

CYP78A1 Preferentially Expressed in Developing Inflorescences of *Zea mays* Encoded a Cytochrome P450-Dependent Lauric Acid 12-Monooxygenase

Hiromasa IMAISHI,¹ Satoshi MATSUO,² Eri SWAI,² and Hideo OHKAWA^{2,†}

¹Center for Cooperative Research and Development, and ²Department of Biological and Environmental Science, Faculty of Agriculture, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe 657-8501, Japan

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Cytochrome P450 (P450 or CYP) monooxygenases play an important role in the oxidation of a number of lipophilic substrates including secondary metabolites in higher plants. Larkin reported that CYP78A1 was preferentially expressed in developing inflorescences of *Zea mays* (Larkin, *Plant Mol. Biol.* 25: 343–353, 1994). However, the enzymatic function of CYP78A1 hasn't been clarified yet. To characterize the enzymatic activity of CYP78A1, in this study, CYP78A1 cDNA and tobacco or yeast NADPH-cytochrome P450 oxidoreductase (P450 reductase) was expressed in the yeast *Saccharomyces cerevisiae* AH22 cells under the control of alcohol dehydrogenase promoter I and terminator. The reduced CO-difference spectrum of a microsomal fraction prepared from the transformed yeast cells expressing CYP78A1 and yeast P450 reductase showed a peak at 449 nm. Based on the spectrum, the content of a P450 molecule was estimated to be 45 pmol P450 equivalent/mg of protein in the microsomal fraction. The recombinant yeast microsomes containing CYP78A1 and yeast P450 reductase were found to catalyze 12-monooxygenation of lauric acid. Based on these results, CYP78A1 preferentially expressed in developing inflorescences of *Zea mays* appeared to have participated in the monooxygenation of fatty acids.

Key words: cytochrome P450; *Zea mays*; lauric acid; *Saccharomyces cerevisiae*

Cytochrome P450 monooxygenases, consisting of a number of cytochrome P450 (P450 or CYP) species and a generic NADPH-cytochrome P450 oxidoreductase (P450 reductase) play an important role in the biosynthesis of secondary metabolites as well as in the metabolism of a wide variety of lipophilic xenobiotics in higher plants.¹⁾ Plant P450 enzymes were reported to be involved in the biosynthesis of sterols,²⁾ glucosinolates,³⁾ phenylpropanoids,⁴⁾ salicylic acid,⁵⁾ jasmonic acid,^{6,7)} gibberellins,⁸⁾ abscisic acid,¹⁾ brassinosteroids,⁹⁾ and alkaloids,¹⁾ and in the

metabolism of herbicides including chlorotoluron, bentazone, and diclofop-methyl.¹⁾

It has been reported that the *cyp78A1* gene is preferentially expressed in developing maize tassels.¹⁰⁾ Nadeau *et al.* have reported that the *cyp78A2* gene was specifically expressed in the pollen tubes of *Phalaenopsis*.¹¹⁾ Recently, it was also reported that the *cyp78A5* gene was strongly expressed in the peripheral regions of the vegetative and reproductive shoot apical meristems.¹²⁾ These reports suggested that some P450 species may be important in the processes of flowering or flower development. However, there are no reports showing that the catalytic activities of P450 species are encoded by the *cyp78A* gene family.

In this study, to analyze the enzyme function of CYP78A1 protein we attempted to express CYP78A1 cDNA in the yeast *Saccharomyces cerevisiae* cells and to assay the microsomal fractions expressing CYP78A1 for the metabolism of endogenous and exogenous substrates.

Materials and Methods

Construction of recombinant plasmids for expression of CYP78A1 and yeast or tobacco P450 reductase in Saccharomyces cerevisiae. The expression vector pAAH5N¹³⁾ containing the *Hind*III site between the yeast alcohol dehydrogenase (ADH) I promoter and terminator was used for the expression of CYP78A1 cDNA and yeast or tobacco P450 reductase cDNAs in *S. cerevisiae*. The full length CYP78A1 cDNA fragment was ligated into the *Hind*III site of pAAH5N to construct the expression plasmid pAMTC1. Then the *Not*I fragment of pAMTC1 was ligated into the *Not*I sites of pAYR and pATR to construct the co-expression plasmids pAMTC1YR and pAMTC1TR (Fig. 1). The expression vector, pAAH5N, pAMTC1, and pAMTCYR, were each introduced into *S. cerevisiae* AH22 cells.

[†] To whom correspondence should be addressed: Tel: +81-78-803-0669; Fax: +81-78-871-3617; E-mail: hohkawa@kobe-u.ac.jp

Abbreviations: dNTP, deoxynucleotide triphosphate; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography; GC-MS, gas chromatography-mass spectrometry

Transformation of AH22 cells with each of the expression plasmids was done by the lithium chloride method as described previously.¹³ The reduced CO-difference spectra of microsomal fractions prepared from the transformed yeast cells were measured by the method of Oeda *et al.*¹³ P450 hemoprotein contents in the microsomal fractions were measured from the spectra by using an extinction coefficient of $91 \text{ mM}^{-1} \text{ cm}^{-1}$.¹⁴ NADPH-cytochrome *c* reductase activity was measured by the method of Imaishi *et al.*¹⁵ Protein was assayed with a Bio-Rad Protein Assay Kit I (Bio-Rad Laboratories, Inc. Tokyo, Japan). BSA was used as a standard.

Assay for monooxygenase activity. A monooxygenase assay mixture contained in a final volume of $400 \mu\text{l}$: 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 of a microsomal fraction prepared from each of the recombinant yeast strains of AH22/pAAH5N, AH22/pAMTC1, AH22/pAMTC1YR, and AH22/pAMTC1TR cells. After incubation of the reaction mixtures for 3 min at 30°C , the enzyme reactions were started by the addition of 5 mM NADPH at the final concentration and then stopped by the addition of $40 \mu\text{l}$ of 1 N HCl after 1 h . The reaction mixtures were extracted twice with $800 \mu\text{l}$ of ethylacetate and the extracts were dried and then dissolved in methanol. Each of these samples were analyzed by HPLC (Model L-7000, Hitachi, Tokyo, Japan) at a flow rate of 1.0 ml/min on a $4.6 \times 150 \text{ mm}$ Cosmosil 5C₁₈-AR column (Nakalai Tesque, Kyoto, Japan). HPLC conditions for each compound were as follows: trans-cinamic acid, acetonitrile/water/acetic acid (40:60:0.2, v/v/v) at 286 nm ; Ferulic acid, acetonitrile/water/acetic acid (10:90:0.2, v/v/v) at 318 nm ; naringenin, methanol/water (40:60, v/v) at 292 nm ; tyrosine, 1.5% isopropanol at 222 nm , and abscisic acid, acetonitrile/acetic acid (75:25, v/v) at 262 nm . For an enzyme assay with a radioactive substrate, $10 \mu\text{M}$ of a radioactive substrate was added to a reaction mixture and then extracted as described above. Each sample containing the total radioactivity of $4,000 \text{ dpm}$ was put on to a TLC plate coated with silica gel 60F₂₅₄ (Merk A.G., Darmstadt, Germany). The TLC conditions for each sample were as follows: benzoic acid, chloroform/acetic acid/water (40:10:10, v/v/v); lauric acid, diethylether/ether/formic acid (70:30:1, v/v/v); chlortruron, hexane/ethylacetate (2:1, v/v); chlorsulfuron, benzene/acetone/formic acid (30:10:1, v/v/v); atrazine, chloroform; simazine, chloroform. The radioactivity on the TLC plate was measured with a BAS 2000 image analyzer (Fuji Co., Tokyo, Japan). Measurements of the enzyme activity were done three times for each enzyme reaction.

GC-MS Analysis. The oxygenated reaction products of lauric acid were methylated with diazomethane, and silylated with a mixture of bis-(trimethylsilyl)trifluoro-acetamide and 1% trimethylchlorosilane and pyridine (1:1, v/v), before gas chromatography and electron impact (70 eV) ionization mass spectrometry. The analysis was monitored on a 1% SE30 capillary column ($30 \text{ m} \times 0.25 \text{ mm}$) programmed to rise from 100°C to 280°C at 5°C/min , on a Hitachi model M-2500 system (Hitachi, Tokyo, Japan).

DNA sequence and computer analysis. DNA sequences were analyzed with ABI 310 (PE Applied Biosystems, Foster, CA, U.S.A.) and 5500L (Hitachi, Tokyo, Japan) instruments. The sequence data was analysed by GENETYX-MAC, version 7.3 (Software Development Co., Tokyo, Japan), and the resulting DNA sequences were searched for in the DDBJ, EMBL, and GenBank Nucleotide Sequences databases.

Results

Expression of a CYP78A1 and P450 reductases in the yeast

In order to analyze the catalytic activity of CYP78A1 protein, we attempted to express the cDNA clone of CYP78A1 in the yeast cells. Figure 1 shows the yeast expression plasmids, pAAH5N,

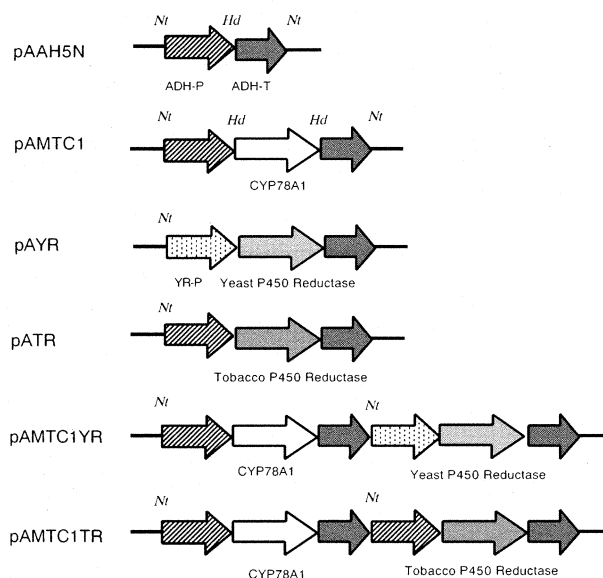


Fig. 1. Schematic Representation of Yeast Expression Plasmids.

The yeast expression vector pAAH5N includes *alcohol dehydrogenase I* promoter (ADH-P) and terminator (ADH-T). pAMTC1 is the expression plasmid for CYP78A1. pAMTCYR is the co-expression plasmid for CYP78A1 and yeast P450 reductase, and pAMTCR is also the co-expression plasmid for CYP78A1 and tobacco P450 reductase. Details of the construction of these plasmids are described in Materials and Methods. Nt, *NotI*; Hd, *HindIII*; YR-P, yeast P450 reductase promoter.

pAMTC1, pAYR, pATR, pAMTC1YR, and pAMTC1TR. To express CYP78A1 protein in yeast cells, the CYP78A1 cDNA was cloned in the yeast expression vector pAAH5N which has an *ADH I* promoter. The co-expression plasmid pAMTC1YR contained the coding sequences of CYP78A1 and yeast P450 reductase. The co-expression plasmid pAMTC1TR also contained the coding sequences of CYP78A1 and tobacco P450 reductase. After the transformation of AH22 cells with each of the expression plasmids by the LiCl method, microsomal fractions were prepared from the cultures of each of the recombinant cells. Then, the reduced CO differential spectra of these microsomal fractions were measured. Figure 2 shows the reduced CO-difference spectrum of the microsomal fraction prepared from the yeast AH22/pAMTC1YR cells with a peak at 449 nm. Based on the spectrum, the content of a P450 molecule was estimated to be 45 pmol P450 equivalent/mg of protein in the microsomal fraction. On the other hand, there was no corresponding absorbance observed with the control yeast strain AH22/pAAH5N. The specific content of a P450 pro-

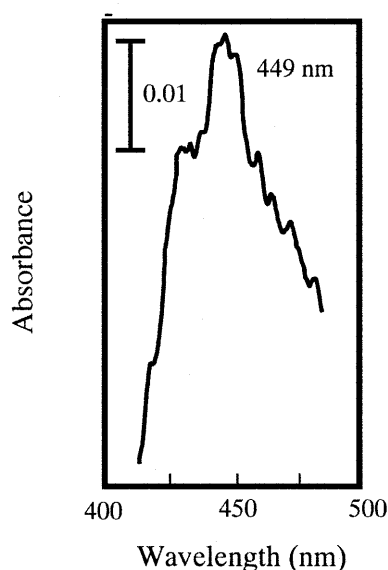


Fig. 2. Reduced CO-difference Spectrum of the Microsomes of the Recombinant Strain AH22/pAMTCYR.

Reduced CO-differential spectrum of the microsomal fraction from recombinant yeast strain AH22/pAMTCYR. The microsomal fraction (10 mg of protein) was reduced by adding solid $\text{Na}_2\text{S}_2\text{O}_4$. After bubbling the reduced microsomal fraction with CO for 1 min, the spectrum was measured.

tein in the microsomal fractions from AH22/pAMTC1, AH22/pAMTC1YR, and AH22/pAMTC1TR cells were 89 pmol/mg microsomal protein, 45 pmol/mg microsomal protein, and 23 pmol/mg microsomal protein, respectively (Table 1). The NADPH-cytochrome *c* reductase activity in the microsomal fractions of the AH22/pAAH5N, H22/pAMTC1, AH22/pAMTC1YR, and AH22/pAMTC1TR were 60 nmol/min/mg microsomal protein, 58 nmol/min/mg microsomal protein, 132 nmol/min/mg microsomal protein and 230 nmol/min/mg microsomal protein, respectively (Table 1). These results indicate that CYP78A1 and yeast or tobacco P450 reductase are functionally expressed in the microsomes of recombinant yeast cells.

Metabolism of endogenous and exogenous substrates with microsomal fractions from recombinant yeast cells

The microsomal fractions prepared from each of the recombinant yeast cells were used for the enzyme assay of a number of endogenous and exogenous compounds. A substrate and NADPH were incubated with the microsomal fraction from each of the recombinant yeast strains. Then, each of the reaction mixtures was analyzed by HPLC or TLC. Table 2 summarizes the results of the metabolism assay. Possible reaction products were found with lauric acids, but not with the other compounds tested. Figure 3(a) shows TLC autoradiograms of the extracts from the reaction mixture containing $[1-^{14}\text{C}]$ lauric acid and the microsomal fraction of AH22/pAMTC1YR. One major reaction product was detected by radio-TLC of reaction mixtures of lauric acid and the microsomal fraction of AH/22pAMTC1YR. On the other hand, no reaction products of $[1-^{14}\text{C}]$ lauric acid were found in the reaction mixtures containing the microsomal fractions prepared from AH22/pAAH5N, AH22/pAMTC1, and AH22/pAMTC1TR. These results suggest that CYP78A1 expressed in the recombinant yeast microsomes with yeast P450 reductase catalyzes the oxidation of lauric acid.

TLC of a reaction product of lauric acid with authentic references was done. The R_f (0.33) value of the lauric acid metabolite was consistent with 12-hydroxy lauric acid. The position of hydroxylation was also located with GC-MS analysis. The methylester trimethylsilyl ether derivatives showed

Table 1. P450 Contents and Cytochrome *c* Reductase Activity in the Microsomal Fractions of the Recombinant Yeast Cells

Yeast strain	P450 content (pmol/mg protein)	Cytochrome <i>c</i> reductase activity (nmol/mg protein)
AH22/pAAH5N	<1	60
AH22/pAMTC1	89	58
AH22/pAMTC1YR	45	132
AH22/pAMTC1TR	23	230

Table 2. The Catalytic Properties of Recombinant Yeast Microsomal Fractions AH22/pAMTC1YR

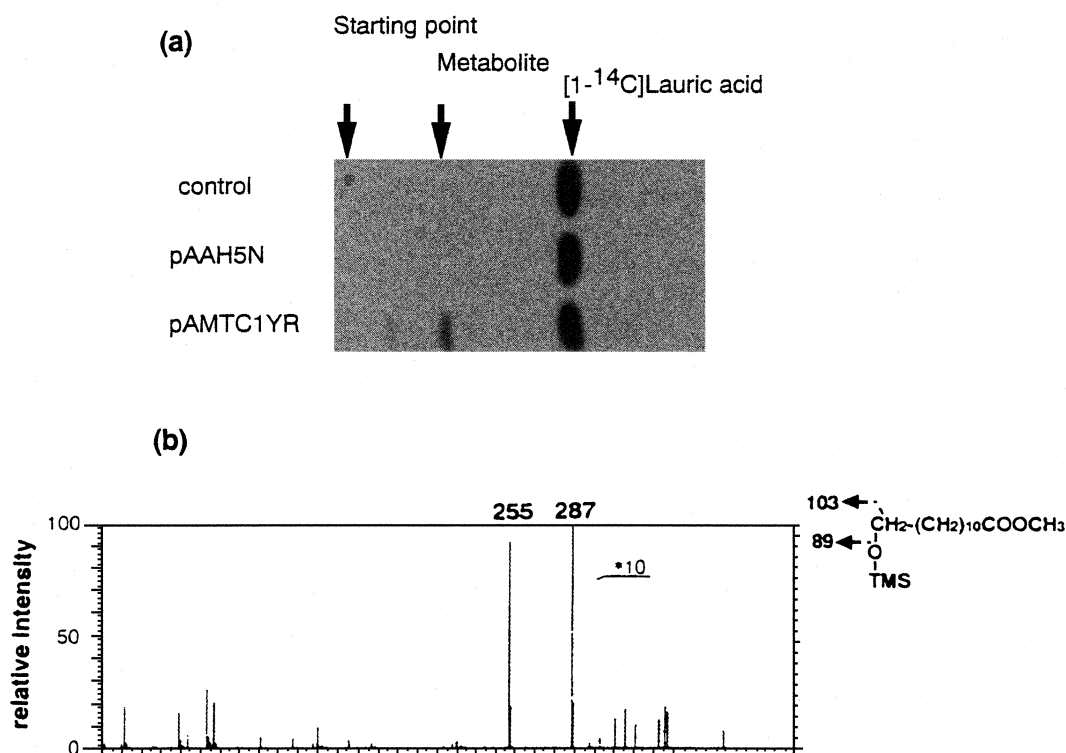
Substrate	Activity (mol/min/mol P450)
Secondary metabolite	
Cinnamic acid	—
Ferulic acid	—
Benzoic acid	—
Naringenin	—
Tyrosine	—
Geraniol	—
HBOA (Benzoxazinone)	—
Abscisic acid	—
Fatty acid	
Lauric acid	6
Myristic acid	<1
Palmitic acid	<1
Sterol	
Testosterone	—
Herbicide	
Chlortoluron	—
Chlorsulfuron	—
Atrazine	—
Simazine	—

—; not metabolized.

the expected mass fragmentation for hydroxylated lauric acid, with characteristic ions resulting from α , β cleavage of trimethylsilyl ether at m/z 89 and 103 for the 12-hydroxy lauric acid (Fig. 3(b)). Thus, CYP78A1 was found to catalyze the 12-hydroxylation of lauric acid.

Sequence analysis

Multiple alignment of the amino acid sequences of CYP78A1 with those of the reported P450 plant species was done. The similarity scores for the amino acid sequences show that the highest sequence similarity of CYP78A1 was 57% with CYP78A4, and the similarity with CYP78A5 (expressed in the peripheral regions of the shoot apical meristems) and CYP78A3 was 49% and 46%, respectively. A dendrogram showed that CYP78A1 belongs to the same cluster of P450 species CYP75A1 (flavonoid-3',5'-hydroxylase), CYP75A2 (flavonoid-3',5'-hydroxylase), CYP75A3 (flavonoid-3',5'-hydroxylase), CYP84A1 (ferulate-5-hydroxylase), CYP80A1 (berbamunine synthase), CYP81B1v1 ((S)-N-methylcoclaurine 3'-hydroxylase), and CYP81B1v2 (fatty

**Fig. 3.** TLC and GC-MS Analyses of the Lauric Acid Metabolites.

(a) TLC analysis of $[1-^{14}\text{C}]$ lauric acid and its reaction products. The oxidation of lauric acid was assayed in the microsomal fractions prepared from the recombinant yeast strains of both AH22/pAAH5 and AH22/pAMTCYR. A reaction mixture in 400 μl of a 0.1 M potassium phosphate buffer (pH 7.4) contained 6.7 mM glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 1 mM NADPH, 60 pM $[1-^{14}\text{C}]$ lauric acid (50 $\mu\text{Ci}/\text{ml}$) and 50 pM P450 protein. After extraction with ethyl acetate, each sample was spotted on to a thin-layer silica gel plate and then developed by a solvent system of diethylether/ether/formic acid (70:30:1, v/v/v). Solid arrows show $[1-^{14}\text{C}]$ lauric acid, main reaction product, and the starting point for TLC analysis. Each lane shows samples as follows: lane 1, without microsomal proteins; lane 2, microsomal proteins (2 mg) from the recombinant yeast strain AH22/pAAH5N; lane 3, microsomal proteins (2 mg) from the recombinant yeast strain AH22/pAMTCYR. The radioactivity on the TLC plate was measured by a BAS 2000 image analyzer. (b) A GC-MS spectrum of the methylated and trimethylsilylated derivative of the major lauric acid metabolite.

acid hydroxylase), which are related to the plants secondary metabolite pathway (Fig. 4), but not closely related to the group of P450 species metabolizing fatty acids such as CYP86A1 (fatty acid hydroxylase) or CYP74A1 (alleneoxide synthase).

Discussion

We analyzed the enzyme function of CYP78A1 that is specifically expressed in the early stage of maize tassel. It was found that the recombinant yeast microsomes containing CYP78A1 and yeast P450 reductase catalyzed 12-hydroxylation of lauric acid.

In order to improve the expression level of P450 proteins in yeast cells or *E. coli*, modification of the N-terminal sequences of P450 species has been done.^{15,16} Although modification of the N-terminal sequence appeared to improve the expression levels of the P450 proteins, their substrate specificity may be affected by a modification of the N-terminal amino acid sequence. To avoid such a problem, CYP78A1 was expressed in the yeast microsomes without any modification of the N-terminal sequence of CYP78A1.

The microsomal fractions obtained from AH22/pAMTC1YR catalyzed the 12-hydroxylation of lauric acids. On the other hand, the microsomal fractions obtained from AH22/pAAH5N and AH22/pAMTC1 did not catalyze the 12-hydroxylation of lauric acids. These results suggest that maize CYP78A1 interacts with yeast P450 reductase, but does not interact with tobacco P450 reductase efficiently. It was also reported that the co-expression of CYP81B1 and *Arabidopsis thaliana* P450 reductase improved the stability of CYP81B1.¹⁶

CYP78A1 shared the highest similarity of 57% in amino acid sequence with that of CYP78A4 from *Pinus radiata*. However, the enzyme function of CYP78A4 is not clear at present. CYP78A1 catalyzed the 12-hydroxylation of lauric acid. Therefore, this plant hydroxylase appears to be functionally similar to CYP4A,¹⁷ CYP86A1,¹⁸ and CYP94A1,¹⁹ which catalyze the hydroxylation of fatty acids. The branching pattern of the dendrogram in Fig. 4 shows that CYP78A1 belongs to a different branch of CYP86A1 (lauric acid 12 hydroxylase),¹⁸ CYP94A1 (lauric acid 12 hydroxylase),¹⁹ CYP51 (obtusifolios 14 α -demethylase),²⁰ CYP90 (teasterone-23-hydroxylase),⁹ CYP88A1 (13-hydroxylase in the gibberellin biosynthesis pathway),⁸ and CYP74A1 (allene oxide synthase).²¹ Therefore, CYP78A1 is likely to have evolved with the P450 family, which is involved in the biosynthesis and metabolism of secondary metabolites, including phenylpropanoids rather than steroids and terpenoids.

It was reported that several P450 species in higher plants metabolize fatty acids, such as CYP86A1, CYP94A1, and CYP81B1v2, however the physiologi-

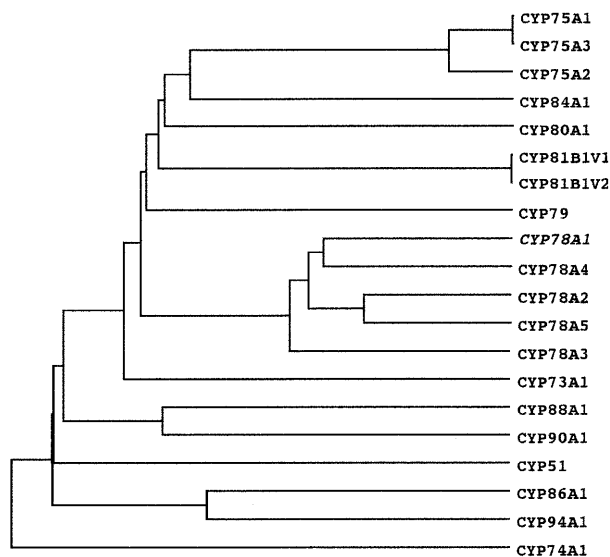


Fig. 4. Dendrogram of CYP78A1 to Other Reported P450 Plant Species.

The dendrogram was generated by using the DDBJ *malign* program. CYP81B1v1, (s)-N-methylcochlorine 3'-hydroxylase; CYP81B1v2, fatty acid hydroxylase; CYP73A1, cinnamate 4-hydroxylase; CYP86A1, fatty acids ω -hydroxylase; CYP94A1, cutin monomers synthase; CYP75A3, flavonoid-3',5'-hydroxylase; CYP75A1, flavonoid-3',5'-hydroxylase; CYP75A2, flavonoid-3',5'-hydroxylase; CYP84A1, ferulate-5-hydroxylase; CYP80A1, berbaminine synthase; CYP79, tyrosine *N*-hydroxylase; CYP51, obtusifolios 14- α demethylase; CYP90A1, teasterone-23-hydroxylase; CYP88A1, 13-hydroxylase in the gibberellin biosynthesis; CYP74A1, allene oxide synthase. The accession numbers of the P450 genes in GeneBank are CYP81B1v1, AJ000477; CYP81B1v2, AJ000478; CYP73A1, Z17369; CYP86A1, X90458; CYP94A1, AF030260; CYP75A3, Z22544; CYP75A1, X71130; CYP75A2, X70824; CYP84A1, U38416; CYP80A1, U09610; CYP79, U32624; CYP51, U74319; CYP90A1, X87367; CYP88A1, U32579; CYP74A1, U00428; CYP78A1, L23209; CYP78A2, U34744; CYP78A3, AF022463; CYP78A4, AF049067.

cal roles of these P450 species have not been well characterized. It is known that the prostaglandin seemed to control some physiological functions such as flowering.²² The presence of a large amount of the ω -hydroxy derivative of the C18 fatty acid family in plant stigmas suggests that they may play a role in the recognition of the stigma by pollen.²³ It has recently been reported that lipids are the essential factor needed for pollen tubes to penetrate the stigma.²⁴ These reports suggest that some oxidized-lipid compounds can control important physiological roles for flowering and flower development in higher plants. Since the CYP78A1, CYP78A2 and CYP78A5 were specifically expressed in the early stages of flower development, these P450 species may play important roles for the flowering or flower development in higher plants.

Further studies on the *cyp78A1* gene should lead to a better understanding of its role in the process of maize floral bud development.

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