Functional expression in yeast and characterization of a clofibrate-inducible plant cytochrome *P*-450 (CYP94A1) involved in cutin monomers synthesis

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The chemical tagging of a cytochrome *P*-450-dependent lauric acid ω-hydroxylase from clofibrate-treated *Vicia sativa* seedlings with [1-^{14}C]11-dodecynoic acid allowed the isolation of a full-length cDNA designated *CYP94A1*. We describe here the functional expression of this novel *P*-450 in two *Saccharomyces cerevisiae* strains overproducing their own NADPH-cytochrome *P*-450 reductase or a reductase from *Arabidopsis thaliana*. The results show a much higher efficiency of the yeast strain overproducing the plant reductase compared with the yeast strain overproducing its own reductase for expressing CYP94A1. The methyl end of saturated (from C-10 to C-16) and unsaturated ($C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) fatty acids was mainly oxidized by CYP94A1. Both *E*/*Z* and *Z*/*E* configurations of 9,12-octadecadienoic acids were ω-hydroxylated. Lauric, myristic and linolenic acids were oxidized with the highest turnover rate (24 min^{-1}). The strong regioselectivity of CYP94A1 was clearly shifted with sulphur-containing substrates, since both 9- and 11-thia laurate analogues were sulphoxidized. Similar to animal ω-hydroxylases, this plant enzyme was strongly induced by clofibrate treatment. Rapid *CYP94A1* transcript accumulation was detected less than 20 min after exposure of seedlings to the hypolipidaemic drug. The involvement of CYP94A1 in the synthesis of cutin monomers and fatty acid detoxification is discussed.

INTRODUCTION

Plants are subjected to chemical, physical and biological stresses, such as exposure to environmental pollutants, wounding (i.e. by herbivores) and pathogenic infections. As a first physical defence barrier, the plant is protected from the outer environment by cuticular layers and epicuticular waxes. Numerous studies related to the biosynthesis [1], the physical properties [2] and the monomeric composition [3] of cuticles have documented the diversity and distribution of the cutin biopolymer. Elongation of the cutin matrix involves the esterification of a carboxylic function by the terminal hydroxyl group of fatty acids (FAs). It has been shown that certain cutin monomers (i.e. 10,16-dihydroxypalmitic and 9,10,18-trihydroxystearic acids), which are liberated by fungal cutinases, may amplify the expression of these cutinases [4], thus favouring the penetration of the fungus. However, in many cases the fungal cutinase seems not to be required for infection [5,6]. On the other hand, recent reports by Schweizer et al. [7] indicate that the same cutin monomers may protect the plant against fungal infection by inducing the plant defence mechanisms [8]. Also, the cutin monomer 9,10-epoxy-18-hydroxyoctadecanoic acid was demonstrated to protect rice against rice blast [9] and also to induce appressorium formation during the pathogen attack [10], a process that facilitates the penetration of the plant cuticle by the pathogen. Recently, we observed a parallel induction of lauric acid ω-hydroxylase and feruloyl-CoA transferase activities in elicitor-treated French bean cells [11]. It is believed that feruloyl-CoA transferase, by linking ferulate to FAs, is responsible for the presence of aromatic residues that are found in cutin and suberin [12]. In addition to their role as constituents of cuticle and as chemical signals, ω-hydroxylated long-chain FAs have been found in large amounts in pistil and pollen from several tobacco species and are believed to play a role in the compatible/incompatible fertilization process between *Nicotania* species [13].

The catalytic capabilities and the biological significance of plant *P*-450 FA hydroxylases remain to a large extent unknown [14–18]. Evidence suggests that long-chain FA ω-hydroxylases, such as C_{18} ω-hydroxylases [19–21], may play a role in plant cuticle biosynthesis. Recently, we have demonstrated that *Vicia sativa* microsomes contain *P*-450 hydroxylases, epoxygenase(s) and epoxide hydrolases [20], which together catalyse the formation of the main C_{18} cutin monomers from oleic acid. In addition to oleic acid, the *P*-450 hydroxylase was able to ω-hydroxylate the stearate 9,10-epoxide and 9,10-diol [21]. The plant FA ω-hydroxylases, but not the epoxygenase and the epoxide hydrolase activities, were found to be strongly inducible by a class of compounds (i.e. clofibrate) known to induce peroxisome proliferation in mammals [22,23] and in plants [24,25]. In rat and humans, the induction of FA ω-hydroxylases (mainly the CYP4 family) by clofibrate and chemically related compounds involves transcriptional gene activation mediated by nuclear peroxisome proliferator-activated receptors [26].

FA analogues containing a terminal acetylenic function have

Abbreviations used: DDT, dichlorodi[U-^{14}C]phenyltrichloroethane; 11-DDYA, 11-dodecynoic acid; 17-ODNYA, *Z*9-octadecen-17-ynoic acid; 8S-LAU, 8-propylsulphanyloctanoic acid; 10S-LAU, 10-methylsulphanyldecanoic acid; FA(s), fatty acid(s); RT-PCR, reverse transcription PCR.

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The nucleotide sequence of *CYP94A1* reported in this paper will appear in GenBank nucleotide Sequence Database under the accession number AF030260.

previously been shown to be potent mechanism-based inactivators of both mammalian and plant FA ω-hydroxylases [27,28]. Inhibition of enzyme activity was usually associated with the activity dependent alkylation of the haem group and/or the apoprotein. We used this approach to achieve the covalent tagging of the laurate ω-hydroxylase apoprotein by [1-^{14}C]11-dodecynoic acid ([1-^{14}C]11-DDYA). An internal peptide sequence from the radiolabelled protein was determined and a full-length cDNA corresponding to a new *P*-450 family, *CYP94A1*, was isolated. Here we describe the functional expression of this cDNA in yeast and demonstrate that the *CYP94A1*-encoded protein has FA oxidation activity of both medium- and long-chain FAs. Regulation at the transcription level of expression of the gene in response to several plant treatments is discussed.

EXPERIMENTAL

Chemicals

Radiolabelled [1-^{14}C]capric acid (12.2 Ci/mol) was obtained from Sigma Chimie (La Verpillière, France). [1-^{14}C]Lauric (45 Ci/mol), [1-^{14}C]octadeca-*Z*9,*E*12-dienoic (54 Ci/mol), [1-^{14}C]octadeca-*E*9,*Z*12-dienoic (54 Ci/mol), [1-^{14}C]10-methylsulphanyldecanoic (42.3 Ci/mol) and [1-^{14}C]8-propylsulphanyloctanoic (42.3 Ci/mol) acids were from CEA (Gif sur Yvette, France). [1-^{14}C]11-DDYA (42.3 Ci/mol) and *Z*9-octadecen-17-ynoic acid (17-ODNYA) were synthesized by established procedures [21,29]. [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) and [1-^{14}C]linolenic acid (52 Ci/mol) were from NEN-Dupont (Stevenage, U.K.). [^{14}C]Trioleylglyceride (56 Ci/mol), 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). [^{14}C]Diclofop-methyl (10 Ci/mol) was a gift from Hoechst (Frankfurt, Germany).

Thin-layer plates (Silica gel G60F254) were from Merck (Darmstadt, Germany). Clofibrate (ethyl *p*-chlorophenoxyisobutyrate) and NADPH were purchased from Sigma Chimie (La Verpillière, France). V8-protease from *Staphylococcus aureus* was from Boehringer (Mannheim, Germany). Silylating reagent *N*,*O*-bistrimethylsilyltrifluoroacetamide containing 1 % (v/v) trimethylchlorosilane (BSTFA + 1 % TMCS) was from Pierce Europe (Ound-Beijerland, The Netherlands).

Heterologous expression of CYP94A1 in yeast

CYP94A1 was expressed in two strains of *Saccharomyces cerevisiae* obtained by Urban et al. [30] and derived from strain W303-1B [*MAT*a; *ade2*-1; *his3*–11,-15; *leu2*-3, -112; *ura3*-1; *trp1*-1]. The W(R) strain was obtained after substitution of the yeast NADPH-cytochrome *P*-450 reductase promoter by the galactose-inducible hybrid promoter GAL10-CYC1. In the WAT11 strain, the yeast reductase was replaced by the ATR1 reductase from *Arabidopsis thaliana*, controlled by the GAL10-CYC1 promoter. The plasmid pYeDP60 [30] used for the heterologous expression contains an *Escherichia coli* replication origin, a yeast 2 μm plasmid replication origin, an *E. coli* ampicillin resistance gene, and the yeast genes URA3 and ADE2. It utilizes an expression cassette including a GAL10-CYC1 promoter and a phosphoglycerate kinase terminator.

Reformatting and cloning *CYP94A1* into the expression vector pYeDP60 was performed by PCR amplification using specific primers to delete the 5′- and 3′-non-coding regions of the *CYP94A1* cDNA. Specific primers were designed to introduce a *Bam*HI restriction site immediately upstream of the initiation codon and an *Eco*RI site immediately downstream of the stop codon: forward primer 5′-cggc**ggatcc**ATGTTTCAATTTCATCTTGAA G-3′; reverse primer 5′-cggc**gaattc**CTAAGAATCCCTCTTCTGAATCG-3′.

CYP94A1 was amplified using 25 thermal cycles (1 min 93 °C, 2 min 56 °C, 3 min 72 °C) with Pfu DNA polymerase (Stratagene) under the recommended conditions. The PCR product was digested with *Bam*HI and *Eco*RI and inserted between the *Bam*HI and *Eco*RI sites of *p*Bluescript. The nucleotide sequence was determined with a Perkin-Elmer model 373 automatic sequencer (Applied Biosystems Division) using T3 and T7 primers and specific oligonucleotide sequences belonging to the sequenced gene. Complete sequencing of both strands of DNA was performed. The reformatted *Bam*HI–*Eco*RI fragment was then subcloned into *Bam*HI and *Eco*RI sites of pYeDP60.

Transformation of yeast was performed according to the method of Schiestl and Gietz [31]. A fresh yeast culture (initial absorbance = 0.2) was grown in complete YPGA medium [10 g/l yeast extract (Difco)/10 g/l bactopeptone (Difco)/20 g/l glucose/200 mg/l adenine) for 5 h. The cells were collected, washed twice with water and resuspended in 1.5 ml of a 0.1 M lithium acetate (LiAc) solution in TE buffer (10 mM Tris/HCl, pH 7.5/1 mM EDTA).

Salmon sperm was added as DNA carrier (100 μg from a 10 mg/ml solution in TE after 10 s sonication and 20 min boiling) to the plasmid DNA (1 μg). Competent yeast cells (50 μl) and 500 μl of 40 % polyethylene glycol 4000 in LiAc solution in TE buffer were added. The mixture was incubated for 30 min at 30 °C, then for 15 min at 42 °C. After centrifugation, the transformed yeast cells were resuspended in YPGA (1 ml), incubated for 2 h at 30 °C, collected and then plated (with 100 μl of water) on minimum medium SGI [7 g/l yeast nitrogen base/20 g/l glucose/20 mg/l tryptophan/20 g/l agar (Difco)]. Strains transformed with pYeDP60 were grown for 3 days at 30 °C on liquid minimum SGI medium. The culture was centrifuged, the pellet resuspended in a complete YPI medium (10 g/l yeast extract/10 g/l bactopeptone/20 g/l galactose) and grown overnight at 30 °C.

Yeast cells were harvested, broken with glass beads (0.45 mm diameter) and microsomes were prepared by differential centrifugation as in [32]. Microsomal membranes were resuspended in 50 mM Tris/HCl, pH 7.4/1.5 mM 2-mercaptoethanol/30 % (v/v) glycerol, and conserved at −20 °C. Microsomal proteins were quantified by a micro-assay procedure from Bio-Rad using BSA as a standard. The total amount of expressed CYP94A1 in WAT11 strain, determined by CO difference spectroscopy [33], was about 15 nmol of *P*-450 per litre of yeast culture.

Enzyme activity

Enzyme activities were measured as previously described [34]. The standard assay contained, in a final volume of 0.2 ml, 0.19–0.43 mg of microsomal protein from transformed yeast, 20 mM phosphate buffer, pH 7.4, and radiolabelled substrate. ω-Hydroxylase activities were measured in the presence of 1 mM NADPH plus a regenerating system (consisting of glucose-6-phosphate at a final concentration of 6.7 mM and 0.4 unit of glucose-6-phosphate dehydrogenase) and 375 μM 2-mercaptoethanol. The reactions were initiated with NADPH at 27 °C and stopped with 0.2 ml of acetonitrile/acetic acid (99.8/0.2, v/v), mixed and aliquots (100 μl) were directly spotted onto silica plates. Products were quantified by liquid-scintillation counting. Enzyme kinetic analyses were fitted using the non-linear re-

gression program DNPEASY derived by Duggleby from DNPRP53 [35].

Chemical labelling

The procedure of Helvig et al. [28] was followed. Microsomes were preincubated at 27 °C with 1 mM NADPH plus a regenerating system in the presence of 100 μM [1-^{14}C]11-DDYA and stopped as described above. Microsomal incubation media were extracted twice with 1 ml of cold acetone to remove unreacted substrate and metabolites. Acetone-insoluble protein (200–400 μg) was collected by centrifugation (15 min at 3000 ***g***), dissolved in 40 μl of Tris/HCl buffer (180 mM, pH 6.8) containing 5 % (v/v) SDS, 5 % (v/v) 2-mercaptoethanol, 30 % (v/v) glycerol and 0.025 % (v/v) Bromophenol Blue and heated for 1 min in boiling water. Aliquots of this SDS solution (10 μl) were counted by liquid scintillation and analysed by SDS/PAGE [36] using a 10 % acrylamide gel. After Coomassie staining, gels were soaked for 20 min in Amplify and dried. Radioactivity was detected on dry gels after a 1 month exposure of Kodak X-ray films (Amersham Hyper film-βmax) or 8–15 days exposure using a Bio Imaging Analyser (Fugix Bas 2000).

Chromatographic analysis

Metabolites were resolved by silica TLC with a mixture of diethylether/light petroleum (b.p. 40–60 °C)/formic acid (70/30/1, by vol.) for C_{10} to C_{14} FAs and a mixture of the same solvents (50/50/1, by vol.) for long-chain FAs. The areas corresponding to polar metabolites generated from each FA were scraped directly into counting vials or eluted with ether and subjected to reverse-phase HPLC analysis using a mixture of water/acetonitrile/acetic acid (25/75/0.2, by vol.) and (45/55/0.2, by vol.) as previously described [20,34]. Radioactivity of reverse-phase HPLC effluents was monitored with a computerized on-line solid-scintillation counter (Ramona-D RAYTEST, Germany).

GC/MS

Metabolites generated from a series of FAs were methylated and silylated as described in [34] and subjected to GC/MS. GC and electron impact (70 eV) ionization MS studies were performed as described elsewhere [20]. The mass spectrum of the methylester trimethylsilyl ether derivative (MeTMSi) of the enzymic product formed from C_{10} to C_{16} saturated FAs showed characteristic ions at m/z 73 (base peak), 103 ($H_2COTMSi$), $M-15$ (loss of CH_3), $M-31$ (loss of OCH_3), $M-47$ (loss of OCH_3O). A similar fragmentation pattern was obtained for compounds generated from the unsaturated C_{18} family, except for the presence in the mass spectra of low-intensity fragments corresponding to molecular ions (M^+). Results that are consistent with the mass fragmentation already described [37] show that in all cases ω-hydroxylated FAs were generated. Minor metabolites, which represent less than 10 % of total metabolites, were present in a microsomal incubation of transformed yeast cells and were characterized as (ω-1)-hydroxylated FAs by selected-ion monitoring [m/z 117, ($M-44$), ($M-47$) and ($M-15$)]. Mass spectra analysis of metabolites generated from (8-propylsulphanyloctanoic acid) (8S-LAU) and 10-methylsulphanyldecanoic acid (10S-LAU) showed ion fragments present in both spectra at m/z 233 ($M-15$), m/z 232 (loss of O), m/z 217 ($M-31$), m/z 201 ($M-47$) and m/z 55 (base peak), a mass fragmentation pattern similar to authentic sulphoxides, 8-propylsulphinyloctanoate and 10-methylsulphinyldecanoate methyl esters. Cleavage at a position α to the sulphoxide groups give rise to prominent fragments at m/z 157 ($M-91$) and m/z 185 ($M-63$) for metabolites generated from 8S-LAU and 10S-LAU respectively.

Northern blot analysis

Total RNAs were isolated using the procedure of detergent and phenol/chloroform extraction [38]. For Northern blot analysis, total RNAs (30 μg/lane) were denaturated, subjected to electrophoresis on 1.2 % agarose gel containing formaldehyde and transferred onto a Hybond N^+ membrane (Amersham). The blot was hybridized with the ^{32}P-labelled *CYP94A1* cDNA corresponding to the coding region at 65 °C for 16 h. An 18 S ribosomal cDNA from radish was used as an internal standard. After hybridization, the blot was washed twice with 2 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1 % SDS at room temperature for 15 min, and twice with 0.2 × SSC/0.1 % SDS at 55 °C for 30 min. Densitometric quantification of mRNA was performed from scanned autoradiography (Agfa Arcus II Scanner) using the free NIH-Image program version 1.5.9. Spot-intensity measurement of mRNA was adjusted as a function of the intensity of the internal standard.

RESULTS

Expression of *CYP94A1* in *S. cerevisiae*

Specific peptide sequences of a lauric acid ω-hydroxylase from *V. sativa* microsomes were obtained by employing a newly developed method based on the alkylation of the *P*-450 apoprotein by radiolabelled [1-^{14}C]11-DDYA [28]. This mechanism-based inhibitor was incubated either with microsomes or with partially purified and reconstituted lauric acid ω-hydroxylase protein fraction. A radiolabelled protein (about 53 kDa) was isolated by successive SDS/PAGE analysis and subjected to 'in-gel' V8 proteolysis. Resulting peptides were sequenced by the Edman degradation method. An oligonucleotide was deduced from an internal peptide sequence and used in association with an oligo(dT) primer to produce a probe, by RT-PCR, on RNAs from clofibrate-treated vetch seedlings. With this probe, a full-length 1545 bp cDNA (*CYP94A1*) which encodes a protein of 514 amino acids, was isolated from a *V. sativa* cDNA library (GenBank accession number AF030260).

In order to characterize the protein encoded by *CYP94A1*, the corresponding cDNA, inserted into the expression vector pYeDP60, was used to transform two distinct yeast strains. Strain W(R) overexpresses its own NADPH-cytochrome *P*-450 reductase, whereas WAT11 overproduces a plant reductase ATR1 from *A. thaliana* [39]. No typical CO-difference spectrum could be measured with microsomes from the transformed W(R) yeast strain, and lauric acid was only poorly metabolized, at rates of about 0.15 nmol·min^{-1}·mg^{-1} of protein. In contrast, laurate was actively metabolized into 12-hydroxylauric acid (3 nmol·min^{-1}·mg^{-1} of protein) by microsomes isolated from the transformed WAT11 yeast. The reaction was NADPH-dependent and control microsomes, prepared from WAT11 cells transformed with the void pYeDP60 plasmid, showed no detectable activity. In contrast with W(R), about 152 pmol *P*-450 per mg of protein were recorded in microsomes from transformed WAT11 (Figure 1). These results indicate that the expression of CYP94A1 is greatly favoured by the presence of a plant *P*-450 reductase in WAT11.

Substrate specificity of CYP94A1

The highly active WAT11-expressed CYP94A1 was used to investigate the capability of this *P*-450 to oxidize five saturated

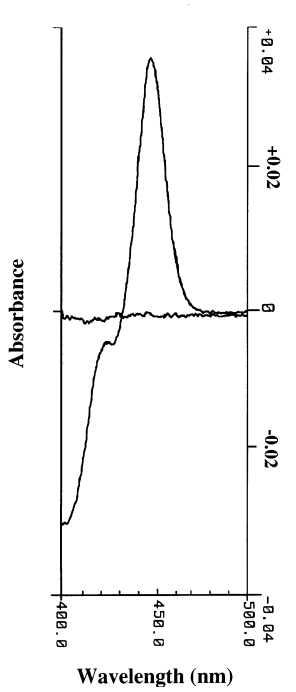

Figure 1 CO difference spectrum of microsomes from WAT11 yeast strain transformed with *CYP94A1* cDNA

Spectra were recorded with 2.6 mg of microsomal protein in Tris/HCl (50 mM, pH 7.4) containing 1 mM EDTA and 30% (v/v) glycerol. A baseline was recorded after addition of an excess of sodium dithionite to both cuvettes. The difference spectrum was obtained after bubbling CO for 1 min in the sample cuvette.

Table 1 Substrate specificity of CYP94A1 expressed in WAT11 yeast strain

Kinetic parameters of FA metabolism by yeast-transformed microsomes. The ω-hydroxylase activities were measured as described in the Experimental section using purified radiolabelled substrates. Results are means $\pm$ S.D. of triplicate measurements.

Substrates	V_{max} (app) (mol/min per mol *P*-450)	K_m (μM)	V_{max}/K_m
Capric acid ($C_{10:0}$)	4.9 $\pm$ 0.1	101 $\pm$ 7.0	0.05
Lauric acid ($C_{12:0}$)	20.0 $\pm$ 1.2	14.7 $\pm$ 3.0	1.36
Myristic acid ($C_{14:0}$)	24.6 $\pm$ 0.1	45.0 $\pm$ 5.5	0.55
Palmitic acid ($C_{16:0}$)	7.2 $\pm$ 0.3	7.2 $\pm$ 0.8	1.0
Stearic acid ($C_{18:0}$)	0	0	–
Oleic acid ($C_{18:1}$)	14.3 $\pm$ 0.5	38.7 $\pm$ 4.3	0.18
Linoleic acid ($C_{18:2}$)	9.1 $\pm$ 0.9	47.0 $\pm$ 1.9	0.19
Linolenic acid ($C_{18:3}$)	24.6 $\pm$ 0.2	70.0 $\pm$ 3.5	0.35

FAs from C_{10} to C_{18} and unsaturated C_{18} FAs (Table 1). Apart from stearic acid, all the FAs tested were converted by CYP94A1 into a major product which was characterized by MS as the corresponding ω-hydroxylated FA.

Based on V_{max}/K_m values, the catalytic efficiency of CYP94A1 was highest for laurate > palmitate > myristate > linolenate > linoleate > oleate > caprate. The K_m for palmitate, the main precursor of cutin monomers in Fabacae, is significantly lower than that for the other substrates. Although our enzyme measurements were performed using very sensitive radiochemical assay methods, no oxidation of stearate ($C_{18:0}$) was observed, whereas

Table 2 Substrate specificity of CYP94A1 expressed in WAT11 yeast strain

Metabolism of *cis* and *trans* unsaturated FAs and thia laurate analogues by microsomes from yeast-transformed cells. The enzyme activities were measured as described in the Experimental section using purified radiolabelled substrates. Values are means $\pm$ S.D. of triplicate measurements (all substrates = 100 μM).

Substrate	Product formed (mol/min per mol *P*-450)
$C_{18:2}$ (*E*9, *Z*12)	52 $\pm$ 1
$C_{18:2}$ (*Z*9, *E*12)	43 $\pm$ 2
Linoleic acid (*Z*9, *Z*12)	25 $\pm$ 2
8-Propylsulphanyloctanoic acid	40 $\pm$ 6
10-Methylsulphanyldecanoic acid	133 $\pm$ 11

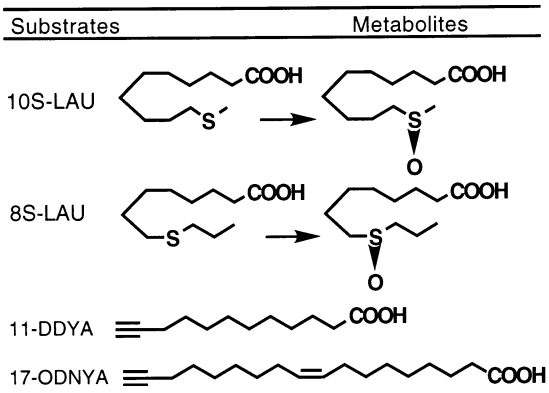

Figure 2 Chemical structure of thia laurate analogues, sulphoxide derivatives and mechanism-based inhibitors

the mono- ($\Delta 9Z$), double- ($\Delta 9Z,12Z$) and especially triple- ($\Delta 9,12,15$ all Z) unsaturated FAs were actively metabolized. In addition, both E/Z and Z/E double-bond configurations of 9,12-octadecadienoic acid were even more actively ω-hydroxylated than linoleic acid, the natural Z/Z isomer (Table 2).

CYP94A1 exhibits remarkable regioselectivity, since hydroxylation was mainly at the terminal carbon position whatever the chain length, the number and the stereochemistry of double bonds. However, recently we had shown that *V. sativa* microsomes catalyse the sulphoxidation of two lauric acid analogues containing a sulphur atom (Figure 2) in their carbon chain [40]. As shown in Table 2, it is the corresponding sulphoxides that were generated by CYP94A1 from both [1-^{14}C]8S-LAU and [1-^{14}C]10S-LAU (Figure 2), whereas no ω-hydroxy-thia acids were detected. NADPH was required for sulphoxidation, which occurred with an apparent reaction rate of 0.6 and 2 nmol$\cdot$min$^{-1}\cdot$mg^{-1} of protein respectively. No sulphoxidation was observed in control incubations with microsomes from WAT11 transformed with the void expression vector. These results demonstrate that the strict regioselectivity of attack with FAs is shifted in-chain when CYP94A1 is confronted with sulphur-containing substrate analogues. The oxidation rate of 10S-LAU, where sulphur is adjacent to the methyl terminus, was three times that of 8S-LAU. It is noteworthy that the reaction did not proceed further, since no sulphone was formed from 8S-LAU or 10S-LAU.

Results presented above demonstrate that in addition to lauric acid, CYP94A1 is also efficient in oxidation of C_{16} and C_{18} FAs.

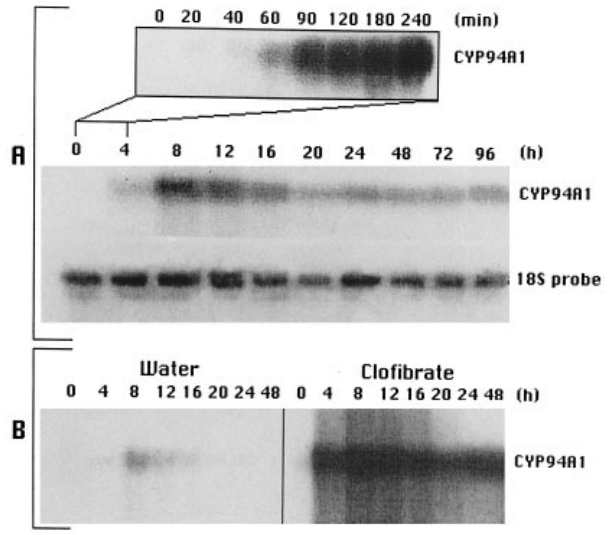

Figure 3 Autoradiography of RNA from *V. sativa* seedlings aged in water or a clofibrate solution analysed with *CYP94A1* and a radish 18 S probe

Total RNA (30 μg) extracted from seedlings aged in water or in 0.5 mM aqueous clofibrate solution for 96 h were analysed in a denaturing formaldehyde gel and transferred by capillarity onto nylon membrane. (**A**) The RNA blot from clofibrate-induced *V. sativa* seedlings was hybridized with *CYP94A1* cDNA and with a radish 18 S cDNA as internal control. Details of CYP94A1 induction from 0 to 240 min is given on top. (**B**) Comparison of induction of *CYP94A1* transcripts in seedlings aged in water or treated with 0.5 mM clofibrate for 48 h.

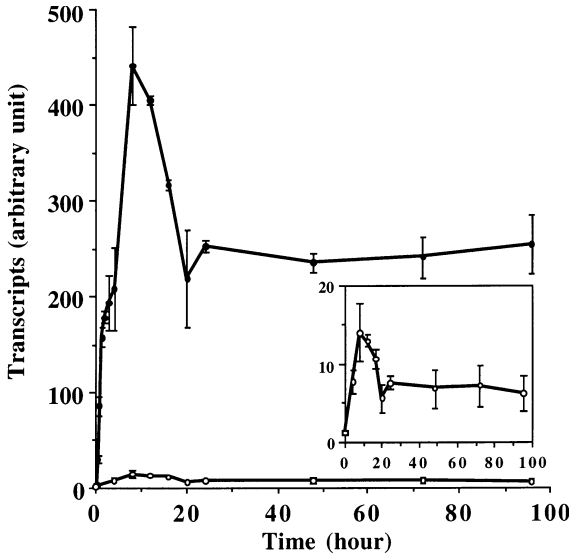

Figure 4 Detailed time course of accumulation of *CYP94A1* transcripts in etiolated *V. sativa* seedlings aged in water (○) and in a 0.5 mM clofibrate solution (●) for 96 h

RNA blots were hybridized, autoradiographed as in Figure 3 and scanned. The densitometric values were corrected using the radish 18 S control. Results are the means of duplicate measurements ± S.D. The insert is a magnification of the values obtained in the water control seedlings.

It was therefore of interest to check whether CYP94A1 was susceptible to autocatalytic inactivation by long-chain FA acetylenic analogues. We used 17-ODNYA (Figure 2) which was previously found to inactivate the oleate ω-hydroxylase from *V. sativa* microsomes [21]. [1-^{14}C]17-ODNYA and NADPH were incubated with microsomes from WAT11 transformed with CYP94A1. After analysis by SDS/PAGE, the radioactivity was found associated with a protein band of about 53 kDa which co-migrated with a Coomassie Blue stained band from microsomes of WAT11 yeast expressing CYP94A1 and that was absent from microsomes of the control WAT11 strain transformed with the void vector.

Furthermore, neither hexadecane nor trioleylglyceride were substrates of CYP94A1, confirming earlier results [41], showing that the carboxyl group of the FA appears essential for the binding of substrate to the enzyme. Although other plant *P*-450s with physiological functions are able to metabolize foreign compounds [42], DDT, testosterone, 7-ethoxycoumarin, 7-ethoxyresorufin, and the herbicide diclofop were not metabolized by CYP94A1.

Regulation of expression of *CYP94A1*

Plant FA ω-hydroxylases share with mammalian and yeast *P*-450s from the CYP4 and CYP52 families the remarkable property of being induced by peroxisome proliferators. In previous studies [21], we have shown that lauric and oleic acid ω-hydroxylase activities are strongly enhanced in microsomes from *V. sativa* seedlings treated with clofibrate. Here, we studied the accumulation of *CYP94A1* transcript during the early development stages of etiolated seedlings (from seed imbibition up to 96 h thereafter) and in seedlings exposed for a further 96 h to clofibrate. Hybridization with a ^{32}P-labelled cDNA probe, corresponding to the whole coding sequence of CYP94A1, detected an mRNA of about 1.9 kb 6 h after imbibition. The transcript level increased 2-fold at 24 h and remained constant until 96 h after imbibition (results not shown). At this time, seedlings were transferred either to water (control) or to a 0.5 mM clofibrate solution. Figure 3(A) and Figure 4 show the very rapid and intense increase in the *CYP94A1* transcript induced by clofibrate. The level of hydroxylase transcript doubled after only 20 min of treatment and was increased 25-fold after 40 min. It reached a peak at 8 h and then decreased until 20 h and remained constant thereafter. A similar evolution of transcript accumulation, but at a much lower level, was measured for the water control [Figure 3B and Figure 4 (insert)]. These results strongly suggest that clofibrate, which is a water-insoluble drug, reaches cellular target(s) which activate the transcription of *CYP94A1*.

DISCUSSION

CYP94A1 is the first *P*-450-dependent FA ω-hydroxylase isolated and cloned from a plant. The enzyme was isolated by tagging of the *P*-450 apoprotein with a radiolabelled mechanism-based inhibitor, a technique based on a mechanism first proposed by Cajacob et al. [27] and recently corroborated in our laboratory [28]. The corresponding cDNA was isolated by screening of a cDNA library from clofibrate-treated seedlings with a gene-specific probe derived from the amino acid sequence. The deduced protein sequence had less than 40% identity with any *P*-450 in the databases and is therefore the first member of CYP94, a new *P*-450 family (D. R. Nelson, personal communication). CYP94A1 displays only weak identity with *P*-450s from families 2, 4, 52 and 102 which catalyse FA oxidation in animals, fungi and bacteria.

Previous studies on the expression of CYP73A1, the cinnamate 4-hydroxylase [32], showed excellent expression and high turnover (over 300) of this plant *P*-450 in W(R), a yeast strain

overexpressing its own *P*-450-reductase. In the present study, we compared the expression of CYP94A1 in W(R) with that in WAT11, a yeast strain recently engineered by Pompon et al. [39], which overexpresses an NADPH-*P*-450 reductase from *A. thaliana* (ATR1). Not only activity, but also expression of CYP94A1, as measured by CO-difference spectrometry, was clearly higher in WAT11 and was very weak in W(R). When considered along with the fact that the *V. sativa P*-450-reductase is phylogenetically closer to ATR1 (expressed in WAT11), and only distantly related to the yeast reductase expressed in W(R) [38], these results suggest that coupling of CYP94A1 with a plant reductase exerts a stabilizing effect, and that this effect is greater with the more similar flavoprotein.

Among the saturated FAs tested, high turnover numbers (about 20 min^{-1}) were measured with lauric and myristic acids, whereas linolenate was the best substrate among the unsaturated C_{18} FAs. Thus, the substrate-specificity of CYP94A1 resembles most that of *P*-450s from the CYP4 family, and primarily that of CYP4A1 which can accommodate both short- and long-chain saturated and unsaturated FAs, and also hydroxylates mainly the ω-position. In addition, the same shift in the regioselectivity as for CYP4A1 [43] was observed for CYP94A1 in the sulphoxidation of 10S-LAU. In the case of CYP4A1, it was proposed that the greater length of the C–S bond would bring the sulphur atom close enough to the ferryl-oxo to be oxidized. Our results, showing that the more in-chain sulphur of 8S-LAU was also readily attacked, suggest that these thia-FA analogues are positioned differently from their FA homologue, at least in the active site of CYP94A1. This is corroborated by the lack of any detectable ω-hydroxylation of the thia-acids and further supported by previous work showing that *V. sativa* microsomes did not catalyse the epoxidation of 9-10 or 10-11 unsaturated laurate analogues. Kinetic parameters for the sulphoxidase activities were not determined, but compared with linoleic acid, the metabolism of 10S-LAU and 8S-LAU was 5.3 and 1.6 times greater respectively (Table 2). As already described for the metabolism of unsaturated laurate analogues by *V. sativa* microsomes [34], both Z/E and E/Z analogues of linoleic acid were ω-hydroxylated by CYP94A1 with a greater efficiency than linoleic acid (Z/Z) itself. In contrast with other plant *P*-450 enzymes from wheat [44] and *Helianthus tuberosus* [45] microsomes, which catalyse in-chain oxidation and display a more relaxed regioselectivity, CYP94A1 is highly selective for the terminal, primary C–H bonds of the FA in preference to the more easily hydroxylated secondary C–H or C = C bonds at internal positions such as ω-1, ω-2 or ω-3. Contrary to the thia-acids, FAs are probably positioned in such a way that the methyl terminus is tightly constrained within reaction distance from the ferryl-oxo. The fact that, (1) a free carboxylic group appears essential for substrate recognition, and (2) FAs of considerably differing chain-length are oxidized, suggests that two or more basic residues, at the entrance or in the substrate channel, may serve as anchoring points for the substrates. A distinct property of the plant ω-hydroxylase is the total lack of oxidation of the $C_{18:0}$ acid, whereas $C_{16:0}$ and $C_{18:n}$ were actively metabolized. This is in contrast with *P*-450s of families 4 and 52 which convert stearic acid into 18-hydroxystearate, and was not expected, since ω-hydroxystearate has been found, generally in minor amounts, in cutin from several plants [46].

The study of the regulation of CYP94A1 at the transcription level clearly shows that the gene is constitutively expressed. We observed a sharp activity increase when the seedlings were transferred and aged in water (Figure 4, insert). This effect is probably due to wound-induction when the seedlings are removed from the germination trays to be transferred to water or clofibrate solution. Wound repair and healing depends on the rapid deposition of an isolating suberin layer. ω-Hydroxylation of C_{16} and unsaturated C_{18} FAs plays a pivotal role in cutin and suberin synthesis, since this reaction controls the polymer elongation process. We demonstrate here the capability of CYP94A1 to generate 16-hydroxypalmitate and 18-hydroxy-oleate, -linoleate and -linolenate, which are the main precursors for cutin biosynthesis. Particularly interesting in this context is the low K_m of CYP94A1 for palmitate, since cutin and suberin are predominantly formed from C_{16} monomers in Fabacae. Furthermore, the rapid increase in *CYP94A1* transcript during the plant development and after wounding of tissues is consistent with the involvement of this enzyme in the formation of the cuticular layers which protect the plant from the outer environment. According to early work by Kolattukudy [1], not only 16-hydroxypalmitate, but also 9,16- and 10,16-dihydroxypalmitic acids, are found in cutin and suberin. It was suggested that the hydroxylation at position 9 or 10 was catalysed by *P*-450. Until now, this enzyme has not been isolated. The *P*-450-dependent in-chain hydroxylase characterized in *H. tuberosus* catalyses hydroxylation at positions 9 and 10 of capric, lauric and myristic acids, but not of palmitic acid [45]. Recently, it was demonstrated that an oleate 12-hydroxylase from *Ricinus communis* [47], which is not a *P*-450 but is related to the desaturase family of non-haem oxidases, can direct the formation of lesquerolic acid, a 14-hydroxylated eicosenoic acid [48], opening the possibility that, given the appropriate substrate, this hydroxylase may catalyse in-chain hydroxylation at other positions.

There is an intriguing similarity between the effects of peroxisome proliferators in mammals and in plants. In addition to the induction of laurate and oleate ω-hydroxylase activities by clofibrate [21,25], Del Rio and co-workers [24,49] have demonstrated in pea seedlings that clofibrate stimulates the production of activated oxygen species as well as lipid peroxidation, whereas catalase and superoxide dismutase were inhibited. Therefore, we have analysed the accumulation of *CYP94A1* transcript in clofibrate-treated plants. Compared with the response of the mammalian CYP4s, the induction of the plant oxygenase was much more rapid and of very high amplitude. The mechanism by which clofibrate may trigger the transcription of *CYP94A1* remains an open question. However, other peroxisomal proliferators, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and di(2-ethylhexyl)-phthalate (DEHP) [25], which bear no structural similarity to clofibrate or auxins, are equally effective in inducing the *Vicia* ω-hydroxylases. This suggests that clofibrate induction may be mediated in plants by a mechanism similar to that in animal systems. In mammals, the induction of transcription by peroxisome proliferators is mediated by members of the steroid hormone receptor superfamily, the peroxisome proliferator activated receptors, which bind to specific target sequences of DNA, the peroxisome proliferator responsive elements, leading to an increase in the transcription rate of the gene (reviewed in [26]). There is recent evidence that the physiological ligands of peroxisome proliferator activated receptors may actually be FAs [50]. On the other hand, several lines of evidence indicate that, in plants, wounding and attack by pathogens result in the rapid activation of phospholipases and the release of FAs [51,52]. It is tempting to propose that clofibrate in our studies mimics FAs liberated upon wounding or infection. The rapid induction of CYP94A1 would ensure that the liberated free FAs, which are cellular toxicants, are effectively transformed into cutin monomers needed for repair and defence.

In addition, there is growing evidence to support a role for hydroxylated FAs in stress-response reactions and as signalling molecules on the plant surface. The loss of the plant cuticle

integrity by wounding, pathogenic attack induces a cascade of events, including the accumulation of phytoalexins and the enhancement of activities of several enzymes involved in detoxification and the production of signalling molecules. It has recently been shown that FA ω-hydroxylation is induced in cell cultures treated with a fungal elicitor [11] and that ω-hydroxy FAs are able to elicit defence responses [7]. This possible role of hydroxylated FAs as natural elicitors of plant defence mechanisms opens unexpected perspectives for investigating new regulatory routes of naturally occurring plant defence compounds.

Finally, recent advances in engineering of FA metabolism for the production of oilseed crops suggest that there are no fundamental barriers towards making modifications in plant FA synthesis [53]. In addition to the control of chain-length and the location or the stereochemistry of unsaturations, production of terminally and in-chain oxidized FAs should be of high commercial value for non-food uses, such as in lubricants and plasticizers.

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