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Molecular cloning of CYP76A3, a novel cytochrome P450 from *Petunia hybrida* catalyzing the ω-hydroxylation of myristic acid

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Abstract

In higher plants, fatty acid hydroperoxides are intermediates in the synthesis of a diverse group of bioactive compounds. We used the reverse-transcriptase polymerase chain reaction to isolate a gene responsible for the oxidization of fatty acids from *Petunia hybrida*. A P450 cDNA that has not previously been isolated (CYP76A3) contained an open reading frame predicted to encode a polypeptide consisting of 507 amino acid residues. The *cyp76A3* cDNA was expressed in *Saccharomyces cerevisiae* AH22 cells under the control of an alcohol dehydrogenase promoter and terminator. The recombinant yeast microsome containing the CYP76A3 hemoprotein was found to specifically catalyze ω-hydroxylation of myristic acid. A high level of the transcripts of the *cyp76A3* gene was found in the leaves and roots of *P. hybrida*, but not in the stems and flowers.

Additional key words: biosynthesis regulation, cytochrome monooxygenases, oxidation of fatty acids.

Introduction

Cytochrome P450 (EC 1.14.14.1) monooxygenases, consisting of a number of cytochrome P450 (P450 or CYP) species and NADPH-cytochrome P450 oxidoreductases (P450 reductase), play an important role in the biosynthesis of a variety of secondary metabolites, as well as in the metabolism of xenobiotics in higher plants.

Estimates from current genome projects imply that the number of P450 genes exceeds 273 in *Arabidopsis thaliana* (*A. thaliana*) (http://www.p450.kvl.dk/). More than 3000 P450 cDNA sequences from higher plants are currently known, but the functions of very few of these genes have been identified. Important P450 enzymes whose functions remains elusive or poorly understood are related to the biosynthesis of hydroxy-fatty acids, sterols, glucosinolates, phenylpropanoids, salicylic acid, jasmonic acid, gibberellins, abscisic acid, brassinosteroids, and alkaloids (Schuler 1996).

Cytochrome P450-dependent monooxygenases can catalyze in-chain and ω-hydroxylation as well as epoxidation of medium and long-chain fatty acids (Salaun and Helvig 1995). Plants, like other eukaryotes, synthesize lipids for membrane biogenesis, as signal molecules, and as a form of stored energy (Schmid and Ohlorogge 1996). The physical properties of the membrane are largely determined by the chain length and polarity of the fatty acid components (Millar *et al.* 1998). In higher plants, a subset of cellular proteins is post-translationally modified by addition of a myristic acid to the N-terminus of the cellular proteins for the regulation of cell growth and development (Thompson and Okuyama 2000, Dumonceaux *et al.* 2004). These reports show that fatty acids and their derivatives play

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Abbreviations: dNTP - deoxynucleotide triphosphate; HPLC - high-performance liquid chromatography; MOPS - 3-(N-morpholino) propanesulfonic acid; PCR - polymerase chain reaction; RT-PCR - reverse-transcriptase polymerase chain reaction; SDS - sodium dodecyl sulfate; SSC - saline-sodium citrate; SSPE - a mixture of 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA at pH 7.5; TLC - thin-layer chromatography.

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The nucleotide sequence reported here is available in the DDBJ, EMBL, and GenBank Nucleotide Sequences Databases under accession number AB016060 (CYP76A3).

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important roles in higher plants.

Several P450s that catalyze the hydroxylation of fatty acids have been proposed for use in crop improvement (Feldmann 2001). For example, CYP78A9 (Ito and Meyerowitz 2000) and LACERATA (encoded by *lcr*; Wellesen *et al.* 2001) seem to control the establishment of apical dominance and senescence in plants, respectively. Ito and Meyerowitz found that CYP78A9 mutants had larger ovaries than wild-type plants (Ito and Meyerowitz 2000). Wellesen *et al*. (2001) described LACERATA mutants of *A. thaliana* with developmental abnormalities. The *lcr* gene encodes CYP86A8, which catalyzes ω-hydroxylation of fatty acids ranging from C12 to C18 in size.

Another group of P450s are allene oxide synthases and hydroperoxide lyases, which are involved in the metabolism of fatty acid hydroperoxides (Howe *et al.* 2000). Unlike P450 monooxygenases, which need molecular oxygen and NADPH-dependent cytochrome P450 reductase for their activity, allene oxide synthase and hydroperoxide lyase use an acyl hydroperoxide both as the oxygen donor and as the substrate on which new carbon-oxygen bonds are formed. The metabolites of fatty acids made by CYP74 serve as the precursor in the enzymatic synthesis of jasmonic acid (Matsui *et al.* 2000). There has been increasing interest in jasmonic acid, a fatty acid derivative, because of evidence for its involvement in plant defences (Song *et al*. 1993), signalling, tuber formation, and fruit ripening (Suzuki *et al.* 1996). Derivatives of fatty acids may participate in plant cuticle biosynthesis and stress induction in *Lemna paucicostata* (Yokohama *et al.* 2000). Recently, it has been reported that proteins having covalently attached lipids such as myristic acid and palmitic acid are of critical physiological importance, especially as participants in cellular signalling in higher plants (Dumonceaux *et al.* 2004). However, little is known about the P450s involved in the oxidation of long-chain fatty acids.

The aim of present study was to isolate a P450 that specifically metabolizes long-chain fatty acids in *Petunia hybrida* and to clone and sequence a cDNA that encodes CYP76A3. The heterologous expression of the gene in yeast allowed to examine its enzymatic function, with NADPH as the electron donor.

Materials and methods

Plants: *Petunia* × *hybrida* cv. Blue Star seeds were purchased from *Sakata Seeds Co*. (Yokohama, Japan) and grown for 9 weeks in a growth chamber at a 10-h photoperiod with irradiance of 100 μmol m^{-2} s^{-1}, temperature of 25 °C and relative humidity of 50 %.

Chemicals: DNA modification enzymes and restriction endonucleases were purchased from *Takara Shuzo Co*., (Shiga, Japan), *New England Biolabs* (Beverly, MA, USA), and *Toyobo Biochemical* (Tokyo, Japan). The *Uni-ZAPII* cDNA synthesis kit was obtained from *Stratagene* (La Jolla, CA, USA). A DNA ligation kit (version 2) and random-primer DNA labeling kit were obtained from *Takara Shuzo*. GeneElute Agarose Spin Columns were purchased from *Supelco* (Bellefonte, PA, USA). The Quick Prep Micro mRNA purification kit was obtained from *Pharmacia P-L Biochemicals* (Milwaukee, WI, USA) and *Nippon Gene Co.* (Tokyo, Japan), respectively. The First-Strand cDNA synthesis kit was obtained from *Amersham Pharmacia Biotech* (Piscataway, NJ, USA). The protein assay I kit was obtained from *Bio-Rad Lab.* (Tokyo, Japan). *Hybond-N*$^+$ nylon membranes were purchased from *Amersham International* (Buckinghamshire, UK). The pT7Blue T-vector was purchased from *Novagene* (Madison, WI, USA). [α-^{32}P]dCTP, [l-^{14}C]lauric acid, and [l-^{14}C]linoleic acid were purchased from *Amersham Life Science* (Tokyo, Japan). [l-^{14}C]Myristic acid, [l-^{14}C]palmitic acid, and [l-^{14}C]testosterone were purchased from *DuPont NEN* (Boston, MA, USA). [l-^{14}C]Stearic acid and [l-^{14}C]oleic acid were purchased from *Moravek Biochemicals* (Brea, CA, USA). Other reagents were obtained from *Nacalai Tesque* (Kyoto, Japan), *Wako Pure Chemicals* (Osaka, Japan), and *Sigma Chemical Co*. (St. Louis, MO, USA).

RT-PCR cDNA cloning of partial sequences of P450 in *P. hybrida*: Poly(A)$^+$RNA was isolated from the 9-week-old leaves of *P. hybrida* using the *Quick Prep Micro* mRNA purification kit. First-strand cDNA was synthesized from 100 ng of poly(A)$^+$RNA using a *First-Strand* cDNA synthesis kit according to the protocol specified by the manufacturer, and 10 ng of the cDNA was used for PCR. A pair of degenerate primers (Oligo #4 and #5), corresponding to the primers used by Holton and Lester (1996) for random cloning of *P. hybrida* P450 species, were used: Oligo #4, 5'-CCIGG(A,G)CAIATIA (G,T)(C,T)(C,T)TICCIGCICC(A,G)AAIGG-3'; Oligo #5, 5'-TT(C,T)(G,T)(G,T)IG(C,G) IGGI(A,T)(C,T)IGAIAC-3'. The temperature program was 3 min at 94 °C, followed by 50 cycles of 50 s at 94 °C, 50 s at 45 °C, and 45 s at 72 °C. PCR products were separated in 2 % (m/v) agarose gels, and the region ranging from 400 to 450 bp was extracted on a *GeneElute* agarose spin column. Purified cDNA fragments were cloned into the pT7Blue T-vector and then transformed into *Escherichia coli* strain JM109. Randomly selected clones were sequenced, and the resulting sequences were compared with those of reported plant P450 species using the protein-protein

BLASTp search program (http://www.ncbi.nlm.nih.gov/blast/index.shtml).

Screening of a cDNA library for the cloning of a full-length novel P450 from *P. hybrida*: A cDNA library was constructed from the poly(A)$^+$RNA fraction prepared from *P. hybrida* leaves using a Uni-ZAPII cDNA synthesis kit according to the manufacturer's instructions. The library was screened with the RT-PCR No-73 of the cDNA clone (Fig. 1; 916 to 1344 bp) as the probe. The nucleotide sequence of No-73 is shown in bold in Fig. 1. For the first screening, the probe was labelled with [α-^{32}P]dCTP with a random-primer oligo-DNA labelling kit. Duplicate filters were treated for 1 h at 42 °C in a mixture of 50 % formamide, 5× SSPE (1× SSPE contained 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA, pH 7.5), 0.5 % SDS, 5× Denhardt's solution, and 100 μg cm^{-3} of denatured salmon sperm DNA, then were incubated for 16 h at 42 °C with the labelled probe. Filters were washed twice in a mixture of 2× SSC and 0.15 % SDS at room temperature for 20 min and once in 0.2× SSC plus 0.1% SDS at 65 °C for 15 min, and radioactivity was measured with a BAS 2000 image analyzer (*Fuji Co.*, Tokyo, Japan). Plaques with positive signals were collected, and the second screening was done under the same conditions. After the second screening, several clones with positive signals were isolated and sequenced.

DNA sequencing and computer analysis: *cyp76A3* cDNA was sequenced automatically with the *ABI PRISM 310* DNA sequencer (*Perkin-Elmer*, Foster City, CA, USA). Sequencing alignment was done with *version 7.3* of the *GENETYX MAC* program (*Software Development Co*., Tokyo, Japan). The phylogenetic tree was created using the *GENETYX WIN version 5.0* computer program by the UPGMA method.

Southern blot analysis: Total DNA was isolated from 5 g of leaves of *P. hybrida* using an *Isoplant* kit from *Wako Chemical Co.* (Osaka, Japan). For Southern blot analysis, 20 μg of the total DNA fraction was digested with *Dra*I, *Hind*III and *Eco*RV, respectively. DNA fragments were separated in a 1.2 % (m/v) agarose gel, and then transferred onto a *Hybond N*$^+$ nylon membrane with 10× SSC. Hybridizations were done at 65 °C in 6× SSC with 7.5× Denhardt's reagent, 200 mg cm^{-3} salmon sperm DNA, and ^{32}P-labeled probes for 18 h. After the hybridization, the filters were washed for 30 min in 300 cm^3 of solution (1× SSC, 0.1 % SDS) at room temperature, followed by a second wash for 30 min in a solution (0.1× SSC, 0.1 % SDS) at 60 °C. Radioactivity on the filter was measured using the *BAS 2000* image analyzer.

Construction of the pA76A3 expression plasmid and transformation of *Saccharomyces cerevisiae*: In order to express CYP76A3 protein into *S. cerevisiae*, yeast expression vector pAAH5N was used (Oeda *et al.* 1985). A PCR method was used to add the *Hin*dIII sites to the DNA sequences corresponding to the N- and C-terminal regions of the *cyp76A3* cDNA. The expression plasmid pA76A3 was constructed by ligating digested modified PCR products with *Hin*dIII and digesting pAAH5N with *Hin*dIII. Transformation of *S. cerevisiae* AH22 cells with the expression plasmid pA76A3 was carried out using the lithium chloride method, as described previously (Oeda *et al.* 1985). Reduced CO-difference spectra of the microsomal fractions prepared from transformed yeast cells were measured according to the method of Oeda *et al*. (1985). P450 hemoprotein contents in the microsomal fractions were determined from reduced CO-difference spectra using a coefficient of absorbance of 91 mM^{-1} cm^{-1} (Omura and Sato 1964). Protein concentrations were measured with a protein assay I kit with bovine serum albumin as the standard (Bradford 1976).

Assay for P450-dependent monooxygenase activities: A P450-dependent monooxygenase assay mixture was created in a final volume of 400 cm^3 using 0.1 M potassium phosphate buffer (pH 7.4), 6.7 mM glucose-6-phosphate, 1 unit glucose-6-phosphate dehydroxylase, 5 mM substrate, and 50 pM P450 protein from a microsomal fraction prepared from recombinant yeast strains. After preincubation of the reaction mixtures for 3 min at 30 °C, enzyme reactions were initiated by the addition of 5 mM NADPH at a final concentration of 10 μM and then stopped after 1 h by the addition of 40 cm^3 of 1 M HCl. Reaction mixtures were each extracted twice with 800 cm^3 of ethylacetate, and extracts were dried and then dissolved in 99 % methanol. Each of these samples was analyzed by means of HPLC (model *L-7000*, *Hitachi*, Tokyo, Japan) at a flow rate of 1.0 cm^3 min^{-1} on a 4.6-mm × 150-mm *Cosmosil 5C18-AR* column (*Nacalai Tesque*, Kyoto, Japan). HPLC conditions for each compound were as follows: *trans*-cinnamic acid in acetonitrile/water/acetic acid (40:60:0.2, v/v/v) at 286 nm; chrysophanol in methanol/water (80:20, v/v) at 254 nm; emodin in methanol/water (70:30, v/v) at 254 nm; bergapten in methanol/water (60:40, v/v) at 254 nm; psolaren in methanol/water (40:60, v/v) at 295 nm; isopimpinellin in methanol/water (45:55, v/v) at 310 nm; xanthotoxin in acetonitrile/water (30:70, v/v) at 254 nm; imperatorin in acetonitrile/water (70:30, v/v) at 254 nm; abscisic acid in acetonitrile/acetic acid (75:25, v/v) at 262 nm; gibberellin (GA_3) in methanol/water (50:50, v/v) at 254 nm; naringenin in methanol/water (40:60, v/v) at 292 nm; apigenin in methanol/water (50:50, v/v) at 263 nm; eriodictyol in methanol/water/acetic acid (30:65:5, v/v/v) at 280 nm; and ferulic acid in

acetonitrile/water/acetic acid (10:90:0.2, v/v/v) at 318 nm.

For enzyme assay with a radiolabeled substrate, 10 μM radiolabeled substrate was added to the reaction mixture and extracted as described above. Each sample, containing a total radioactivity of 37 kBq was applied to a 60F254 thin-layer chromatography (TLC) plate precoated with silica gel (*Merck AG*, Darmstadt, Germany). TLC conditions for each sample were as follows: testosterone in chloroform/ethylacetate (66:34, v/v); lauric acid, myristic acid, and palmitic acid in diethyl ether/ether/formic acid (70:30:1, v/v/v); and oleic acid, stearic acid, and linolenic acid in n-hexane/diethyl ether/acetic acid (40:60:1, v/v/v). Radioactivity on each TLC plate was measured using the *BAS 2000* image analyzer. Hydroxylated myristic acid (100 nmol) without radioactivity was spotted onto the same positions that were spotted with the radioactive samples, and the TLC plate was developed as described above. The radioactivity was measured, and then to detect the hydroxylated myristic acids, 50 % H_2SO_4 was sprayed onto the TLC plate, which was then baked at 180 °C for 10 min. Measurements of enzyme activity were carried out three times for each enzyme reaction.

Poly(A)$^+$RNA extraction from flowers, leaves, stems and roots and Northern blotting: RNA prepared as described above was separated on a 1.0 % (m/v) agarose gel containing 0.66 M formaldehyde in MOPS buffer (0.02 M MOPS, containing 0.01 M sodium acetate and 2 mM EDTA) and transferred to a *Hybond N*$^+$ membrane with 20× SSC (3.0 M sodium chloride and 0.3 M sodium citrate at pH 7.0). After transfer, the membranes were cross-linked using UV radiation with a *Stratalinker* (*Funakoshi Co*., Tokyo, Japan). Hybridization was done at 65 °C in a mixture of 6× SSC, 7.5× Denhardt's reagent, 200 μg cm^{-3} salmon sperm DNA, and labeled probes for 18 h. The membranes were washed twice in 2× SSC and 0.1 % SDS at room temperature for 20 min and once in 2× SSC and 0.1 % SDS at 65 °C for 15 min. Radioactivity was measured with the *BAS 2000* image analyzer.

Results

Isolation of a partial sequence of novel P450 cDNA from *P. hybrida*: We isolated a cDNA clone of a novel P450 species that was expressed in *P. hybrida* by means of RT-PCR using a pair of degenerate PCR primers (Oligo #4 and Oligo #5) that corresponded to the highly conserved domains A and D (Fig. 1; Holton and Lester 1996). PCR amplification was carried out with these primers and with the template first-strand cDNA that had been synthesized from a poly(A)$^+$RNA fraction prepared from *P. hybrida* leaves. The amplified DNA fragments migrated in a 2.0 % agarose gel as diffuse bands ranging between 400 and 450 bp. The PCR products at around 430 bp were cloned into the pTBlue T-vector. Two hundred randomly selected clones were each sequenced, and the sequences were compared with those of previously reported P450 species from plant using the *BLASTp* search program. Only one clone (No-73) was found to encode a novel P450-like sequence (Fig. 1).

cDNA cloning of CYP76A3 from *P. hybrida*: After secondary screening from the *P. hybrida* cDNA library, the largest cDNA clone (designated P450-3) was found to contain a 1521-bp insert with an uninterrupted open reading frame that encoded a polypeptide consisting of 507 amino acid residues. A search of the *SWISS-PROT* database (http://www.ebi.ac.uk/swissprot/) showed that the deduced amino-acid sequence of P450-3 contained domains A through D (Fig. 1), which are highly conserved among P450 gene families. P450-3 contained the N-terminal hydrophobic region (a signal-anchor sequence), followed by a proline-rich region that suggests P450-3 is a microsomal-type P450, and contains the binding motif for heme. The cysteine residue that serves as the fifth ligand for heme-Fe was found at position 1340 counting from the first methionine (Poulos 1995). P450-3 was officially assigned to the name CYP76A3 by Dr. D.R. Nelson (Department of biochemistry, University of Tennessee, personal communication) and the Committee for Standardized Cytochrome P450 Nomenclature.

Sequence analysis of CYP76A3: Sequencing alignment was done with the *GENETYX MAC* program. Multiple alignment of the amino-acid sequence of CYP76A3 with those of previously reported plant P450 species was carried out. The similarity scores for the amino-acid sequences showed the highest sequence similarity, of 64 %, with CYP76A2 (unknown function from *Solanum melongena*; Toguri *et al.* 1993), followed by 63 % with CYP76A1 (unknown function from *Solanum melongena*; Toguri *et al.* 1993) and 39 % with CYP76B1 (7-ethoxycoumarin *O*-deethylase from *Helianthus tuberosus*; Batard *et al.* 1998, Robineau *et al.* 1998). A dendrogram obtained with *GENETYX WIN* computer program by the UPGMA method showed that CYP76A3 belongs to the same cluster as flavonoid-3′,5′-hydroxylase (CYP76A1, CYP76A2, CYP75A1 from *P. hybrida*, Holton *et al.* 1993; CYP75A2 from *Solanum melongena*; Toguri *et al.* 1993b; CYP75A3 from *P. hybrida*; Holton *et al.* 1993) (Fig. 2). However, CYP76A3 is not closely related to CYP86A1 (fatty acid hydroxylase from *A. thaliana*; Benveniste *et al.* 1998), CYP94A1 (cutin monomers synthesis from *Vicia sativa*; Tijet *et al.* 1998), or CYP74A1 (allene oxide synthase from *Linum usitatissimum*; Laudert *et al.* 1996).

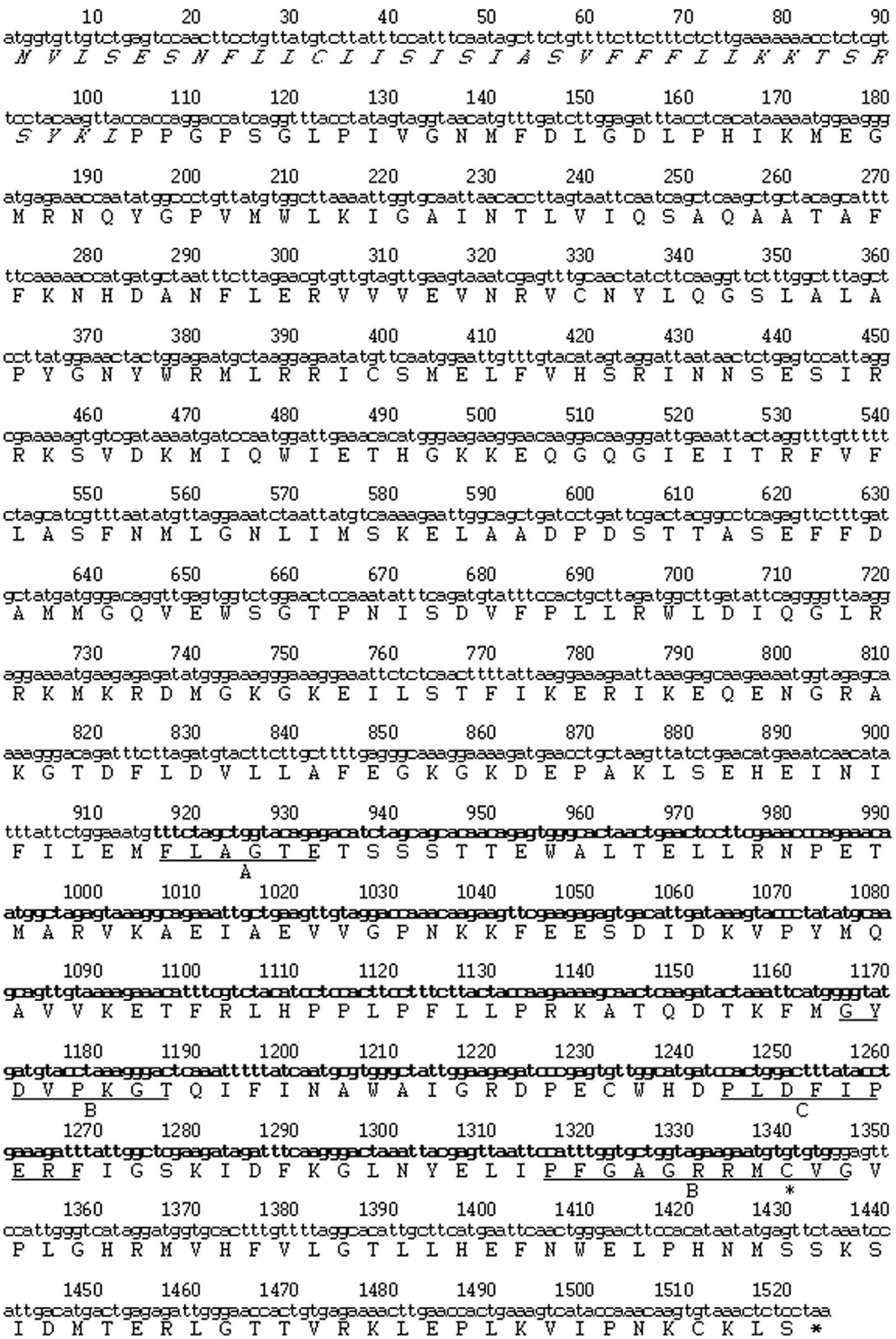

Fig. 1. Nucleotide and deduced amino-acid sequences of CYP76A3: Amino acids are designated using the standard single-letter code. The nucleotide sequence of clone No-73 is shown in bold. Four domains (A to D) that are highly conserved among P450 families are shown with underlines. N-terminal hydrophobic region is shown in italic. The cystein residue that serves as the fifth ligand for heme-Fe was shown with asterisk at position 1340 counting from the first methionine.

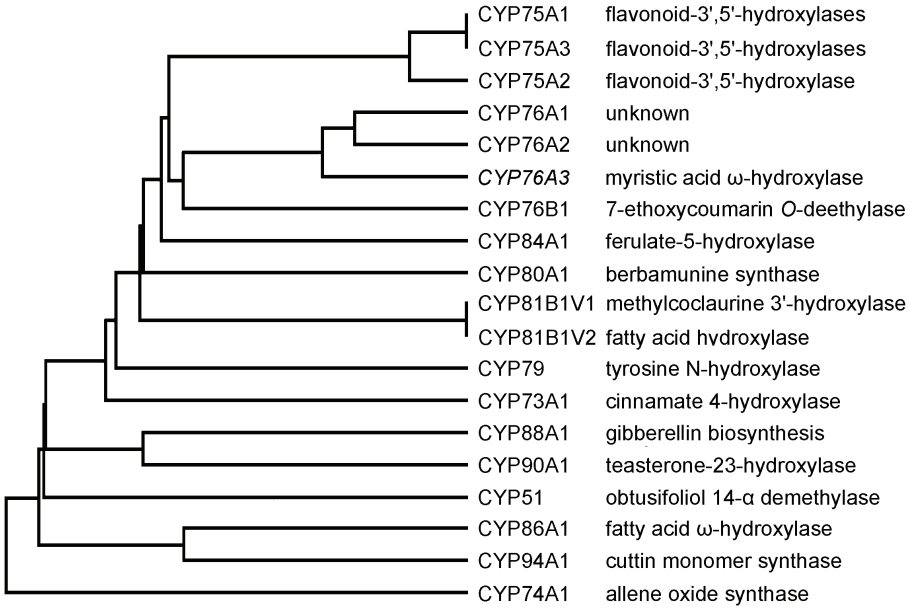

Fig. 2. Dendrogram showing the relatedness of CYP76A3 to previously reported plant P450 species: The enzyme functions and accession numbers of the P450 genes are shown as follows: flavonoid-3′,5′-hydroxylases (CYP75A1, X71130; CYP75A3, Z22544; CYP75A2, X70824); unknown function (CYP76A1, X71658; CYP76A2, X71657); 7-ethoxycoumarin *O*-deethylase (CYP76B1, Y09920); ferulate-5-hydroxylase (CYP84A1, U38416); berbamunine synthase (CYP80A1, U09610); (s)-N-methylcoclaurine 3′-hydroxylase (CYP81B1v1, AJ000477); fatty acid hydroxylase (CYP81B1v2, AJ000478); tyrosine N-hydroxylase (CYP79, U32624); cinnamate 4-hydroxylase (CYP73A1, Z17369); 13-hydroxylase in gibberellin biosynthesis (CYP88A1, U32579); teasterone-23-hydroxylase (CYP90A1, X87367); obtusifoliol 14-α-demethylase (CYP51, U74319); fatty acid ω-hydroxylase (CYP86A1, X90458; cutin monomer synthase (CYP94A1, AF030260); allene oxide synthase (CYP74A1, U00428).

Southern blotting analysis of *P. hybrida* genomic DNA with CYP76A3: Southern blot hybridization was carried out to examine the number of *cyp76A3* genes in the genome of *P. hybrida.* There was one band in each of the three digests (*Dra*I, *Hin*dIII, and *Eco*RV; Fig. 3), indicating that the *P. hybrida* genome contains one gene, although a high degree of sequence similarity to P450 family was found in the *cyp76A3* gene.

Expression of CYP76A3 cDNA in yeast: In order to analyze the enzyme functions of CYP76A3, at first we attempted to express the cDNA clone of CYP76A3 in yeast. The multicopy plasmid pA76A3 inserted the coding region of CYP76A3 corresponding to the first methionine to the C-terminus between the alcohol dehydrogenase (ADH) I promoter and the terminator of expression vector pAAH5N. The expression plasmid was transformed into AH22 cells by means of the LiCl method. CO-difference spectrum of the microsomal fraction prepared from the transformed yeast AH22/pA76A3 cells showed a peak at 448 nm; the content of the P450 was estimated to be 25 pmol(P450 equivalent) mg^{-1}(protein) in the microsomal fraction.

Metabolism of substrates by CYP76A3 in microsomal fractions from the recombinant yeast cells: The microsomal fraction prepared from the recombinant yeast cells was used for an enzyme assay with a number of endogenous compounds as substrates. A substrate and NADPH were incubated with the microsomal fractions from the recombinant yeast strains and then the reaction

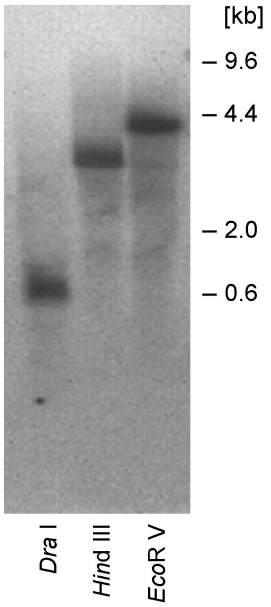

Fig. 3. Southern blot analysis of genomic DNA with CYP76A3. Genomic DNA prepared from petunia leaves was digested with *Dra*I, *Hin*dIII, and *Eco*RV. cDNA clone No-73, 916-1344 bp of CYP76A3 was used as a DNA probe.

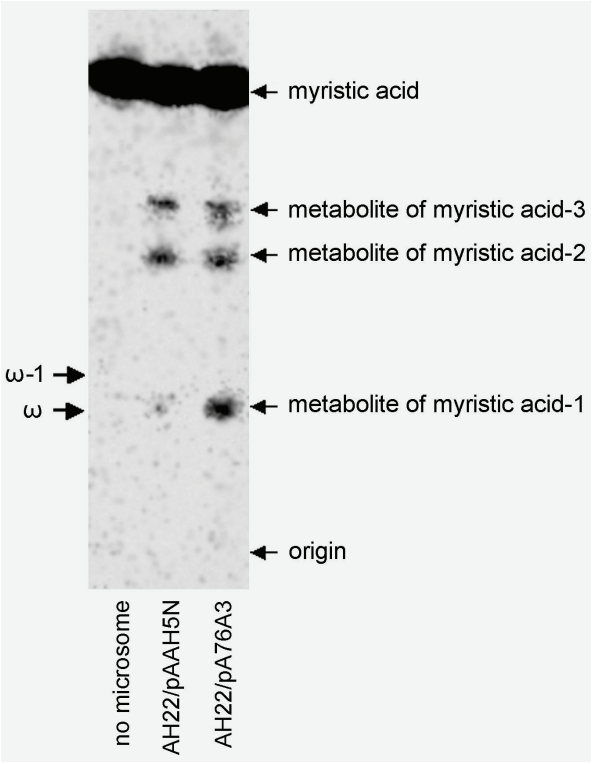

Fig. 4. TLC analysis of myristic acid and its metabolites. Co-chromatography of several hydroxylated myristic acid and [1-^{14}C]myristic acid metabolites is shown. The metabolism of myristic acid was assayed using microsomal fractions prepared from the recombinant yeast strains AH22/pAAH5N and AH22/pA76A3. A reaction mixture in 200 cm^3 of 100 mM potassium phosphate buffer (pH 7.4) contained 0.5 mM NADPH, 60 pM [1-^{14}C]myristic acid (1.85 MBq cm^{-3}), and 50 pM P450 protein. After extraction with ethyl acetate, each sample was spotted onto a thin-layer silica gel plate and then developed by a solvent system of diethyl ether/petroleum ether/formic acid (70:30:1 v/v/v). The radioactivity on the TLC plate was measured with a BAS 2000 image analyzer. The *arrows* on the left show the positions of ω- and (ω-1)-hydroxylated myristic acid that resulted from the non-radioactive TLC.

mixtures were analyzed for metabolites by means of HPLC or TLC. Possible metabolites were found with myristic acid, but not with the other compounds (Fig. 4). From [1-^{14}C]myristic acid, three metabolites were observed on the TLC plate. The main product (metabolite of myristic acid-1) was detected only in the reaction mixtures containing NADPH, [1-^{14}C]myristic acid, and the microsomal fraction of AH22/pA76A3. Thus, CYP76A3 appears to metabolize myristic acid. Other metabolites seem to come from the original yeast microsomal proteins. To determine the hydroxyl position of the product, we used CO-chromatography. Several control hydroxy myristic acids without radioactivity were spotted onto the same positions used to spot the metabolites of [1-^{14}C]myristic acid. The result showed that metabolite of myristic acid-1 produced by CYP76A3 was at the same position as the standard ω-hydroxy myristic acid (Fig. 5). Metabolite of myristic acid-1 was thus determined to be ω-hydroxy-myristic acid. An enzyme activity for CYP76A3 against myristic acid was determined to 30 mol min^{-1} per mol of P450.

Northern blot analysis: Northern blot analysis was carried out by using the cDNA clone No-73, 916-1344 bp of CYP76A3 as a probe for flowers, leaves, stems, and roots of *P. hybrida*. The transcripts of *cyp76B3* gene were shown with expected lengths. Only one band of transcripts of *cyp76B3* gene was obtained. The contents of transcripts hybridized with the *cyp76A3* cDNA were highest in the leaves, followed by roots. On the other hand, a low content of transcripts was found in the flowers, and no transcripts were found in the stems (Fig. 5).

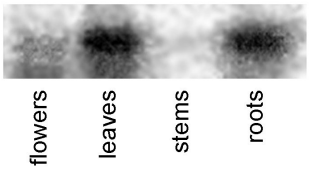

Fig. 5. Northern blot analysis of the poly (A)$^+$RNA from *P. hybrida*. Poly(A)$^+$RNA fractions obtained from flowers, leaves, stems, and roots were electrophoresed and transferred to a *Hybond-N* membrane, and 2 μg of the poly(A)$^+$RNA fraction was applied to each lane. cDNA clone No-73, 916-1344 bp of CYP76A3 was used as a DNA probe.

Discussion

We isolated the cDNA clone of a novel P450 (CYP76A3) from the leaves of *P. hybrida* by means of RT-PCR using primers specific to the domains conserved among the plant P450 species that have been previously reported (Holton *et al.* 1996). CYP76A3 showed the highest similarity (64 %) in the amino-acid sequence with that of CYP76A2 from eggplant seedlings (Toguri *et al.* 1993a), but also showed similarities of 63 % with CYP76A1 (Toguri *et al.* 1993) and 39 % with CYP76B1 (Batard *et al.* 1998, Robineau *et al.* 1998). The enzymatic functions of CYP76A1 and CYP76A2 have not yet been assigned, but CYP76B1 has been identified as 7-ethoxy-coumarin *O*-deethylase. The branching patterns of the dendrogram (Fig. 2) reveal that CYP76A3 belongs to a branch containing the CYP76A1, CYP76A2, and CYP75A (A1, A2, and A3) family (flavonoid-3′,5′-hydroxylase) and CYP76B1 (7-ethoxycoumarin *O*-deethylase), but not to the branch containing CYP88A1 (13-hydroxylase in the gibberellin biosynthesis pathway; Winkler and Helentjaris 1995), CYP90A1 (teasterone-23-hydroxylase;

Szekeres *et al.* 1996), CYP51 (obtusifoliol 14 α-demethylase; Bak *et al.* 1997), and CYP74A1 (allene oxide synthase; Song *et al.* 1991). Therefore, CYP76A3 is likely to have evolved within the family that is involved in the biosynthesis and metabolism of secondary metabolites, including phenylpropanoids rather than steroids and terpenoids. The phenylpropanoid biosynthesis pathway produces cell wall constituents (lignin), pigments (flavonoids), ultraviolet protectants (coumarins and flavonoids), and plant defence compounds (phytoalexins). Since the hypersensitivity response in plants is associated with the activation of a wide range of defence-related genes involved in the biosynthesis of plant secondary metabolites, CYP76A3 may also play a role in the biosynthesis of plant defence compounds.

High contents of the *cyp76A3* gene transcripts were found in the leaves and roots, but not in the stems or flowers. For protection against attack by fungi, plants constitutively express defence genes in their roots (Nomura *et al.* 2002) and the transcripts of *cyp76A3* were observed in *P. hybrida* roots (Fig. 5). These results suggest that CYP76A3 participates in the plant protection systems that defend against attack by fungi.

To analyze the enzymatic function of CYP76A3, we used the yeast expression system and a subsequent enzyme assay. We found that CYP76A3 in the yeast microsomes specifically metabolized myristic acid. P450 species in plants catalyze in-chain and ω-hydroxylation as well as epoxidation of fatty acids (Salaun and Helvig 1995). The biological roles and substrate specificity of the plant P450 species involved in fatty acids are not well understood. However, evidence suggests that some P450s play an important role in the biosynthesis of plant cuticles (Le Bouquin *et al.* 2001) and plant signalling compounds (Schuler 1996). Cutins and suberins, which prevent water loss and chemical penetration and protect plants from microbial attack, are mainly constituted of hydroxylated fatty acids. On the other hand, it is known that CYP74A1 participates in the biosynthesis pathway of jasmonates (Laudert *et al.* 1996). These reports suggest that CYP76A3 plays an important role in the biosynthesis of plant defence compounds and plant signalling compounds.

In conclusion, CYP76A3 is a novel P450 that differs significantly (a maximum similarity of 64 %) from previously characterized fatty acid oxygenases. CYP76A3 is the first reported P450 to be expressed in leaves and roots, and it is involved in the metabolism of myristic acid. CYP76A3 is highly specific for myristic acid and does not metabolize any of the other 21 fatty acids, plant secondary metabolites, and plant hormones that we tested. The biological roles of this newly reported plant P450 relate to the ω-hydroxylation of myristic acid, but details of these roles are not yet known. Further studies of *P. hybrida* CYP76A3 should lead to a better understanding of its physiological roles in this species.

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