprising and uncommon regiospecificity of hydroxylation which varied with the metabolized substrate. CYP94A2 produced the corresponding ω- and (ω-1)-hydroxy FAs with C12 and C14 but in inverse proportion and (ω-1)- and (ω-2)-hydroxy palmitate with C16 (Fig. 3). This is in contrast to the mammalian CYP4 hydroxylases which convert FAs to a 16/1 to 20/1 mixture of ω/(ω-1) hydroxy FAs (29) and the CYP2 which mainly catalyzes (ω-1)-hydroxylation of FAs with an increasing proportion of ω-hydroxylation correlated to elongation of the FA chain (30). Furthermore, CYP94A2 appears highly

selective for FAs since other known physiological or xenobiotic substrates of plant P450s were not oxidized.

In summary, we have cloned and characterized the first dedicated medium chain fatty acid hydroxylase. This finding confirms previous biochemical studies suggesting that *Vicia* microsomes contain at least two different fatty acid ω-hydroxylases (28). The isolation of this myristate hydroxylase, and particularly its unusual regioselectivity, raises many questions as to the physiological roles of hydroxy FAs in plants. Preliminary analyses show that myristate is at best a minor component of *V. sativa* cutin which appears to be derived mainly from C16 fatty acids [Schreiber & Pinot, unpublished]. However, it is now recognized that the structure and composition of the cuticle is not uniform and constant but may vary largely depending on the age of the plant, but also between leaf, fruit and stems, and even between the upper and lower surfaces of individual leaves (31–33). The substrate specificity of CYP94A2 could play a role in the local diversification and adaptation of the cuticle to developmental or environmental signals, but such a role is probably limited since the enzyme preferentially oxidizes the (ω-1)-position in myristic acid. Alternatively, CYP94A2 may be engaged in metabolic or signalling pathways which remain to be characterized. One potential role is the participation of this type of enzyme in plant defense against pathogen attack. It has recently been shown that free hydroxy FAs can induce the resistance of barley against pathogens and induce defense-related genes in cultured potato cells (4, 5). In addition, ferulic acid may be trans-esterified to hydroxy FAs and incorporated into polymeric material such as suberin, which is involved in wound-healing and pathogen containment (3).

Altogether the present study confirms that similar to mammals, a single plant has several forms of P450-dependent FA-hydroxylases. CYP94A1 and A2 share 76% similarity. The stark difference between both accounts for the substrate selectivity which is very sharp for CYP94A2, and the regioselectivity of hydroxylation, which is more relaxed in CYP94A2. Our results suggest that the FA is positioned *via* interaction of the carboxyl function with a positively charged residue of the P450 apoprotein, and that this enzyme "counts" starting from this carboxyl group of the molecule. Thermodynamic considerations favor oxidation of in-chain carbons over oxidation at the terminal methyl because the energy of the C-H bond is higher at the methyl terminus than at the in-chain methylene groups (34). It is therefore believed that in hydroxylases which attack the ω-position independently of substrate chain-length, such as CYP94A1, the positioning of the terminal methyl is strictly controlled by steric hindrance in the vicinity of the active ferryl-oxo species. It is evident that this is not the case with CYP94A2, where the position of hydroxylation is strictly chain length-dependent. Modelling and site-directed mutagenesis studies which are underway should help to understand the mechanisms which determine substrate preference and regioselectivity in these plant FA hydroxylases.

ACKNOWLEDGMENTS

R. Le Bouquin was supported by a fellowship from Ministère de la l'Education Nationale de la Recherche et de la Technologie. We are grateful to David Nelson and the Committee for Standardized Cytochrome P450 Nomenclature for naming the P450 gene family. We thank A. Hoeft for exellent technical assistance with GC-MS analysis.

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