

Metabolomics for the characterization of cytochromes P450-dependent fatty acid hydroxylation reactions in *Arabidopsis*

Kosuke Kai^{1,2}, Hiroko Hashidzume¹, Kazuya Yoshimura¹, Hideyuki Suzuki³,
Nozomu Sakurai³, Daisuke Shibata³, Daisaku Ohta^{1,*}

¹ Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan;

² Research Association for Biotechnology, Tokyo 105-0003, Japan; ³ Kazusa DNA Research Institute, Kisarazu, Chiba 292-0818, Japan

* E-mail: ohtad@bioinfo.osakafu-u.ac.jp Tel & Fax: +81-72-254-9409

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Abstract Genome sequence analysis has revealed the presence of almost infinite numbers of cytochrome P450 genes in a variety of organisms. To establish a robust experimental platform from which to explore the catalytic potential of those putative P450 proteins, we have developed a comprehensive metabolic profiling system based on Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR/MS) analysis, together with recombinant enzyme studies. Here, we report the enzymatic properties of CYP78A5, CYP78A7, CYP78A10, and CYP86C3 of *Arabidopsis* as short-chain fatty acid hydroxylases, with substrates including lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), and myristoleic acid (C14:1). The fatty acid hydroxylation activity of CYP78A7 was confirmed using FT-ICR/MS metabolic profiling of P450-gene-overexpressing lines of cultured *Arabidopsis* T87 cells. The practical application of this metabolic profiling scheme is discussed as a P450 functional characterization platform.

Key words: Fatty acids, metabolomics, P450.

The cytochromes P450 (P450s) of higher plants play crucial roles in both primary metabolism and a wide variety of secondary metabolic processes, such as in the phenylpropanoid, terpenoid, and alkaloid pathways, which produce, for example, lignin monomers, flavonoids, anthocyanins, and species-specific phytoalexins (Schuler and Werck-Reichhart 2003). Plant P450s are also involved in the detoxification of xenobiotic chemicals such as herbicides (Schuler and Werck-Reichhart 2003; Nelson et al. 2004). Both the biosynthesis and degradation of plant growth regulators cannot be completed without the involvement of specific P450 protein families (Schuler and Werck-Reichhart 2003). In general, serious phenotypic changes including dwarfism, abnormal morphology, and differential hormonal responses have been the major driving forces of studies to clarify these P450 functions. A good background of natural product chemistry is also indispensable for the functional characterization of P450s. However, irrespective of the importance of plant P450 functions in both understanding plant biological processes and their possible applications as biocatalysts, no general key technology has been established to clarify the diverse enzymatic properties of P450s of unknown functions. For example, even in *Arabidopsis thaliana* the physiological functions of more than 70% of the P450

proteins remain unknown (Nelson et al. 2004).

To develop a versatile experimental platform for P450 characterization, we streamlined a metabolic profiling system based on Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR/MS). Full-length cDNA clones of several *Arabidopsis* P450 genes encoding unknown functions were selected for the preparation of recombinant enzymes. Those P450s were constitutively expressed in parallel in a cultured cell system and whole plants. Reverse genetics approaches with T-DNA knockout mutants and RNA interference were also incorporated into this metabolic profiling scheme. Changes in the metabolic profiles of T87 cells resulting from P450 overexpression, if any, were evaluated with a comprehensive metabolomics approach (Oikawa et al. 2006; Nakamura et al. 2007; Ohta et al. 2007).

Here, we report examples of our metabolic profiling study of the *CYP78A* and *CYP86C* genes in *Arabidopsis*. There are six genes for CYP78A subfamily proteins in *Arabidopsis*, the catalytic properties of which are unknown. It has been reported that the CYP78A proteins are involved in plant developmental processes. Thus, CYP78A11 has been shown to be the *PLASTOCHRON1* in rice, which controls the timing of lateral organ formation from the apical meristem (Miyoshi et al.

2004). CYP78A5 (Zondlo and Irish 1999) and CYP78A9 (Ito and Meyerowitz 2000) in *Arabidopsis* are considered to be involved in directional growth in the peripheral region of the shoot apical meristem and in fruit development, respectively. Conversely, there are five genes for CYP86A, two genes for CYP86B, and four genes for CYP86C in *Arabidopsis*. The CYP86A subfamily proteins share some sequence homology with the animal and fungal fatty acid hydroxylases, especially with the fatty acid ω -hydroxylases. The *lcr* (*cyp86a8*) mutant has been implicated in the synthesis of epidermal cutin in *Arabidopsis* (Wellesen et al. 2001). The *Arabidopsis att1* (*cyp86a2*) mutant is characterized by a cutin content of only 30% compared with that of wild-type plants, leading to its enhanced vulnerability *Pseudomonas syringae* (Xiao et al. 2004).

In this study, we characterized the enzymatic properties of recombinant CYP78A5, CYP78A7, CYP78A10, and CYP86C3 proteins with short-chain fatty acids as substrates, including lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), and myristoleic acid (C14:1). The fatty acid hydroxylation activities of these proteins in cells are discussed in terms of the results of a metabolomics study of CYP78A-overexpressing lines of cultured *Arabidopsis* T87 cells.

Materials and methods

Materials

Arabidopsis thaliana ecotype Columbia (Col-0) seedlings (Lehle Seeds, Round Rock, TX, USA) were grown at 22°C under continuous light, as described previously (Mizutani and Ohta 1998). For the lipid analysis and RNA isolation, the *Arabidopsis* seedlings were grown under a sterile conditions on 0.8% (w/v) agar plates containing germination medium supplemented with 1×Murashige and Skoog salts and 1% (w/v) sucrose (Valvekens et al. 1998). An *Arabidopsis* T87 cell suspension culture (Axelos et al. 1992) was maintained with weekly subculture (1.5 ml/25 ml dilution) in 25 ml of JPL medium (Jouanneau et al. 1967) under continuous light at 22°C on a rotary shaker (120 rpm).

Isolation of CYP78A cDNA clones

The cDNA sequences of *CYP78A5*, *CYP78A7*, and *CYP78A10* were amplified by reverse transcription-PCR using total RNA from *Arabidopsis* seedlings, according to the method described previously (Morikawa et al. 2006). Total RNA was isolated with an RNeasy Plant Mini Kit (Qiagen). The PCR was programmed in 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, followed by an extension period of 10 min at 72°C.

A primer set consisting of A5F (5'-CGCTCTAGAAATGTCT-CCGGAAGCTTAC-3', added *Xba*I site underlined) and A5R (5'-CTGCGGCCGCTCAAGCGAAACCAACAT-3', added *Not*I site underlined) was used to amplify the *CYP78A5* cDNA, and a set consisting of A7F (5'-CGATCTAGAAATGGAGTTG-ATGAATTT-3', added *Xba*I site underlined) and A7R (5'-CA-

GCGGCCGCTTAAAATATTTCCGTTACAAC-3', added *Not*I site underlined) was used to amplify the *CYP78A7* cDNA. *CYP78A10* cDNA was amplified using the primer set A10F (5'-CGATCTAGAAATGACTATTGATATGTAT-3', added *Xba*I site underlined) and A10R (5'-CAGCGGCCGCTTAACCCCTAGGGATTGC-3', added *Not*I site underlined). The entire coding region of the *CYP86C3* cDNA sequence was amplified with the set of primers C3F (5'-CGTCTAGAAATGTCTGAAA-TATCATCTT-3', added *Xba*I site underlined) and C3R (5'-CAGCGGCCGCTAGTCTTGAACGTGTAT-3', added *Not*I site underlined). The PCR products were cloned into the pDrive cloning vector (Qiagen K.K., Tokyo, Japan) to generate pD78A5, pD78A7, pD78A10, and pD86C3, respectively. The complete cloned sequences were determined for the recombinant protein expression studies.

Heterologous expression in insect cells

Recombinant CYP78A proteins were prepared by expressing the full-length cDNAs of *CYP78A5*, *CYP78A7*, *CYP78A10*, and *CYP86C3* in a baculovirus expression vector system, as described previously (Mizutani et al. 1997), using the Bac-to-Bac baculovirus expression system (Invitrogen) and *Spodoptera frugiperda* cells (Sf9; Invitrogen). Briefly, pD78A5, pD78A7, pD78A10, and pD86C3 were digested with *Eco*RI and *Not*I, and the isolated coding sequences were cloned into an *Eco*RI-*Not*I double-digested pFastBac1 plasmid. The pFastBac1 constructs were then used for the preparation of recombinant bacmid DNAs by the transformation of *Escherichia coli* strain DH10Bac (Invitrogen). The insect cells were transfected according to the manufacturer's instructions (Invitrogen). To express the recombinant P450 proteins, the Sf9 cells were maintained in Sf900II serum-free medium (Invitrogen) supplemented with 200 μ M 5-aminolevulinic acid and 200 μ M ferrous citrate to increase the low heme-synthesis capacity of the insect cells (Morikawa et al. 2006).

Overexpression in cultured cells

The cDNA sequences encoding *CYP78A5*, *CYP78A7*, and *CYP78A10* were excised from pD78A5, pD78A7, and pD78A10, respectively, with *Xba*I and *Sal*I. The coding sequence for *CYP86C3* was isolated from pD86C3 by digestion with *Xba*I. These cDNA clones were inserted into the corresponding restriction sites of a pBin-based vector (Clontech, Palo Alto, CA) to yield pBin78A5, pBin78A7, pBin78A10, and pBin86C3, respectively. These plasmids were transformed into *Agrobacterium tumefaciens* EHA105. The EHA105 cells transformed with the binary plasmids were cultured in LB medium (10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, and 10 g l⁻¹ NaCl) containing 50 mg l⁻¹ kanamycin. The stationary-phase EHA105 cells were inoculated into T87 cells, and the T87 cells were transferred so that the transformants could be screened on JPL solid medium containing 25 mg l⁻¹ meropenem (Sumitomo Pharmaceuticals Co., Ltd. Osaka, Japan), 10 mg l⁻¹ geneticin (Sigma-Aldrich K.K., Tokyo, Japan), and 3 g l⁻¹ gellan gum (Wako).

RNA gel blot analysis

RNA gels were blotted, hybridized, and washed basically as described previously (Ohta et al. 2000). Total RNA was extracted from T87 suspension-cultured cells using Sepasol

(R)-RNA I Super (Nacalai Tesque Inc.). Samples of total RNA (10 μg) were subjected to RNA gel blot analysis using the chemiluminescent detection method of the Gene Images AlkPhos Direct Labeling and Detection System (Amersham Biosciences K.K., Tokyo, Japan), and the blots were hybridized with a probe prepared from full-length *CYP78A5* cDNA. Hybridization signals were analyzed with the Bio Imaging Analyzer, FLA3000 (Fuji Photo Film, Tokyo, Japan).

Enzyme assays

To prepare microsomal fractions, the infected cells (60 ml of suspension-cultured cells) were washed with PBS and suspended in buffer A (20 mM potassium phosphate [pH 7.25], 20% [v/v] glycerol, 1 mM EDTA, 1 mM DTT). The cells were sonicated and the cell debris was removed by centrifugation at $10,000\times g$ for 15 min. The supernatant was further centrifuged at $100,000\times g$ for 1 h, and the pellet was homogenized with buffer A to produce the microsomal fraction. The microsomal fractions were stored at -80°C until use. The complete reaction mixture (0.5 ml) consisted of 50 mM potassium phosphate (pH 7.25), recombinant CYP78A microsomes (50–500 μg protein ml^{-1}), 100 μM NADPH, and substrates at different concentrations ranging from 1 to 100 μM . To monitor the full P450 activities, microsome assays were supplemented with a 0.1 unit ml^{-1} purified recombinant *Arabidopsis* NADPH-P450 reductase preparation (Mizutani and Ohta 1998). After 5–90 min at 30°C , the reactions were stopped by the addition of 50 μl of 1 N HCl. To calculate the enzyme kinetic parameters, linear regression was used in double-reciprocal analyses of the activity data (Morikawa et al. 2006).

Assay methods

All spectrophotometric determinations were made at room temperature using a Cary 300 spectrophotometer (Varian). P450 was estimated from the CO difference spectrum using the extinction coefficient $\Sigma=91\text{ mM}^{-1}\text{ cm}^{-1}$. Protein was determined using the Coomassie Protein Assay reagent (Bio-Rad Laboratories).

GC-MS analysis

The enzyme reaction mixtures were extracted with CH_3Cl :methanol: H_2O (1:2:1) for 2 h at room temperature and were then further extracted in CH_3Cl :methanol: H_2O (1:1:1) containing 2 M KCl. After centrifugation at $216\times g$ for 5 min, the CH_3Cl layer was dehydrated for 12 h in the presence of Na_2SO_4 and evaporated to dryness under an N_2 stream at 40°C . The plant materials were frozen and homogenized in liquid N_2 , and the total lipid fractions were extracted for 30 min at room temperature in 5 ml of chloroform:methanol (1:2, v/v). The lipids were recovered from the organic phase with the addition of 2 ml of 1% (w/v) KCl and 1 ml of chloroform. After 2 μl of methanol:water (10:9, v/v) had been added to the organic phase, it was evaporated to dryness under an N_2 stream. The sample was saponified in 2.5 ml of 1 M KOH in methanol at 90°C for 1 h, and 2 ml of chloroform and 2.5 ml of water were added to recover the organic phase. After the addition of 1.25 ml of 0.5 M KOH and 6 ml of water, the organic phase was evaporated to dryness in vacuo. TMS ether derivatives for GC-MS analysis were prepared in a 1:1 mixture (40 μl) of pyridine and *N,O*-bis(trimethylsilyl)trifluoroacetamide (Wako Pure

Chemical Industries) containing 1% (v/v) trimethylchlorosilane (Nacalai Tesque) at 90°C for 1 h. The fatty acids were analyzed with a CP-3800 gas chromatograph (Saturn 2200, Varian) with a CP-SIL5 CB Low Bleed/MS column (30 m \times 0.25 mm; Varian). The port temperature for split injections (a split ratio of 50%) was 130°C , and helium was used as the carrier gas at a flow rate of 1.5 ml min^{-1} . The thermal program began with a slow rise from 130°C to 200°C ($2.5^\circ\text{C min}^{-1}$), 5 min at 200°C , from 200°C to 320°C ($20^\circ\text{C min}^{-1}$), and finally 10 min at 320°C . In the GC-MS analysis, we monitored lauric acid (C12; retention time=10.6 min), 12-hydroxy lauric acid (C12-OH; retention time=22.9 min), myristic acid (C14; retention time=16.8 min), pentadecylic acid as the internal standard (C15; retention time=21.2 min), palmitic acid (C16; retention time=23.6 min), 16-hydroxypalmitic acid (C16-OH; retention time=35.8 min), stearic acid (C18; retention time=30.3 min), myristoleic acid (C14:1 Δ^9 ; retention time=16.4 min), and palmitoleic acid (C16:1 Δ^9 ; retention time=23.2 min).

FT-ICR/MS analysis

The T87 cells were collected by filtration, washed with distilled water, and immediately frozen in liquid nitrogen. The frozen cells were ground to a powder in liquid N_2 and extracted with methanol. The extracts were filtered through disposable membrane filter units (DISMIC-13JP, Advantec, Tokyo, Japan), evaporated under an N_2 atmosphere, and stored at -80°C until use. For FT-ICR/MS analyses, the sample extracts were dissolved in 50% (v/v) acetonitrile:water (Oikawa et al. 2006; Nakamura et al. 2007). Metabolomics data were acquired with a previously described method (Oikawa et al. 2006; Nakamura et al. 2007) using an IonSpec Explorer FT-ICR/MS (IonSpec Inc., Lake Forest, CA) equipped with an actively shielded superconducting magnet of 7 T. The positive electrospray solution consisted of 50% (v/v) acetonitrile:water plus 0.1% (v/v) formic acid and the negative electrospray solution of 50% (v/v) acetonitrile:water plus 0.1% (v/v) ammonium hydroxide. All the experimental events were controlled by the Omega8 software (IonSpec Inc.). Raw MS data sets from the FT-ICR/MS analyses were processed using the Dr.DMASS software and the KNApSACk database (Oikawa et al. 2006; Shinbo et al. 2006; Nakamura et al. 2007; Ohta et al. 2007).

Results

P450 genes for metabolic profiling

We listed P450 subfamilies, each represented by a single gene in the *Arabidopsis* genome, which are expected to be involved in nonredundant metabolic functions in the plants. These genes include *CYP703A2*, *CYP706A3*, *CYP711*, *CYP714A1*, *CYP716A3*, *CYP718*, and *CYP720*. Some other gene families were also listed, such as *CYP78A* and *CYP98A*, in which genetic dysfunctions lead to distinctive phenotypic changes. For example, *CYP711* corresponds to the locus *MAX1* and is involved in the production of branching-inhibitor compounds in plants (Booker et al. 2005), and the *CYP78A* gene family has been implicated in the production of an unidentified plant signaling molecule (Zondlo and Irish 1999; Ito and

Meyerowitz 2000; Miyoshi et al. 2004). We isolated the cDNAs of these selected P450s and used cultured T87 cells for their heterologous overexpression as recombinant enzymes. In this study, we examined the catalytic properties of CYP78A and CYP86C from *Arabidopsis*.

CYP78A and CYP86C sequences in *Arabidopsis*

The *Arabidopsis* genome contains six genes encoding the CYP78A subfamily of P450 proteins. These P450s share 47%–80% sequence homology at the amino acid level. In this study, we isolated full-length cDNA clones for *CYP78A5* (At1g13710), *CYP78A7* (At5g09970), and *CYP78A10* (At1g74110) from the flowers, rosette leaves, and callus tissues of *Arabidopsis*, respectively. The coding sequences of *CYP78A5*, *CYP78A7*, and *CYP78A10* were predicted to encode polypeptides of 517, 532, and 537 amino acids, respectively. The deduced primary structure of *CYP78A5* is 49.1%, 48.8%, and 50.0% identical to those of *CYP78A1* from maize, *CYP78A9* from *Arabidopsis*, and *CYP78A11* (PLASTOCHRON) from rice, respectively. In contrast, there are four genes for CYP86C in *Arabidopsis* encoding proteins *CYP86C1* (At1g24540), *CYP86C2* (At3g26125), *CYP86C3* (At1g13140), and *CYP86C4* (At1g13150), which have sequence similarities of 55%–85% at the amino acid level. The precise physiological roles of these P450s have not been reported.

Heterologous expression of P450 proteins

The cDNA clones encoding *CYP78A5*, *CYP78A7*, *CYP78A10*, and *CYP86C3* were expressed in insect cells using a baculovirus expression vector system. Proteins of 54–58 kDa accumulated in the recombinant-virus-infected insect cells, and the apparent molecular masses corresponded well to those predicted from the cDNA sequences (Figure 1). The microsomal membrane fractions (100,000×g pellet) were subjected to spectrophotometric determination of P450 (Figure 2). The microsomal fractions expressing the recombinant P450 proteins showed the expected reduced-CO difference spectra with an absorption maximum at 450 nm, and the specific contents of *CYP78A5*, *CYP78A8*, *CYP78A10*, and *CYP86C3* were 25, 120, 85, and 32 pmol P450/mg microsomal protein, respectively. Relatively large Soret absorption peaks were detected at around 420 nm for the *CYP78A5*- and *CYP86C3*-expressing cells (Figure 2), indicating that recombinant *CYP78A5* and *CYP86C3* were not stable in the reduced CO-bound form. No significant P450 accumulation was detected in the mock-transfected insect cells under the same experimental conditions (data not shown).

Because maize *CYP78A1* and *Arabidopsis* *CYP86A* are active lauric acid ω -hydroxylases (Benveniste et al. 1998; Imaishi et al. 2000), we investigated whether

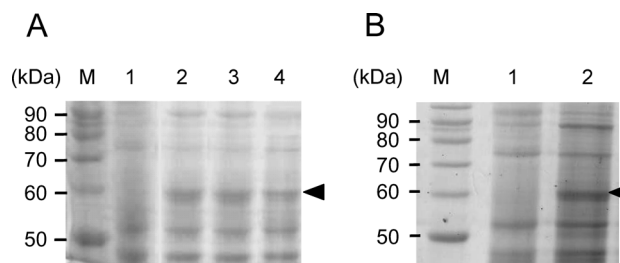


Figure 1. Heterologous expression of recombinant P450 proteins in insect cells. (A) SDS-PAGE analysis of recombinantly expressed P450 proteins. Lane 1, microsomes of mock-infected Sf9 cells; lane 2, microsomes of Sf9 cells infected with recombinant virus containing *CYP78A5* cDNA; lane 3, microsomes of Sf9 cells infected with recombinant virus containing *CYP78A7* cDNA; lane 4, microsomes of Sf9 cells infected with recombinant virus containing *CYP78A10* cDNA. (B) SDS-PAGE analysis of recombinantly expressed P450 proteins. Lane 1, microsomes of mock-infected Sf9 cells; lane 2, microsomes of Sf9 cells infected with recombinant virus containing *CYP86C3* cDNA. The positions of the molecular markers are shown at the left, and the arrows indicate the positions of the recombinantly expressed P450 proteins.

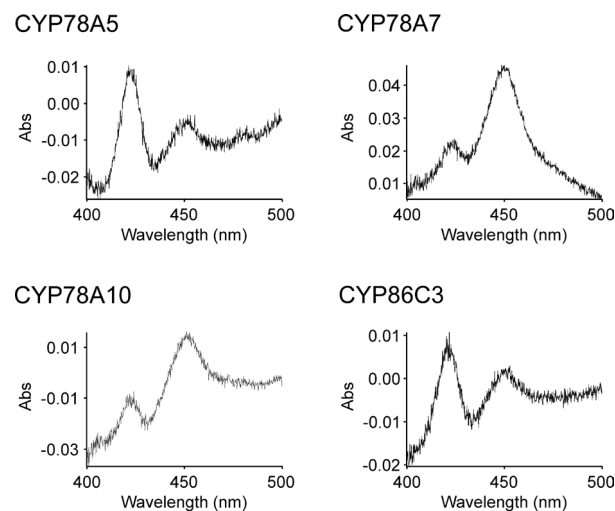


Figure 2. Reduced-CO difference spectra of recombinant *CYP78A5*, *CYP78A7*, *CYP78A10*, and *CYP86C3*. All spectrophotometric measurements were made at room temperature using a Cary 300 spectrophotometer (Varian). P450 was estimated from the CO difference spectrum using the extinction coefficient $\Sigma=91 \text{ mM}^{-1} \text{ cm}^{-1}$.

the recombinant *CYP78A5* (rCYP78A5), *CYP78A7* (rCYP78A7), *CYP78A10* (rCYP78A10), and *CYP86C3* (rCYP86C3) proteins were also active fatty acid hydroxylases. GC-MS analysis of the microsomal fractions from the insect cells showed that lauric acid (C12) is accepted as a substrate by rCYP78A5, rCYP78A7, rCYP78A10, and rCYP86C3. Figure 3 shows the GC-MS results for rCYP78A7, with a specific peak at the same retention time (22.9 min, indicated by the asterisk) as that of ω -hydroxylauric acid. The GC-MS fragmentation pattern obtained for the peak (Figure 3) matched that of authentic ω -hydroxylauric acid, and no other hydroxylation product was detected. These results

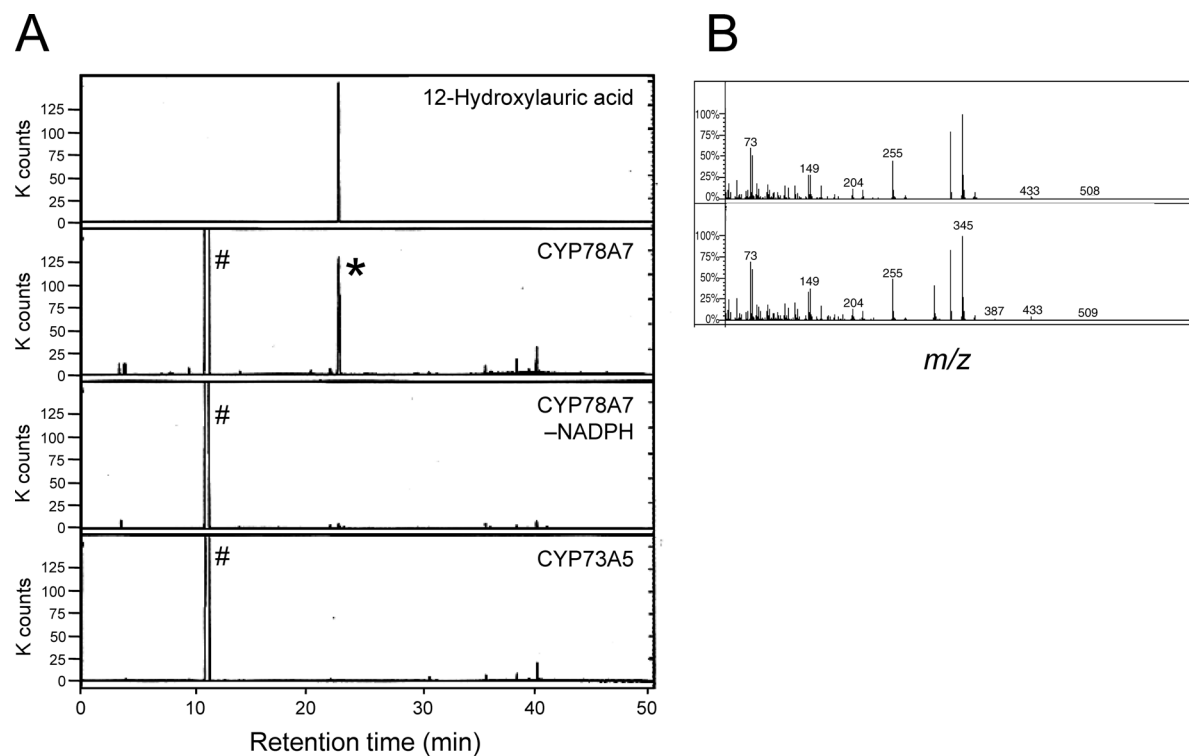


Figure 3. GC-MS analysis of the enzyme reaction products of the recombinant P450s. (A) GC-MS analysis of the reaction products from CYP78A7 with lauric acid as the substrate. The top panel represents the m/z 345 chromatogram of 12-hydroxy-lauric acid-TMS standard. The positions of the reaction product and the substrate were indicated by the asterisk and pound signs, respectively. CYP73A5, *Arabidopsis* cinnate 4-hydroxylase. (B) GC-MS fragmentation patterns of the TMS derivative of 12-hydroxy-lauric acid (upper panel) and the enzyme reaction product (lower panel) with lauric acid as the substrate.

were common to all the GC-MS analyses of rCYP78A5, rCYP78A10, and rCYP86C3 proteins (data not shown). Differentially hydroxylated fatty acids eluted independently at different retention times in the GC-MS analyses; the subterminally hydroxylated form eluted earlier than the ω -hydroxylated form. Thus, a GC-MS comparison with ω -1 and ω -2 hydroxylated lauric acids indicated that the reaction product was ω -hydroxy-lauric acid (data not shown). Next, we tested myristic acid (C14) as a substrate of these P450s. The GC-MS analyses indicated that rCYP78A5, rCYP78A7, rCYP78A10, and rCYP86C3 reactions produced new peaks with the same retention times. No authentic compounds for hydroxylated myristic acid were available for direct comparison with the enzyme reaction products. However, the fragment ions from the enzyme reaction products showed a clear reduction and increase of $m/z=28$ as in the corresponding ions from 12-hydroxy-lauric acid and 16-hydroxypalmitic acid, respectively. These results indicate that rCYP78A5, rCYP78A7, rCYP78A10, and rCYP86C3 accept myristic acid as a substrate. A comparison of the GC-MS retention times, as described for lauric acid hydroxylation, indicated that the reaction with myristic acid specifically yielded the ω -hydroxylated form. Further experiments indicated that only rCYP78A7 used myristoleic acid as a substrate.

Table 1. Comparison of the relative fatty acid hydroxylase activities on fatty acid substrates of different chain lengths.

Enzyme	C12	C14	C14:1	C16
CYP78A5	72	60	5	ND
CYP78A7	100 ^a	3	7	15
CYP78A10	31	10	ND	ND
CYP86C3	20	15	5	16

The enzyme reaction mixture (4 ml) consisted of 50 mM KH_2PO_4 (pH 7.25), 100 pmol of the recombinant P450 protein, 0.1 unit ml^{-1} recombinant NADPH-P450 reductase, 0.1 mM NADPH, and 0.1 mM fatty acid substrate (C12, C14, C14:1, or C16). Relative activities were compared for CYP78A5, CYP78A7, CYP78A10, and CYP86C3 from the peak areas of the reaction products calculated with the 2200 Varian Workstation. The relative amounts of fatty acids were estimated from the ratio of the peak area of each sterol to that of pentadecylic acid, used as the internal standard.

^aThe rCYP78A5, rCYP78A7, rCYP78A10, and rCYP86C3 activities were compared after assigning a score of 100 to the activity of rCYP78A7 with lauric acid as substrate.

ND: no activity detected.

Both rCYP78A5 and rCYP78A7 were also active hydroxylases for palmitic acid, whereas palmitoleic acid, stearic acid, oleic acid, and linoleic acid were not substrates of these three CYP78A proteins.

Table 1 shows a comparison of the relative hydroxylation activities of rCYP78A5, rCYP78A7, rCYP78A10, and rCYP86C3 on fatty acids of different

Table 2. Catalytic properties of the recombinant CYP78A proteins.

Enzyme	K_m	V_{max}	k_{cat}
	μM	pmol min^{-1}	min^{-1}
CYP78A5	14±2.2	2.4±0.150	0.25±0.015
CYP78A7	12±2.1	13±0.250	1.3±0.25
CYP78A10	22±3.8	2.5±0.100	0.13±0.10
CYP86C3	35±5.1	3.1±0.100	0.15±0.0050

chain lengths. These assays were performed under the same conditions with rCYP78A5, rCYP78A7, rCYP78A10, and rCYP86C3 to produce the linear progression of the reactions (Table 1). The results suggest clear differences in the substrate preferences of these P450 enzymes. Further characterization studies were performed using lauric acid as the substrate (Table 2). Whereas the K_m values for lauric acid were not dramatically different for rCYP78A5, rCYP78A7, rCYP78A10, and rCYP86C3, rCYP78A7 was by far the most efficient lauric acid hydroxylase.

Fatty acid hydroxylation activities in the plant cell

The fatty acid hydroxylation catalytic activities of CYP78A7 were studied using T87 transformant cells. The T87 cells were infected with *A. tumefaciens* transformed with the expression plasmid to constitutively express the full-length cDNA of CYP78A7 under the control of the Cauliflower mosaic virus (CaMV)-35S promoter. Independent clones of the transformed T87 cells were isolated on agar plates and maintained in suspension culture (Figure 4). An FT-ICR/MS analysis demonstrated the presence of an ion peak unique to the transformed cells. The m/z value of the ion was 215.1649, which completely matched the theoretical m/z of hydroxylated lauric acid. The MS/MS fragmentation pattern of the new peak was exactly the same as that of ω -hydroxylauric acid (data not shown). These results indicate that CYP78A7 protein functions as a fatty acid hydroxylase in plant cells.

Discussion

Plants have evolved a variety of enzymes for the in-chain, α -, β -, and ω -hydroxylation of fatty acids. Hydroxylated fatty acids are the biosynthetic intermediates of plant biopolymers such as cutin and suberin, which function as barriers against abiotic and biotic stresses, including water loss, mechanical damage, pathogen infection, and insect attack. Accumulating evidence indicates that hydroxylated fatty acids are also involved in plant defense reactions and developmental processes. Thus, fatty acid metabolic enzymes contribute to comprehensive survival strategies under environmental stress conditions.

Among the fatty acid hydroxylases in plants, only P450s perform hydroxylation reactions at the ω position

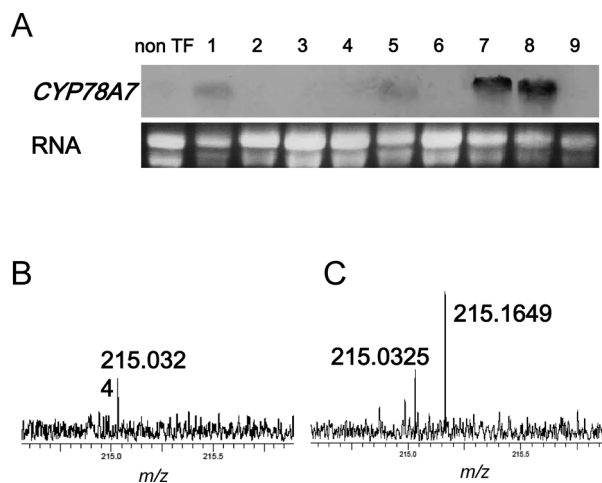


Figure 4. Fatty acid hydroxylation activity in T87 suspension-cultured cells overexpressing CYP78A7. (A) RNA gel blot analysis of the transgene expression levels in 35S::CYP78A suspension-cultured T87 cells. 35S::CYP78A7 transformant clones are shown in lanes 1–9. Endogenous CYP78A7 expression is shown in nontransformed cells (non TF). The transformant clone #7 was used for the metabolite analysis. The metabolomes of nontransformed cells (B) and the #7 line of 35S::CYP78A7 transformant cells (C) were compared by direct-infusion ESI-FT-ICR/MS analysis. The ion $m/z=215.1649$ corresponding to 12-hydroxylauric acid was detected only in the #7 line. No metabolite candidate was identified for the ions $m/z=215.0324$ or $m/z=215.0325$ in either cell type.

(Duan and Schuler 2005). The catalytic properties of a variety of P450 protein families, including CYP86A of *Arabidopsis* (Benveniste et al. 1998), CYP94A of *Vicia sativa* and tobacco (Tijet et al. 1998; Kahn et al. 2001; Le Bouquin et al. 2001), CYP94B and CYP94C of *V. sativa* and *Arabidopsis* (Tijet et al. 1998; Benveniste et al. 2005), and CYP92B1 from a petunia hybrid (Petkova-Andonova et al. 2002), have been extensively characterized in terms of both the chain lengths of their substrates and their hydroxylation positions. CYP709C1 can hydroxylate fatty acids at the $\omega-1$ and $\omega-2$ positions. CYP709C1 transcripts accumulated after treatment with a combination of the safener naphthalic acid anhydride and phenobarbital, indicating a possible detoxifying function for CYP709C1 (Kandel et al. 2005). CYP78A1 from maize has also been reported to be a lauric acid ω -hydroxylase, and CYP703A2 in *Arabidopsis* catalyzes the in-chain hydroxylation of lauric acid during sporopollenin biosynthesis (Morant et al. 2007).

It has been suggested that the CYP78A genes of *Arabidopsis* and rice are involved in plant developmental processes (Zondlo and Irish 1999; Ito and Meyerowitz 2000; Miyoshi et al. 2004). However, the catalytic properties of none of these CYP78A proteins have so far been characterized. In this report, we demonstrate with both enzyme assays and metabolic profiling studies that the CYP78A family proteins catalyze fatty acid hydroxylation reactions. Among the six different CYP78A genes, only a *cyp78a5* mutant has been

reported to display a distinct phenotype, with slightly reduced apical dominance. In contrast, a double mutant, *cyp78a5/a7*, has a pleiotropic phenotype, with short hypocotyls, round rosette leaves, a short petiole, dwarfism, and sterility. Transgenic lines overexpressing CYP78A7 show strong apical dominance and defects in floral development. The defects in floral development are similar to those in transgenic lines overexpressing CYP78A5. These results suggest that CYP78A5 and CYP78A7 are involved in the same metabolic network. The major phenotype of the *cyp78a5/a7* double mutant line was not rescued by the addition of exogenous 12-hydroxylated lauric acid (Mizutani, personal communication).

An FT-ICR/MS based metabolomics scheme was successfully implemented to clarify the P450 functions at the enzyme level and in a cell-culture system. In this study, we demonstrated that rCYP78A5, rCYP78A7, rCYP78A10, and rCYP86C3 proteins catalyze fatty acid hydroxylase reactions in substrates of different chain lengths. These P450s were also specific for hydroxylation at the terminal positions of the corresponding fatty acids. It remains to be clarified whether these fatty acid hydroxylase activities are in fact involved in the physiological functions of this P450 protein family, which have been demonstrated in molecular genetic studies. Further studies should be directed towards clarifying whether fatty-acid-derived metabolites are actually involved in the biosynthesis of a plant branching inhibitor.

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