Molecular Cloning of CYP76B9, a Cytochrome P450 from Petunia hybrida, Catalyzing the ω-Hydroxylation of Capric Acid and Lauric Acid

Hiromasa IMAISHI* and Mariana PETKOVA-ANDONOVA

Research Center for Environmental Genomics, Kobe University, Rokkodaicho 1-1, Nada-ku, Kobe 657-8501, Japan

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A cDNA encoding a cytochrome P450 (CYP76B9) was isolated from Petunia hybrida. Northern blot analysis revealed preferential expression of the gene in flowers and leaves. The recombinant yeast microsomes expressing CYP76B9 was allowed to react with capric acid and lauric acid as substrates. One major metabolite was produced from each fatty acid after incubation with yeast microsomes expressing CYP76B9. The metabolites were identified by gas chromatography–mass spectrometry (GC–MS) as ω-hydroxy capric acid and ω-hydroxy lauric acid. The kinetic parameters of the reactions were $K_{m} = 9.4 \mu\text{M}$ and $V_{\text{max}} = 13.6 \text{ mol min}^{-1}$ per mol of P450 for capric acid, and $K_{m} = 5.7 \mu\text{M}$ and $V_{\text{max}} = 19.1 \text{ mol min}^{-1}$ per mol of P450 for lauric acid. We found that the ω-hydroxy metabolites of capric acid and lauric acid can affect the plant growth of Arabidopsis thaliana. Plants grown in the presence of ω-hydroxy fatty acids exhibited shorter root length than control plants with the corresponding non-hydroxylated fatty acids.

Key words: cytochrome P450; Petunia hybrida; ω-hydroxylation; lauric acid; capric acid

Cytochrome P450 monoxygenases catalyze the oxidative reactions of a wide variety of endogenous and exogenous lipophilic compounds. In higher plants, P450 enzymes are mainly involved in the biosynthesis of secondary metabolites, such as fatty acids, phenylpropanoids, terpenoids, and plant signal molecules.1,2) Normal development in a model plant such as Arabidopsis thaliana (A. thaliana) has three overlapping developmental phases: vegetative development, inflorescence development, and flower development.3) A few cytochrome P450-mediated reactions give products necessary for control of cell expansion in plants. The mutations of P450 species in A. thaliana known as dwarf 4 and lcr give dwarfed plants.4,5) Another example is CYP78A5, reported possibly to have a role in controlling growth of the shoot apical meristem.6) Fatty acids are major components of cell membranes and are responsible for the majority of permeability functions of membranes.7,8) Plants synthesize many fatty acid derivatives, several of which have important regulatory roles such as jasmonates.9) The first enzyme in the biosynthesis of jasmonic acid is allene oxide synthase, an enzyme that converts fatty acid hydroperoxides to allene oxides. Laudert et al. reported the cDNA cloning and functional characterization of A. thaliana allene oxide synthase (CYP 74A1).10) Mammalian fatty acid ω-hydroxylases have been extensively studied. They belong to the CYP4 family of cytochrome P450 species.11) These species are induced by compounds that are known to stimulate peroxisomal proliferation.12) In yeast species, the CYP52 family is induced by aliphatic hydrocarbons.13) CYP52A3 and CYP52A4 catalyze terminal hydroxylation of n-alkanes in the assimilation pathway.14)

A limited number of fatty acid ω-hydroxylases have been characterized in higher plants. CYP86A1 was the first fatty acid ω-hydroxylase isolated from A. thaliana.15) It was found to catalyz e the ω-hydroxylation of saturated and unsaturated fatty acids with chain lengths from C12 to C18. Microsomes from apical buds of pea seedlings hydroxylate lauric acid at the ω-position.16) CYP94A1 from Vicia sativa (V. sativa) is capable of hydroxylating the methyl terminus of 9,10-epoxysearic and 9,10-dihydroxysearic acids to form the corresponding 18-hydroxy derivatives.17,18) CYP94A2 from V. sativa catalyzes the hydroxylation of myristic, lauric, and palmitic acids.19) The metabolites formed from lauric and myristic acids have been identified by GC–MS as the corresponding ω- and (ω-1)-hydroxy fatty acids, and from palmitic acid as the corresponding (ω-1)- and (ω-2)-hydroxy fatty acids.20) CYP94A5 from tobacco

* To whom correspondence should be addressed. Tel/Fax: +81-78-803-5940; E-mail: himaish@kobe-u.ac.jp

Abbreviations: AHD, alcohol dehydrogenase; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; GC–MS, gas chromatography–mass spectrometry; GSP, gene-specific primer; RACE-PCR, rapid amplification of cDNA-end polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; SSC, saline-sodium citrate; TLC, thin-layer chromatograph
catalyze the $\omega$-hydroxylation of 9,10-epoxysearic acid.\textsuperscript{20} CYP78A1 from Zea mays has been found to catalyze 12-monoxygenation of lauric acid.\textsuperscript{21} 

CYP703A1\textsuperscript{22,23} and CYP92B1\textsuperscript{23} have been cloned from P. hybrida. CYP703A1 was found to metabolize lauric acid. CYP92B1 was also found to metabolize lauric, linoletic, and linolenic acid. Functional analysis of a cytochrome P450 monoxygenase, CYP86A8 in A. thaliana, which catalyzes $\omega$-hydroxylation of fatty acids, revealed several different roles of fatty acid $\omega$-hydroxylation in the development of Arabidopsis thaliana.\textsuperscript{5,24} These reports show that the P450 species involved in the in-chain hydroxylation and/or $\omega$-hydroxylation of fatty acids participate in the growth and development of A. thaliana. However, the cDNA sequences and enzyme function of these P450 forms are poorly understood in higher plants.\textsuperscript{1,25}

To analyze the cDNA structure and enzyme function of P450 species involved in the metabolism of fatty acids, we attempted to isolate a novel P450 from Petunia hybrida. In this paper, we describe the cloning of a novel P450 species, CYP76B9, isolated from P. hybrida, characterize its enzyme properties, and investigate the effects of medium-chain fatty acids on plant growth, using wild-type A. thaliana as a model higher plant.

Materials and Methods

Materials. Radiolabeled [1-\textsuperscript{14}C] capric acid and [1-\textsuperscript{14}C] lauric acid were obtained from Amersham International. (Buckinghamshire, UK), and $\omega$-hydroxy capric acid and $\omega$-hydroxy lauric acid were from by Nacalai Tesque (Kyoto, Japan). Thin-layer plates (Silica Gel G60 F254; 0.25 mm) were from Merck (Darmstadt, Germany). All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

Growth of plants. P. hybrida was grown as previously described.\textsuperscript{22,23} A. thaliana ecotype columba (Col-0) was used. To analyze the physiological effects of fatty acids on the growth of A. thaliana, plants were grown in the presence or absence of fatty acids (capric acid, $\omega$-hydroxy capric acid, lauric acid, and $\omega$-hydroxy lauric acid) for 8 weeks under a 10-h light, 14-h dark photoperiod at 25°C in a growth chamber. The pH of culture medium was adjusted to 5.8–6.0 before autoclaving.

RT-PCR cloning of the partial sequence of the P450 species. The P450 gene was cloned by the RT-PCR technique.\textsuperscript{22,23} A Quick Micro mRNA purification kit (GE Healthcare, Milwaukee, WI) was used to isolate poly(A)$^+$ RNA from a mixture of petunia flowers, leaves, stems, and roots. A pair of degenerate primers (primers 4 and 5B) corresponding to the primers used by Holton and Lester\textsuperscript{26} for random cloning of petunia P450 species were used: primer 4, 5'-CTAGTGGACCGTTAGGCGAATTCGGA-3' and primer 5B, 5'-TTA(C.G)(C.G)IGAI(T.C)-T(T.A).TICG(T.)H(A.C)A(T,C).-3'. The temperature program was 3 min at 94°C, 50 cycles of 50 s at 94°C, 50 s at 45°C, and 45 s at 72°C. PCR products were separated on 2% agarose gels, and a major band was extracted on a GeneElute agarose spin column (Supelco, Bellefonte, PA). Purified cDNA fragments were cloned into the EcoRI/HindIII site of pBluescript II SK+ (Stratagene, La Jolla, CA), transformed into Escherichia coli strain JM109, and sequenced. Several randomly selected clones were sequenced, and the resulting sequences were compared with those of reported plant P450 species using the Protein-protein BLAST search program.

5', 3' RACE-PCR method. To obtain cDNA from low-abundance mRNA, we used the RACE-PCR method,\textsuperscript{27} employing the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). Poly(A)$^+$ RNA was isolated from petunia leaves. An adapter ligated cDNA library was made following the instruction manual. Using sequences of the 5'-terminal region and the 3'-terminal region of clone-8, we designed GSP-1 and GSP-2. The following RACE-PCR primers were used (see Fig. 1): GSP-1, 5'-CGACCTCGGATATCTATTTCTGACTCCCA-3', and GSP-2, 5'-CTAGTGGACCGTTAGGCGAATTCGGAATTCGGA-3'. PCR amplification of full-length cDNA was performed using the adapter ligated cDNA library as a template and one pair of GSP-1, GSP-2 primers. The PCR conditions for RACE-PCR were 1 min at 94°C, 25 cycles of 30 s at 94°C, 4 min at 68°C. The RACE-PCR specific products were separated on 1% agarose gels, specific bands were extracted on a GeneElute agarase spin column, and the purified fragments were subcloned into pgT7Blue vector (Novagen, Madison, WI) and sequenced.

Inverse PCR cloning. Genomic DNA from leaves of petunia was prepared as described by Imaishi et al.\textsuperscript{22} Restriction digestion was carried out using 1$\mu$g of petunia genomic DNA treated with 10 units of XhoI for 16 h at 37°C. The restriction enzyme reaction mixture was extracted once with an equal volume of phenol: chloroform (1:1). DNA fragments were precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol and kept at −80°C for 2 h. The DNA pellet was washed once in 70% ethanol, dried, and resuspended in 10$\mu$L of sterile water. For circularization, 0.5$\mu$g of the restriction fragments was diluted to a concentration of 1 ng/ml in ligation buffer (50 mM Tris HCl, pH 7.4, 10 mM MgCl$_2$, 10 mM dithiothreitol, 1 mM adenosine triphosphate). The ligation reaction was initiated by the addition of T4 DNA ligase to a concentration of 20 unit/ml, and the reaction was allowed to proceed for 12 h at 15°C. The PCR reaction was performed with a mixture (50$\mu$L) of 0.5 ng of circularized DNA obtained as described above, 50 pmol of each primer, 500$\mu$L dNTPs, and 2.5 units Taq-
polymerase (Parkin-Elmer, Norwalk, CT). We carried out two PCR reactions with the PCR primers corresponding to the N-terminal regions of IMT-8 cDNA. In the first reaction, primers-1 and -2 were used. Then the second reaction was performed using primers-3, -4 and 1 μl of sample from the first PCR reaction. The nucleotide sequences of primers used are as follows: (see Fig. 1): primer-1, 5'-ACTTCTCTGGCAGCGACTGATGAAATG-3'; primer-2, 5'-TCTTGCAAAAACAAGCAGCATTAATTTTCC-3'; primer-3, 5'-TGAC-CACCGTGATTAGTTGGCCTAATTTGAG-3'; primer-4, 5'-TCCAATAGGTTTGTCCCGGACGTAGTCC-3'. The positions of the inverse PCR primers are indicated by arrows in Fig. 1. The cycling conditions were: 1 min 95°C, 2 min 65°C, 3 min 72°C, for 35 cycles. The DNA products from the second PCR reaction were separated on a 0.7% agarose gel, and DNA bands were purified and ligated to the pT7Blue T-Vector and sequenced.

RT-PCR cloning of full-length cDNA. To obtain full-length cDNA from petunia leaves, RT-PCR was carried out. Poly(A)+ RNA were isolated from petunia leaves using a Quick-Prep Micro mRNA Purification Kit (Pharmacia Biotech, Piscataway, NJ, USA). First-strand cDNA was synthesized from 100 ng of poly(A)+ RNA using a First-Strand cDNA Synthesis Kit (Pharmacia Biotech) according to the protocol of the manufacturer, and 10 ng were used for PCR reactions. RT-PCR was performed with 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 0.25 mM dNTP each, 25 pmol of

Fig. 1. Nucleotide Sequence and Deduced Amino Acid Sequence of CYP76B9.

Domains A, B, C, and D, which are highly conserved among P450 genes, are underlined. Arrows indicate the positions of GSP-1, GSP-2, primer-1, primer-2, primer-3, primer-4, primer-U and primer-L. Clone-8 is designated in bold face.
primers, 2.5 units Taq-polymerase (Parkin-Elmer, Norwalk, CT). The temperature program was 30s 94°C, 2min 50°C, 3min 72°C, 30 cycles. The following RT-PCR primers were used (see Fig. 1): primer-U, 5'-ATGGACATCGTAATTTTGCAGACTG-3'; primer-L, 5'-CTACAGGAGATGTTGGATAGCTAGCAG-3'. The PCR products obtained were separated on 1% agarose gel, and the PCR product around 1.7 kbp was extracted using a GeneElute Agarose Spin Columns (Supelco, Bellefonte, PA). Purified cDNA fragments were cloned to the Smal site on pBluescript II SK+ and sequenced.

Northern blot analysis. Total RNA from petunia leaves, flowers, roots, and stems was extracted with Sepasol RNA I Super Reagent (Takara Shuzo, Otsu, Japan). After isolation, RNA was precipitated with 70% ethanol, and stored at −80°C. The RNA samples were separated by gel electrophoresis (0.8% agarose, 1% formaldehyde) and capillary transferred onto a Hybond-N+ nylon membrane (Amersham International, Buckinghamshire, UK). Equal loading of RNA was checked by ethidium bromide staining of the gel. The immobilized RNA was hybrized at 65°C for 12 h in hybridization solution (2× SSC, 7.5× Denhardt’s reagent, 200 µg/ml salmon sperm DNA), with the 32P-labeled 389-bp PCR product created by clone-8 as a probe. The hybridized blots were washed twice with 2× SSC/0.1% SDS at room temperature for 15 min. An FLA-2000 bioimage analyzer (Fuji, Tokyo) was used to detect radioactivity on the membrane.

Southern blot analysis. Genomic DNA from leaves of petunia was prepared as described by Imaishi et al.22) EcoRI, HindIII, and DraI were used to digest the purified genomic DNA. The digested DNA was separated by 0.7% agarose gel electrophoresis and transferred to a Hybond-N+ nylon membrane with 20× SSC buffer. The hybridization and washing conditions were the same as those described for RNA blot analysis.

DNA sequence and computer analysis. DNA sequencing was carried out as described in Ref. 22.

Expression of CYP76B9 in S. cerevisiae. Cloning of CYP76B9 into pAUR123 (Takara Bio, Otsu, Japan) was performed by PCR amplification using two specific primers to introduce a SalI site upstream of the initiation codon and an XbaI site downstream of the stop codon. The upstream primer sequence was AAAATGGACATCGTAATTTTGCAGACTG, and the downstream primer was AAAATGCTTTACAGGGGAGTGGGATAGCTAGCAG. The upstream primer sequence was inserted into the expression plasmid pAUR76J1. After digestion with SalI/XbaI to construct the expression plasmid pAUR76J1. The resulting plasmids, pAUR123 and pAUR76J1, were introduced into S. cerevisiae by the lithium chloride method of Oeda.28) The yeast culture was grown in 2 ml of YPD medium at 30°C for 16 h, and the cells were collected and resuspended in 0.1 M lithium chloride. The cells were then incubated with 1 µg of the plasmid DNA, 10 mg of denatured salmon sperm DNA as a carrier, and 40% (w/v) polyethylene glycol at room temperature for 30 min. Finally, the transformed cells were cultured in YPD medium at 30°C for 60 min and spread onto YPD plates containing 0.5 µg/ml of Aureobasidine A for selection.

Yeast cells were cultured in YPD medium at 30°C for 48 h.29) After shaking, yeast cells were collected by centrifugation at 7,000 g for 10 min and washed with 0.1 M potassium phosphate buffer (pH 7.4). The collected pellets were resuspended in 40 ml of zymoloyase buffer (10 mM Tris–HCl, pH 7.5, 2 mM sorbitol, 0.1 mM DTT, 0.1 mM EDTA) containing 300 µg/ml of zymolyase-100T (Seikagaku Kougyo, Tokyo), and incubated with gentle shaking at 30°C for 1 h. After centrifugation, the spheroplasts, washed with zymoloyase buffer, were resuspended in 15 ml of sonication buffer (10 mM Tris–HCl, pH 7.5, 0.65 mM Sorbitol, 0.1 mM DTT, 0.1 mM EDTA, 1 mM PMSP) and placed on ice for brief sonication (50 W for 2 min, 3 times). The lysate was centrifuged to precipitate cell debris. The resulting supernatant was transferred to centrifuge tubes and centrifuged at a setting of 10,000 g for 60 min at 4°C. The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.5) containing 10% glycerol and 4.2 mM 2-mercaptoethanol, and stored at −80°C. Reduced CO-difference spectra of microsomal fractions prepared from the transformed yeast cells were measured by the method of Oeda et al.28) The P450 hemoprotein content in the microsomal fraction was estimated on using an extinction coefficient of 91 mmol−1 cm−1.28) Protein assay was carried out by the method of Bradford using Bio-Rad Protein Assay Kit I (Bio-Rad Laboratories, Tokyo) with BSA as a standard.

Enzyme assay. Fifty picomoles of P450 protein in a microsomal fraction prepared from recombinant yeast strain AH22/pAUR123 or AH22/pAUR76J1 was added to a final volume of 400 µl of buffer solution (0.1 M potassium phosphate buffer, pH 7.4, 2 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 10 µM radiolabeled [1-14C] capric acid or [1-14C] lauric acid). After preincubation for 2 min at 30°C, reactions were initiated by the addition of 5 mM NADPH, allowed to proceed for 1 h, and then stopped by the addition of 40 µl of HCl. The reaction mixtures were extracted twice with 800 µl of ethyl acetate, and the extracts were dried and then dissolved in methanol. The total radioactivity in the extracts was measured directly in a liquid
scintillation counter (LSC 3500, Aloka, Tokyo), using Clear-Sol (Nakalai Tesque, Kyoto, Japan) for measurement of radioactivity. Samples containing a total radioactivity of 30,000 dpm were applied to TLC plates precoated with silica gel 60F254 (Merck, Darmstadt, Germany). The TLC solvent system for capric acid and lauric acid was diethyl ether:pentane ether:formic acid (70:30:1 by volume). Radioactivity on the TLC plate was measured with a FLA-2000 biomass analyzer. Kinetic parameters (V_{max} and K_{m}) were estimated by nonlinear regression of the collected data (n = 3) using substrate concentrations ranging from 0.3 to 100 μM.

Identification of capric acid and lauric acid metabolites. For GC–MS analysis, nonradioabeled capric acid and lauric acid were used. Lauric acid metabolism was assayed as described above, except that in this experiment the reaction mixture was prepared in a final volume of 5 ml. Metabolites were resolved by silica TLC, and the solvent system was diethyl ether:pentane ether:formic acid (70:30:1 by volume). The area corresponding to polar metabolites generated from the 5-ml reaction mixture was scraped and eluted twice from silica with 4 ml of ethyl acetate. Eluted compounds were evaporated under nitrogen and dehydrated over P_{2}O_{5} and silica gel for 16 h. The dried residues were resuspended in 125 μl of a 5% hydrogen chloride: methanol solution, heated at 70 °C for 1 h, evaporated under nitrogen, and dehydrated again over P_{2}O_{5} and silica gel for 16 h. The residues were resuspended in 100 μl of hexane and submitted to gas chromatography and electron impact (70 eV) ionization mass spectrometry (Hitachi Model M-9000 System (Hitachi, Tokyo) on a 1% SE30 capillary column, 30 × 0.25 mm programmed from 130 to 250 °C at 3 °C/min.

Effect of medium-chain fatty acids on the growth of plants. Wild-type A. thaliana seeds were sterilized in 70% ethanol for 1 min, and then in a mixture of 5% NaClO and 0.05% Tween-20 for 10 min. Seeds were grown on MS medium with or without 100 μM of fatty acids or hydroxy fatty acids for 20 d at 23 °C under continuous light.

Results

Cloning of full-length cDNA of a novel P450 species

RT-PCR cloning of the partial sequence of the P450 species was carried out. One PCR fragment, designated clone-8 (389 bp, see Fig. 1), encoded a novel P450-like sequence. The 5'- and 3'-RACE-PCR methods were employed to obtain the partial cDNA fragments of P450 in petunia. A 5'-terminal fragment (1,450 bp) and a 3'-terminal fragment (763 bp) were obtained. These products were subcloned for DNA sequencing. Analysis of the deduced amino acid sequences of nested RACE PCR products showed that the cDNA clone, designated IMT-8, lacked the 5'-region.

Hence inverse PCR was carried out to obtain information on the amino-terminal region of IMT-8. A 1,1-kb fragment containing the 5'-region was isolated. The full-length cDNA was obtained by RT-PCR with 5'- and 3'-gene-specific primers, primer-U and primer-L, designed on sequences of RACE-PCR and inverse PCR products (see Fig. 1). We found that the full-length cDNA clone called P450-8 consisted of a 1,719-bp insert with an open reading frame encoding polypeptides of 573 amino acids (Fig. 1). All of the structural and functional motifs common to P450 proteins, including the N-terminal membrane anchor sequence, the proline-rich region, and the heme-binding region, are highly conserved in P450-8. P450-8 was officially named CYP76B9 by D. R. Nelson (Committee for Standardized Cytochrome P450 Nomenclature). The similarity scores for the amino acid sequences showed that the highest sequence similarity of CYP76B9 was 54% with CYP76B1, a xenobiotic-inducible 7-ethoxycoumarin O-deethylase from Helianthus tuberosus. The similarities of CYP76B9 to CYP92B1 and CYP703A1, P450 species preferentially expressed in petunia flower buds, were 30% and 24% respectively.

Northern blot analysis

Northern blot analysis of CYP76B9 was carried out by the use of clone-8 as a DNA probe for the flowers, roots, stems, and leaves of petunia (Fig. 2). As shown in Fig. 2, the level of transcripts hybridized with CYP76B9 was highest in the leaves. On the other hand, low levels of mRNA for CYP76B9 were detected in stems and roots. Thus, CYP76B9 was found to be expressed mainly in flowers and leaves of petunias.

Southern blot analysis

Southern blot analysis of CYP76B9 revealed three bands after EcoRI digestion, but only one band in restriction digests with HindIII and DraI, indicating that the petunia genome contains one copy of the cyp76b9 gene (Fig. 3).
Expression of CYP76B9 in a yeast

To characterize the enzymatic properties of CYP76B9, this P450 species was expressed in the yeast *S. cerevisiae* under the control of the AHD promoter. *S. cerevisiae* AH22 cells were transformed with expression plasmids pAUR123 and pAUR76J1. The reduced CO-difference spectrum of the microsomal fractions from AH22/pAUR76J1 showed an absorption peak at 449 nm (Fig. 4). The P450 content was estimated to be 43 pmol of P450 equivalent per milligram of microsomal protein in each fraction. In contrast, the yeast cells transformed with AH22/pAUR123 showed no characteristic P450 spectrum. These results suggest that CYP76B9 was functionally expressed in the microsomes of recombinant yeast cells and was localized in the yeast microsomes.

Metabolism of fatty acids in yeast microsomes expressing CYP76B9

The microsomal fractions prepared from the recombinant yeast cells were used for metabolism assays of endogenous fatty acids. The substrates and NADPH were incubated with microsomal fractions from recombinant yeast strains AH22/pAUR123 and AH22/pAUR76J1. TLC analysis of the reaction mixtures revealed that CYP76B9 recombinant yeast microsomes metabolized capric acid and lauric acid (Fig. 5A, C). The *R*<sub>f</sub> value (0.34) of the lauric acid metabolite was coincident with that of *ω*-hydroxylated lauric acid. No activities were found in the microsomal fraction of AH22/pAUR123. These results suggest that CYP76B9 expressed in recombinant yeast microsomes catalyzed the metabolism of the C10 and C12 families of fatty acids. GC–MS analysis led to characterization of the metabolites of capric acid and lauric acid. A single polar metabolite was detected after the recombinant yeast microsomes expressing CYP76B9 were incubated with capric acid (Fig. 5A). The metabolite was purified by TLC, analyzed by gas chromatography, and identified as *ω*-hydroxy capric acid by comparison with the authentic compound. The mass spectrum showed characteristic ions at *m/z* 203 (M<sup>+</sup>, parent peak), 185 (M<sup>+</sup> − H<sub>2</sub>O), 172 (M<sup>+</sup> − OCH<sub>3</sub>), 144 (M<sup>+</sup> − H<sub>2</sub>CO − C=O), and 74 and 87 (methyl esters of McLafferty fragment ions) (Fig. 5B). In a similar way, the lauric acid metabolite purified by TLC (Fig. 5C) and analyzed by GC–MS was identified as *ω*-hydroxy lauric acid by comparison with the authentic compound. The mass spectrum showed characteristic ions at *m/z* 231 (M<sup>+</sup>, parent peak), 213 (M<sup>+</sup> − H<sub>2</sub>O), 200 (M<sup>+</sup> − OCH<sub>3</sub>), and 74 and 87 (methyl esters of McLafferty fragment ions) (Fig. 5D).

The enzyme properties of CYP76B9 were determined using various substrate concentrations (from 0.3 to 10 μM). The kinetic parameters of the reactions were *K<sub>m</sub> = 9.4 μM and *V<sub>max</sub> = 13.6 mol min<sup>−1</sup> per mol of P450 for capric acid, and *K<sub>m</sub> = 5.7 μM and *V<sub>max</sub> = 19.1 mol min<sup>−1</sup> per mol of P450 for lauric acid (Fig. 6).

Effects of fatty acids on plant growth and development

Figure 7 shows the result for *A. thaliana* roots treated with *ω*-hydroxy capric acid and *ω*-hydroxy lauric acid. In treatment with 1, 10, and 100 μM *ω*-hydroxy capric acid, the root lengths were 5.10, 3.30, and 0.41 cm, respectively (Fig. 7), indicating that the root length was reduced by 13.4-fold with respect to that of the control.
(5.50 cm) on medium supplemented with 100 μM β-hydroxy capric acid. One hundred μM β-hydroxy lauric acid similarly reduced the root length to 8.1% of the control (1 μM, 5.20; 10 μM, 3.20; and 100 μM, 0.45 cm; Fig. 7). However, as shown in Fig. 7, CA and LA, capric acid and lauric acid, did not inhibit root elongation.

**Discussion**

Cytochrome P450 species from plants are involved in the oxidation of a large number of compounds, including fatty acids.\(^{32}\) We report here the cDNA cloning of CYP76B9 from petunia. The amino acid sequence similarity of CYP76B9 was 30% and 24% with
CYP92B1 and CYP703A1 respectively in petunia. Amino acid sequence comparison showed that the highest sequence similarity of CYP76B9 was 54% with CYP76B1. CYP76B1 was expressed in yeast cells. Microsomes from the transformed yeast catalyzed the \( \text{O}^- \)-dealkylation of 7-ethoxycoumarin. 31) CYP76B9 did not metabolize 7-ethoxycoumarin (data not shown). In order to analyze the enzyme function of CYP76B9, CYP76B9 was expressed in yeast AH22 cells. CYP76B9 expressed in recombinant yeast cells catalyzed \( \omega \)-hydroxylation of capric acid and lauric acid. Imaishi et al. previously demonstrated the oxidation of lauric acid to \( \omega \)-hydroxy lauric acid by CYP78A1. 21) CYP92B1 from petunia, expressed in a yeast, converted linoleic acid to two polar metabolites. 22)

The interplay of multiple enzymes in the oxidative metabolism of fatty acids makes it difficult to understand the biological role and effects of individual metabolites generated during the oxidative cascade of saturated and unsaturated fatty acids. 33) Cutin covers the aerial part of leaves and fruits and acts as a barrier against mechanical stress and pathogen attack. 7) Cutin is generated mainly from hydroxylated and epoxidized derivatives of medium- and long-chain fatty acids such as palmitic acid, oleic acid, linoleic acid, and linolenic acid. 33) In this study, it was found that CYP76B9 metabolized medium-chain fatty acids (capric acid and lauric acid). These results therefore suggest that the role of CYP76B9 in cutin biosynthesis is not of primary importance.

We studied the possibility that \( \omega \)-hydroxy capric acid and hydroxy lauric acid affect plant development. For this purpose, we used wild-type A. thaliana as a model plant. Much of the progress in the genetic dissection of plant lipid metabolism has come from extensive studies of A. thaliana by Sommerville et al., who attempted to understand the relationships between membrane fatty acid composition and cell physiology. 34) We found that growth of A. thaliana, especially root growth and elongation, was inhibited by the addition of \( \omega \)-hydroxy capric acid and \( \omega \)-hydroxy lauric acid (Fig. 7). In contrast, capric acid and lauric acid did not affect the growth of A. thaliana roots. We speculate that CYP76B9-catalyzed oxygenation of capric acid and lauric acid produces derivatives that participate in pathways that regulate plant growth phenomena. Lee has shown that aliphatic fatty acids (oleic acid) can cause the loss, through changes in root permeability, of ions such as K\(^+\), Ca\(^+\), and NO\(^-\)/CO\(^2-\), resulting in inhibition of root growth of Hordeum vulgare. 35) We think that there is a possibility that the difference in permeability among lauric acid, capric acid, and these \( \omega \)-hydroxides influences the root growth of A. thaliana.

We have shown that CYP76B9 is a capric acid \( \omega \)-hydroxylase and a lauric acid \( \omega \)-hydroxylase, but the biological roles of fatty acid monooxygenases, including
this newly reported plant P450 cytochrome, are not well understood. Additional studies on this P450 cytochrome are needed to help clarify the biological roles of fatty acid hydroxylases in the regulation of growth and development in higher plants.

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References


