CYP92B1, A Cytochrome P450, Expressed in Petunia Flower Buds, That Catalyzes Monooxidation of Long-Chain Fatty Acids

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In higher plants, long-chain 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 oxidation of fatty acids from Petunia hybrida. A P450 cDNA not isolated earlier, CYP92B1, contained an open reading frame predicted to encode a polypeptide consisting of 510 amino acid residues. The transcript of the cyp92B1 gene was expressed at a high level in the early stage of flower development. CYP92B1 cDNA was expressed in a yeast, Saccharomyces cerevisiae, and recombinant yeast microsomes containing CYP92B1, a hemoprotein, metabolized lauric acid, linoleic acid, and linolenic acid.

Key words: cytochrome P450; Petunia hybrida; lauric acid; linoleic acid; linolenic acid

Cytochrome-P450-dependent monooxygenases can catalyze in-chain and ω-hydroxylation as well as the epoxidation of medium- and long-chain fatty acids. Plants, like other eukaryotes, synthesize lipids for membrane biogenesis, as signal molecules, and as a form of stored energy. The physical properties of such membranes are largely governed by chain length, polarity, and the degree of fatty acid unsaturation. The C16 and C18 families of fatty acids, containing one to three double bonds, are common in plants.

The physiological role and substrate specificity of cytochromes P450 are poorly understood. Several P450s that catalyze the hydroxylation of fatty acids have been proposed for use in crop improvement. For example, CYP78A9 and LACERATA (lcr), seem to control the establishment of apical dominance and senescence in plants, respectively. Ito and Meyerowitz found that CYP78A9 mutants had larger ovaries than the wild type. Wellesen et al. described LACERATA mutants of Arabidopsis thaliana with developmental abnormalities. The lcr gene encodes a cytochrome P450, CYP86A8, that catalyzes ω-hydroxylation of fatty acids. In Nicotiana tabacum, a cytochrome-P450-dependent fatty acid hydroxylase, CYP94A5, oxidizes a terminal methyl group to the corresponding carboxyl of saturated and unsaturated fatty acids with aliphatic chains ranging from C12 to C18.

Another group of P450s are allene oxide synthase and hydroperoxide lyase, involved in the metabolism of fatty acid hydroperoxides. Unlike, P450 monoxygenases, which need molecular oxygen and NADPH-dependent cytochrome P450 reductase for activity, allene oxide synthase and hydroperoxide lyase use an acyl hydroperoxide both as the oxygen donor and as the substrate in which new carbon-oxygen bonds are formed. The metabolites of unsaturated fatty acids made by CYP74 serve as the precursor in the enzymatic synthesis of jasmonic acid. There has been increasing interest in jasmonic acid, a derivative of linolenic acid (C18:3), because of evidence, of its involvement in plant defense, signaling, tuber formation, and fruit ripening. Large amounts of ω-hydroxylated long-chain fatty acids have been found in pistils and pollen from several tobacco species. They seem to be involved in fertilization. Derivatives of linoleic acid and linolenic acid (jasmonic acid and ω-ketols) may participate in plant cuticle biosynthesis and stress induction in Lemna paucicostata.

Little is known about P450s involved in the oxidation of long-chain fatty acids. Recently, we have reported that CYP703A1 from Petunia hybrida and CYP78A1 from Zea mays catalyze the monoxygenation of lauric acid. Lauric acid has been used as a model substrate for hydroxylation catalyzed by P450s. There are no reports about the metabolism of the C18 family of fatty acids by P450s specifically expressed in the early stage of flower development.

Cytochrome P450 proteins are membrane-bound monooxygenases that catalyze many reactions of secondary metabolism. In this study, we undertook the
isolation of P450 expressed only during flower development in *P. hybrida*. Here, we report the cloning and sequencing of a cDNA, that encoded CYP92B1. The heterologous expression in a yeast allowed examination of its enzymatic function, with NADPH as the electron donor. We found that CYP92B1 metabolized medium- and long-chain fatty acids: lauric acid, linoleic acid, and linolenic acid.

**Materials and Methods**

**Plant materials.** *P. hybrida* seeds cv Blue Star were purchased from Sakata Seeds Co., (Yokohama, Japan) and grown for 9 weeks with a 10-h light and 14-h dark photoperiod at 25°C in a growth chamber.

**Chemicals and biochemicals.** DNA modification enzymes and restriction endonucleases were purchased from Takara Shuzo Co., Ltd. (Shiga, Japan), New England Biolabs, (Beverly, MA), and Toyobo Biochemical Inc. (Tokyo, Japan). Uni-ZAPII cDNA synthesis kit was obtained from Stratagene (La Jalla, CA). A DNA ligation kit (version 2), random primer DNA labelling kit, and yeast expression vector pAUR123 were obtained from Takara Shuzo. GeneElute Agarose Spin Columns were purchased from Supelco, Inc. (Bellefonte, PA). Quick Prep Micro mRNA purification kit and Isoplant kit were obtained from Pharmacia P-L Biochemicals, Inc. (Milwaukee, WI) and Nippon Gene Co., Ltd. (Tokyo, respectively. Hybond-N+ nylon membranes were purchased from Amersham International Inc. (Buckinghamshire, U.K). The Expand High Fidelity PCR System was obtained from Roche Diagnostics Co. (Indianapolis, IN). pT7Blue T-vector was purchased from Novagen, Inc. (Madison, WI). [32P]dCTP, [1-14C]lauric acid, [1-14C]linoleic acid, and [1-14C]arachidonic acid were purchased from Amersham Life Science (Tokyo, Japan). [1-14C]Myristic acid, [1-14C]palmitic acid, [1-14C]linolenic acid, and [1-14C]testosterone were purchased from DuPont NEN (Boston, MA). [1-14C]Stearic acid and [1-14C]oleic acid were purchased from Moravek Biochemicals, Inc. (Brea, CA). [1-14C]Pelargonic acid was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). [1-14C]Chlortoluron, [1-14C]atrazine and [1-14C]simazine were obtained from Novartis International Inc. (Basle, Switzerland). Other reagents were obtained from Nakalai Tesque, Inc. (Kyoto, Japan), Wako Pure Chemicals, Inc. (Osaka, Japan), and Sigma Chemical Co., (St. Louis, MO).

**RT-PCR cDNA cloning.** The flower development of *P. hybrida* was divided into five stages: stage 1, flower buds 1 to 2 mm; stage 2, floral buds more than 2 to 4 mm; stage 3, floral buds more than 4 to 12 mm; stage 4, floral buds more than 12 to 30 mm; and stage 5, mature flower. 15 Poly(A)+RNA was isolated from flower buds of *P. hybrida* (Stage 1 to 5) with mRNA purification kit. First-strand cDNA was synthesized from 100 ng of poly(A)+RNA using a First-Strand cDNA synthesis kit (Pharmacia Biochem.) according to the protocol of the manufacturer and 10 ng were used for PCR. RT-PCR was done with 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 0.25 mM each dNTP, 25 pmol of primers, and 2.5 units of Taq polymerase (Perkin-Elmer, Foster City, CA). The temperature program was 30 sec at 94°C, 2 min at 50°C, 3 min at 72°C, for 30 cycles. The primers were: upstream, 5’-ATGAAATCCAGAAAAATTCATTCA-AAGCACCC-3’ (primer A), and downstream, 5’-ATAAGCTTAAATCTTCGTCCAGCACCAGTA-3’ (primer D). The PCR products obtained were separated on a 2% agarose gel and the region ranging from 200 to 300 bp was extracted with an agarose spin column. Purified cDNA fragments were cloned into the EcoRI/HindIII site of pBluescript II SK+ (Stratagene), and used to transform *Escherichia coli* strain JM109. Sequence analysis showed that one clone C-1 contained a P450-like sequence.

**Poly(A)+RNA extraction and Northern blotting.** RNA prepared above was separated on a 1.0% (v/v) agarose gel containing 0.66 M formaldehyde in MOPS buffer (0.02 M MOPS, containing 0.01 M sodium acetate and 2 M EDTA) and transferred to a Hybond N+ membrane with 20×SSC, 0.15 M sodium chloride and 0.015 M sodium citrate at pH 7.0. After transfer, the membranes were cross-linked with UV light with a Stratallinker (Funakoshi Co., Tokyo). The probes were labeled with [32P]dCTP with the Klenow fragment. Hybridization was at 65°C, in a mixture of 6×SSC, 7.5× Denhardt’s reagent, 200 μg/ml 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 an image analyzer BAS 2000 (Fuji Film Co., Tokyo).

**Construction and screening of a cDNA library from *P. hybrida* flower buds.** After northern blotting, a cDNA library was constructed from the poly(A)+RNA fraction prepared from stage 2 of *P. hybrida* flower buds using a Uni-ZAPII cDNA synthesis kit according to the manufacturer’s instructions. The library was screened with the RT-PCR fragment of the cDNA clone C-1 as the probe. The nucleotide sequence of C-1 is shown in bold in fig. 1. For the first screening the probe was labeled with 32P with a random primer oligo DNA labeling kit (Takara). 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 NaPO4, and 1 mM EDTA, pH7.5), 0.5% SDS, 5× Denhardt’s solution, and
100 µg/ml denatured salmon sperm DNA and incubated for 16 h at 42°C with the labeled 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, 0.1% SDS at 65°C for 15 min, and radioactivity was measured with image analyzer. 

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. The largest clone, IMT-3 (1.8 kb), contained the full length of P450 cDNA.

**Isolation of genomic DNA and Southern blotting.** Genomic DNA was isolated from 5 g P. hybrida leaves with an Isoplant kit. For Southern blotting, 20 µg of genomic DNA was digested with EcoRI, HindIII, or Dral, separated on a 1.2% (w/v) agarose gel, and transferred to a Hybond-N+ nylon membrane with 10× SSC. Hybridization and washing were the same as for the northern blotting. Digested with HindIII 902 bp long (from 459 to 1361 bp) a cDNA fragment of CYP92B1 was used as the DNA probe. For hybridization, the probes were labeled with 32P-labeled dCTP with the Klenow fragment. The radioactivity on the filter was measured with the image analyzer.

**Cloning of the 5'-flanking region of cyp92B1.** A 1-µg portion of genomic DNA from P. hybrida was treated with 10 units of Dral for 16 h at 37°C. The reaction mixture was extracted once with an equal volume of phenol:chloroform (1:1). DNA fragments were precipitated by the addition of a 1× precipitation buffer (50 mM Tris-HCl, pH 7.4, containing 10 mM MgCl2, 20 units of Taq polymerase. We did two reactions with the PCR primers corresponding to the N-terminal region of cDNA clone of CYP92B1. In the first reaction, primers 1 and 2 were used. In the second reaction primers 3 and 4 were used with 1 µl of mixture from the first PCR. The nucleotide sequence of the primers were: primer 1, 5'-CTCTTTTGGGGATTATTTTTGGATAAACCTT-3'; primer 2, 5'-TAGTTG-GTCAAAACCCCATGAGTTTGGC-3'. The positions of the inverse PCR primers are shown with arrows in Fig. 1. The cycling conditions were 1 min at 95°C, 2 min at 65°C, and 3 min at 72°C, for 35 cycles. The DNA products from the second PCR were separated on a 0.7% agarose gel, DNA bands were purified, and the DNA obtained was ligated to the pT7Blue T-vector and sequenced with the T7 promoter primer, U19, and gene specific primer 4 as the sequencing primers.

**DNA sequencing and computer analysis.** CYP92B1 cDNA was sequenced automatically with DNA sequencer 550L, Hitachi, Tokyo and ABI PRISM 310, Perkin-Elmer, Foster city, CA. Sequencing alignment was done with a GENETIX-MAC program version 7.3 (Software Development Co., Tokyo), and similar DNA or amino acid sequences were searched for in EMBL, GenBank, or SWISS-PROT and NBRF databases.

**Expression of CYP92B1 in a yeast.** S. cerevisiae AH22 cells and the expression plasmid pAUR123 were used for expression of CYP92B1 cDNA as the host and vector, respectively. The expression vector pAUR123 contains the SalI/SacI site between the alcohol dehydrogenase I gene promoter and alcohol dehydrogenase promoter terminator. The full length cDNA fragment of CYP92B1 was digested with SalI and SacI and then ligated into pAUR123, giving expression plasmid pAUR92B1. The expression vectors pAUR123 and pAUR92B1 were introduced separately into S. cerevisiae AH22 cells by the lithium chloride method. Reduced CO-diʃerence spectra of the microsomal fractions prepared from the transformed yeast cells were measured by the method of Oeda et al. P450 hemoprotein in the microsomal fractions was assayed by the method of Omura and Sato(20) from the spectra with an extinction coefficient of 91 mM-1cm-1. Protein concentrations were measured with a protein assay kit (Bio-Rad Lab., Inc., Tokyo) with bovine serum albumin as the standard.

**Metabolism of endogenous and exogenous compounds in yeast microsomes expressing CYP92B1.** Metabolism was assayed of a number of fatty acids, testosterone, and herbicides. The reaction contained, in a total volume of 400 µl, mixture of 0.1 M potassium phosphate buffer (pH 7.4), 2 mM glucose 6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 10 µM radioactive substrate, and 50 pmol of P450 protein from the microsomal fraction, prepared from each of the recombinant yeast strains AH22/pAUR123 or AH22/pAUR92B1. The substrate and NADPH were incubated with the microsomal fraction from each of the recombinant yeast strains. After incubation first for 2 min at
28°C, the reaction was started by the addition of 5 mm NADPH. After 1 h it was stopped by addition of 40 ml of 4N HCl. The reaction mixture was extracted twice with 800 ml of ethyl acetate and the extract was dried under nitrogen and then dissolved in methanol. Products were measured with liquid scintillation counting. A portion, containing radioactivity of 20,000 dpm was put on to a TLC plate coated with silica gel 60F254 (Merck, Darmstadt, Germany). The TLC solvent system for linoleic acid, linolenic acid, and oleic acid was a mixture of diethyl ether, petroleum ether, and formic acid (50:50:1, by vol.). For the other fatty acids lauric acid, myristic acid, palmitic acid, stearic acid, and pelargonic acid the solvent system was a mixture of diethyl ether, petroleum ether, and formic acid (70:30:1, by vol.). For arachidonic acid, we used a mixture of chloroform, methanol, and acetic acid (90:5:2, by vol.). For chlortoluron a mixture of hexane and ethyl acetate (2:1, by vol.) was used and for the other herbicides, chloroform was used alone. The radioactivity on the TLC plate was measured with the image analyzer and also bioimaging analyzer (FLA-2000, Fuji Photo Film).

GC-MS of lauric acid metabolites produced by CYP92B1 was done by the method of Imaishi et al.16

Results

cDNA cloning of CYP92B1 from P. hybrida

The PCR primers corresponded to the highly conserved domains A and D were used (Fig. 1). Fifty randomly selected clones were sequenced, and compared with the sequences of P450s using the BLAST search program. One clone, C-1, encoded a P450-like sequence not published before. After the secondary screening from the cDNA library from P. hybrida flower buds in stage 2, the largest cDNA clone, designated IMT-3, was found to contain a 1533-bp insert with an uninterrupted open reading frame encoding a polypeptide consisting of 510 amino acid residues. A search of the SWISS-PROT database showed that the deduced amino acid sequence of IMT-3 contained domains A-D which are highly conserved among P450 gene families. IMT-3 contained the N-terminal hydrophobic region (signal-anchor sequence), followed by a proline-rich region which suggest that IMT-3 is a microsomal-type P450, and has the binding motif for heme. The cystein residue that serves as the fifth ligand for heme-Fe was found at position 1340, counting from the first methionine.22 The central region of the sequence deviated from other known cytochrome P450 proteins. Recently, Nelson and the Committee for Standardized Cytochrome P450 Nomenclature have officially named IMT-3 as CYP92B1 (D. R. Nelson, personal communication).

For the amino acid sequence of CYP92B1 the most similar sequence was that of CYP92A2, with a similarity of 50.6%.
Northern blotting
The level of cyp92B1 transcripts was the highest at stages 1 and 2, and then decreased as floral buds developed further (Fig. 2). cyp92B1 transcripts were not detected in leaves, stems, or roots.

Southern blotting
There were three bands in each of the three digests EcoRI, HindIII, or DraI, indicating that the genome of P. hybrida contains few copy numbers of cyp92B1 (Fig. 3).

Cloning of 5' flanking region of cyp92B1
Genomic DNA was digested with several restriction enzymes (DraI, KpnI, HindIII, and Smal), some with and some without restriction sites within the 5' proximal region of the cyp92B1. Oligonucleotide primers 1 and 4 which anneal to the 5' terminal sequence of cyp92B1, were used for inverse PCR. DraI, produced the longest fragment, of approximately 1600 bp, which was cloned into pT7Blue T-vector and yielding a clone containing 1057 bp of 5' flanking DNA (Fig. 4). The promoter region of the cyp92B1 gene contained several DNA binding motifs including AthB1, PBF-1, SBF-1,26 GATA box,27–28 Myb.Pb3,29 GAMyb,30–31 Myb.ST1, GBP, P,32 and bZIP33–34 as well as a putative TATA box at position –88, which is needed for efficient and correct initiation of transcription.

Expression of CYP92B1 in a yeast
The reduced CO-diifference spectrum of the microsomal fraction from AH22/pAUR92B1 had an absorption peak at 448 nm (Fig. 5). There was 37 pmol of P450-equivalent per milligram of microsomal protein. Yeast cells transformed with an empty vector pAUR123 lacked this characteristic peak of P450s.

Metabolism of endogenous and exogenous compounds in yeast microsomes expressing CYP92B1
Table 1 gives the results of the metabolism assays. CYP92B1 caused metabolism of a medium-chain fatty acid, lauric acid, and two long-chain fatty acids, linoleic and linolenic acids. CYP92B1 did not degrade the herbicides or testosterone. Only one possible metabolic product was formed after incubation with the standard substrate, lauric acid (data not shown). The metabolites of lauric acid produced by CYP92B1 and CYP78A1 were analyzed simultaneously by TLC. CYP78A1 was found by Imaishi et al.16 to be involved in the ω-hydroxylation of lauric acid. The metabolite of lauric acid produced by CYP78A1 and the metabolite of lauric acid produced by CYP92B1 appeared at the same position on the TLC plate with an Rf of 0.34. The metabolite of lauric acid produced by CYP92B1 was purified and identified by GC-MS as 12-hydroxydodecanoic acid (data not shown).

Figure 6 shows the results of the metabolism assays of unsaturated fatty acids. Two metabolites were found in both reaction mixtures containing either linoleic acid or linolenic acid and microsomes from the recombinant yeast strain expressing CYP92B1 cDNA. We used ω-hydroxylated linoleic acid and linolenic acid as standards, and than we compared the Rf value of metabolites of the fatty acids formed by CYP92B1. Rf values were different, so there were no ω-hydroxylated linoleic or linolenic acid in the reaction mixture. The concentration of this two metabolites from linoleic acid and linolenic acid produced by CYP92B1 was very low to be an-
Fig. 4. The 1057-bp 5′-Flanking Region of cyp92B1 Gene from P. hybrida.

The nucleotide sequence is numbered from the putative translation initiation site of the cyp92B1. The corresponding N-terminal nucleotide sequence of CYP92B1 cDNA is shown in italics. The TATA box and bZIP are marked in bold face. Putative DNA binding sequences are underlined: AthB1, PBF-1, GAMyb, GATA box, Myb.Ph3, bZIP, Myb.ST1, GBP, SBF-1, and P.

Fig. 5. Reduced CO-Differential Spectra of Recombinant Yeast Microsomes Expressing CYP92B1 cDNA.

The microsomal fraction (10 μg of protein) was bubbled with CO for 1 min and reduced by the addition of solid Na2S3O4. A typical P450 absorption peak at 448 nm was observed for AH22/pAUR92B1 (solid line). The recombinant yeast microsomes that expressed only empty vector AH22/pAUR123 did not produce this characteristic peak (dotted line).

Table 1. Catalytic Activity of CYP92B1 in Yeast Cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme activity, pmol/min/per mol of P450</th>
</tr>
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<tbody>
<tr>
<td>1. Fatty acids</td>
<td></td>
</tr>
<tr>
<td>Lauric acid (12:0)</td>
<td>2.5</td>
</tr>
<tr>
<td>Myristic acid (14:0)</td>
<td>ND</td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>ND</td>
</tr>
<tr>
<td>Linoleic acid (18:2)</td>
<td>14.6</td>
</tr>
<tr>
<td>Linolenic acid (18:3)</td>
<td>27</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>ND</td>
</tr>
<tr>
<td>Oleic acid (18:0)</td>
<td>ND</td>
</tr>
<tr>
<td>Arachidonic acid (20:0)</td>
<td>ND</td>
</tr>
<tr>
<td>2. Herbicides</td>
<td></td>
</tr>
<tr>
<td>Pelargonic acid (9:0)</td>
<td>ND</td>
</tr>
<tr>
<td>Chlortoluron</td>
<td>ND</td>
</tr>
<tr>
<td>Atrazine</td>
<td>ND</td>
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<tr>
<td>Simazine</td>
<td>ND</td>
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<tr>
<td>3. Hormone</td>
<td></td>
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<tr>
<td>Testosterone</td>
<td>ND</td>
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ND, not detected.
The metabolism of linoleic acid (left) and linolenic acid (right) was assayed in microsomal fractions prepared from the recombinant yeast strains of the \( \text{AH22} \) \( \text{W} \) \( \text{pAUR92B1} \) and \( \text{AH22} \) \( \text{W} \) \( \text{pAUR123} \). A reaction mixture had in a total volume of 400 \( \mu \text{L} \) and contained 100 \( \mu \text{M} \) potassium phosphate buffer (pH 7.4), 0.5 mM NADPH, 50 pmol of a substrate, and 50 pmol of P450 protein. After the extraction with ethyl acetate, each reaction mixture was spotted on a TLC plate, and developed with a solvent system. Solvent systems for linoleic acid and linolenic acid were a mixture of diethyl ether, petroleum ether, formic acid (50:50:1, by vol.). Arrows show [\( ^{14}\text{C} \)]linoleic acid and [\( ^{14}\text{C} \)]linolenic acid, their metabolites: metabolite 1 (M1), metabolite 2 (M2), and the starting point (origin) for the TLC. Each line shows samples as follows: Left: lane 1, control, containing only [\( ^{14}\text{C} \)]linoleic acid; line 2, microsomal protein from recombinant yeast strain \( \text{AH22} \) \( \text{W} \) \( \text{pAUR123} \) incubated with [\( ^{14}\text{C} \)]linoleic acid; line 3, microsomal protein from recombinant yeast strain \( \text{AH22} \) \( \text{W} \) \( \text{pAUR92B1} \) incubated with [\( ^{14}\text{C} \)]linoleic acid. Right: line 1, control, containing only [\( ^{14}\text{C} \)]linolenic acid; line 2, microsomal protein from recombinant yeast strain \( \text{AH22} \) \( \text{W} \) \( \text{pAUR123} \) incubated with [\( ^{14}\text{C} \)]linolenic acid; line 3, microsomal protein from recombinant yeast strain \( \text{AH22} \) \( \text{W} \) \( \text{pAUR92B1} \) incubated with [\( ^{14}\text{C} \)]linolenic acid.

Fig. 6. TLC Analysis of [\( ^{14}\text{C} \)]Linoleic Acid and [\( ^{14}\text{C} \)]Linolenic Acid and Their Metabolites.

alyzed by GC-MS. No metabolites appeared in the control incubation with a substrate or with the microsomes transformed with the empty expression vector.

**Discussion**

In higher plants, linoleic acid oxide and linolenic acid oxide may be important in plant protection, flowering, and development.\(^{35,36}\) The C18 family of fatty acids (especially \( \alpha \)-linolenic acid) are the most prevalent fatty acids in higher plants. C18 fatty acids are particularly enriched in the galactolipids of the photosynthetic membranes in plastids and storage in the seed proteins.\(^{37}\) Cutin, the first barrier against mechanical stress or pathogen attack, is a polymer consisting of hydroxylated and epoxidized fatty acids mainly derived from palmitic, oleic, linoleic and linolenic acids. This evidence suggests that long-chain fatty acid hydroxylases play an important role in the biosynthesis of plant cuticles, by generating terminal and internal hydroxy functions which yield to the polymerization of cutin monomers.\(^{37}\) The major C18 cutin monomers like 18-hydroxy-9,10-epoxyoctadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid are messengers in plant-pathogen interactions.\(^{35,36}\) P450s involved in the epoxidation or hydroxylation of unsaturated fatty acids inhibit the growth of pathogens.\(^{37}\) In contrast, in *Erysiphe graminis*, a certain monomer 9,10-epoxy-18-hydroxyoctadecanoic acid, has been found to induce appressorium formation during pathogen attack.\(^{38}\)

We isolated the cDNA clone IMT-3 of CYP92B1 from *P. hybrida* flower buds by RT-PCR, using two primers specific for the conserved domains among the plant P450 species reported. Database searches confirmed that the polypeptide is a cytochrome P450, with all of the structural and functional domains conserved among plant P450 proteins. The *cyp92B1* gene was found to be expressed in the early stage of flower development of petunia, but not in leaves, stems, or roots, and had a low copy number.

The 1057-bp 5' flanking region of the *cyp92B1* gene contained a putative TATA box and several kinds of DNA binding motifs: AthB1, PBF-1, SBF-1 binding sites, PBF-1 binding sites, GATA motif, Myb.Ph3, GAMyb, P, bZIP. SBF-1 binds to the promoter region of the gene encoding chalcone synthase gene, *chs*, of the bean *Phaseolus vulgaris* L., and regulates the synthesis pathways of flower pigments.\(^{35,36}\) Many plant genes involved in the biosynthesis of flower pigments and plant hormones are expressed in the early or middle stage of flower development. Three GATA boxes were found in the promoter of petunia chlorophyll a/b binding protein, required for tissue-specific expression and light regulation.\(^{27,28}\) The DNA binding transcription factor, Myb.Ph3, from petunia petal
The biological roles of this newly reported plant P450 involved in fatty acids biosynthesis are not known. Some essential P450 functions are conservative among plant species, including metabolism of hormones, sterol, and oxygenated fatty acids. Therefore, CYP92B1 may have a functions similar with CYP703A1, CYP78A1, CYP94A1, CYP94A5, CYPC81B1, and CYP86A1, which catalyze hydroxylation of fatty acids. The dendrogram in the fig. 7 suggested that P450s forms with different substrate specificity, share a common catalytic mechanism of oxygen activation. CYP81B1 catalyzes the in-chain the hydroxylation of capric (C10:0), lauric (C12:0) and myristic (C14:0) acids. Among saturated fatty acids, CYP92B1 oxidizes ω-hydroxylation only of lauric acid. The substrate specificity of CYP92B1 most resembles that of CYP94A1 and CYP94A5. CYP94A1, when expressed in a yeast, catalyzes the ω-hydroxylation of oleic, linoleic, and linolenic acid, with possible involvement in plant defense. Tobacco CYP94A5, also has been reported to be involved in the ω-hydroxylation of saturated and unsaturated fatty acids with aliphatic chain ranging from C12 to C18.
In conclusion, CYP92B1 completely differs from the fatty acid oxygenases already characterized. CYP92B1 is the first reported P450 to be expressed only in an early stage in flower development, and it is involved in the metabolism of the lauric acid and C18-fatty acids family. CYP92B1 is highly specific for fatty acids and does not metabolize the herbicides and hormone tested.

Acknowledgments

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References


25) Werck-Reichart, D., Cytochrome P450 in phenil-


