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GENETIC ENGINEERING STUDIES ON CYTOCHROME P450 MONOOXYGENASES IN HIGHER PLANTS

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博士論文

GENETIC ENGINEERING STUDIES ON CYTOCHROME P450 MONOOXYGENASES IN HIGHER PLANTS

高等植物におけるチトクローム P450 モノオキシゲナーゼに関する遺伝子工学的研究

平成１０年１月

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GENETIC ENGINEERING STUDIES ON CYTOCHROME P450 MONOOXYGENASES IN HIGHER PLANTS

A dissertation for partial fulfillment of a Doctoral Degree
at the Graduate School of Science and Technology,
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ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolylphosphate *p*-toluidine
BY2	bright yellow 2
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CNBr	cyanogen bromide
CTP	cytidine 5'-triphosphate
2,4-D	2,4-dichlorophenoxyacetic acid
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
MOPS	3-morpholinopropanesulfonic acid
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NBT	nitro-blue tetrazolium
PCR	polymerase chain reaction
PVDF	polyvinylide difluoride
rpm	rounds per minute
SDS	sodium dodecyl sulfate
TPCK	N-tosyl-L-phenylalanyl chloromethyl ketone

CHAPTER I

GENERAL INTRODUCTION

Cytochrome P450 Monooxygenases

Cytochrome P450 (P450) was first identified as a carbon monooxide-binding pigment in rat liver microsomes [1, 2] and showed an absorption maximum at about 450 nm in a reduced CO difference spectrum. A number of P450 species were found to be distributed into prokaryotes, plants and mammals. The enzymes catalyze oxidative reactions toward a wide range of lipophilic endogenous and exogenous compounds. Most of P450 species constitute a P450-linked electron transfer system termed P450 monooxygenase as shown in Fig. 1. In bacteria, the enzyme system consists of a flavoprotein reductase, a flavoprotein and soluble P450 species catabolizing organic compounds including camphor [3]. In mammalian mitochondria, the enzyme system consists of a NADPH-adrenodoxin reductase, adrenodoxin and membrane bound P450 species catalyzing oxidative reactions of endogenous steroids. In mammalian microsomes, the enzyme system consists of a generic NADPH-cytochrome P450 oxidoreductase (P450 reductase) and various P450 species catalyzing monooxygenase reactions of exogeneous compounds including drugs and pesticides as well as endogenous compounds such as steroids and fatty acids. In these enzyme systems, electrons from NADPH or NADH are transferred to electron transfer protein(s) and then to a P450 molecule on which molecular oxygen is activated and one active oxygen is inserted into a substrate to yield a hydroxylation product. In higher plants, P450 monooxygenases are involved in secondary metabolism as well as in xenobiotic metabolism. The enzyme system is supposed to be localized on the microsomes [4].

P450 species consist of one of the most structurally and functionally diverse superfamily (Table 1-1). Naming of a newly discovered P450 species is determined based on the amino acid sequence by P450 Nomenclature Committee [5]. The amino acid sequence identity of 40% is required to be placed in the same family. The number of P450 sequences increased dramatically within these five years. So far, more than 700 deduced amino acid sequences of P450 cDNAs or genes were determined from various organisms including vertebrates, invertebrates, fungi, plants and bacteria, and classified into more than 120 families. Estimates from current genome project imply that the number of P450 genes exceeds 80 in humans, 60 in *Drosophila melanogaster* and *Caenorhabditis*

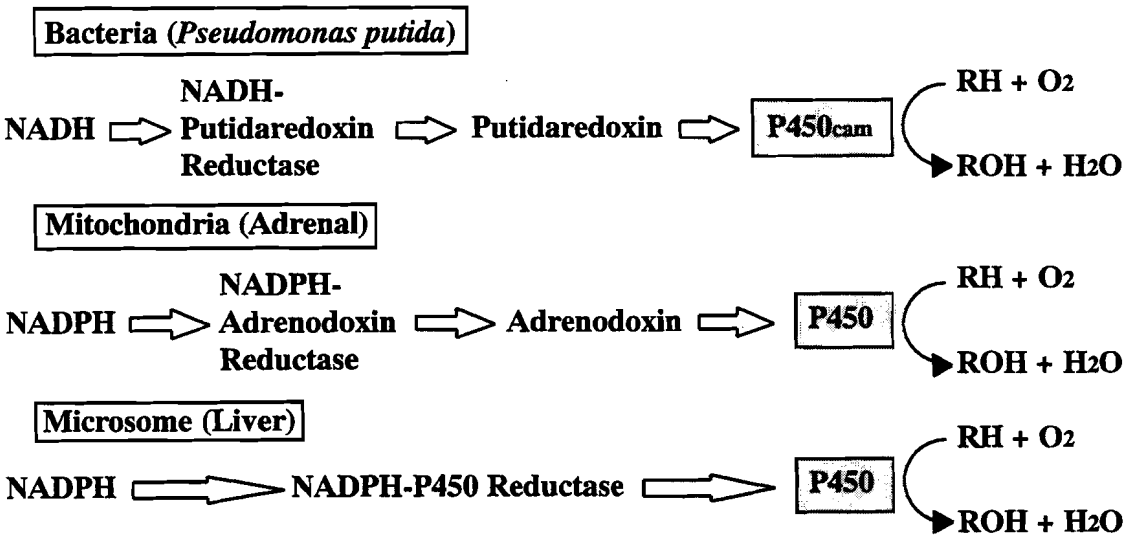

Fig. 1. P450 monooxygenase systems in bacteria and mammals.

Table 1-1 P450 families and enzyme functions in various species

Family	Source	Enzyme function
CYP1	vertebrates	Metabolism of drugs and environmental chemicals
CYP2	vertebrates	Metabolism of drugs and environmental chemicals
CYP3	vertebrates	Metabolism of drugs and environmental chemicals
CYP4	vertebrates	fatty acid hydroxylase
CYP5	vertebrates	thromboxane synthase
CYP6	insects	metabolism of plant chemicals and pesticides
CYP11	vertebrates	steroid biosynthesis (mitochondrial enzyme)
CYP17	vertebrates	steroid 17α-hydroxylase
CYP27	vertebrates	steroid 27-hydroxylase (mitochondrial enzyme)
CYP51	animal, fungi, yeast, plants	steroid biosynthesis
CYP52	yeast	alkane hydroxylases
CYP73	plants	cinnamic acid 4-hydroxylase
CYP101	bacteria	camphor hydroxylase
CYP105	bacteria	metabolism of herbicides

elegans, and 160 in *Arabidopsis thaliana* [6]. It was supposed that all P450 genes have diversed from a common ancestor gene through molecular evolution. The diversity of P450 species may result from co-evolution among different organisms [7]. For example, plants have evolved a variety of P450-linked biosynthetic pathways producing secondary metabolites which affected against the attack of animals and microbes, while animals have evolved P450-linked xenobiotic metabolism systems to detoxify toxic compounds. This is an attractive hypothesis to explain a divergence of P450 superfamily.

P450 Monooxygenases in Higher Plants

Plant P450 monooxygenases participate in broad and divergent biosynthetic pathways to produce secondary metabolites as well as xenobiotic metabolism. Important P450 enzymes are known to be involved in biosynthesis of sterol, glucosinolates, phenylpropanoid / flavonoid, signal molecules including salicylic acid and jasmonic acid, phytohormones including gibberellic acids, abscisic acid and plant growth regulator including brassinosteroids as well as in the metabolism of herbicides (reviewed in [8]).

Characterization of plant P450 enzymes contributes to understand molecular mechanisms of plant defense against pathogens and herbivores, regulation of plant development as well as herbicide selectivity and resistance. The elucidation of molecular mechanisms would promise many applications including molecular breeding for herbicide and disease resistant crops, and for dwarf crops, bioconversion to produce pharmaceutical and industrial compounds, phytoremediation to remove environmental pollutants and so on. These are important for agriculture, human health as well as solution of environmental problems.

Recently the number of plant P450 sequences determined has been increased rapidly. Over 60 sequences of plant P450 species have been determined [6]. However, the number of plant P450 species whose physiological substrates were identified was quite limited as shown in Table 1-2. Most of P450 species which participated in oxidative reactions for the biosynthesis of defense related compounds and phytohormones, and herbicide detoxification remained unknown.

Table 1-2 Plant P450 species and the physiological functions

Species	Source	Function	Reference
CYP51	*Sorghum bicolor*	obtsusifoliol 14α-demethylase	[9]
CYP51	*Triticum aestirum*	obtsusifoliol 14α-demethylase	[10]
CYP71C1	*Zea mays*	DIMBOA biosynthesis	[11]
CYP71C2	*Zea mays*	DIMBOA biosynthesis	[11]
CYP71C3	*Zea mays*	DIMBOA biosynthesis	[11]
CYP71C4	*Zea mays*	DIMBOA biosynthesis	[11]
CYP73A1	*Helianthus tuberosus*	cinnamic acid 4-hydroxylase	[12]
CYP73A2	*Phaseolus aureus*	cinnamic acid 4-hydroxylase	[13]
CYP73A3	*Medicago sativa*	cinnamic acid 4-hydroxylase	[14]
CYP73A4	*Catharanthus roseus*	cinnamic acid 4-hydroxylase	[15]
CYP73A5	*Arabidopsis thaliana*	cinnamic acid 4-hydroxylase	[16 - 18]
CYP73A9	*Pisum sativum*	cinnamic acid 4-hydroxylase	[19]
CYP74A1	*Linum usitatissimum*	allene oxide synthase	[20]
CYP74A3	*Arabidopsis thaliana*	allene oxide synthase	[21]
CYP75A1	*Petunia hybrida*	flavonoid 3',5'-hydroxylase	[22]
CYP75A2	*Solanum melongena*	flavonoid 3',5'-hydroxylase	[23]
CYP75A3	*Petunia hybrida*	flavonoid 3',5'-hydroxylase	[22]
CYP79	*Sorghum bicolor*	tyrosine *N*-hydroxylase	[24]
CYP80	*Berberis stolonifera*	berbamunine synthase	[25]
CYP84	*Arabidopsis thaliana*	ferulate-5-hydroxylase	[26]
CYP88A1	*Zea mays*	gibberellin biosynthesis	[27]
CYP90	*Arabidopsis thaliana*	brassinosteroid biosynthesis	[28]
CYP93B1	*Glycyrrhiza echinata*	(2*S*)-flavanone 2-hydroxylase	[29]

The reasons are why purification of a plant P450 enzyme is difficult because of low content, instability and interference with pigments and phenolic compounds, and why multiple species are present. In addition, many substrates are not commercially available. Thus genetic engineering, reverse genetics and bioorganic chemistry would contribute to study on plant P450 species.

Objective of This Study

In the present study, There are three research targets; 1) tobacco P450 reductase, 2) 5-*epi*-aristolochene 3-hydroxylase involved in the biosynthesis of the phytoalexin capsidiol in green pepper, and 3) tobacco P450 species involved in herbicide metabolism. The structure and function of these enzymes remained unknown. However, characterization of three enzymes are important for understanding molecular mechanism of disease and herbicide resistance in higher plants. Purification of a plant P450 enzyme is very difficult because a P450 species is membrane-bound, low content and unstable. Therefore, cloning and heterologous expression of cloned cDNAs would support to clarify the structure and function relationship of these enzymes.

In chapter II, it was attempted to purify P450 reductase from tobacco BY2 cells and to clarify biochemical and immunochemical characteristics of the purified enzyme. It is important to purify the enzyme for isolation of a cDNA encoding the corresponding enzyme as well as for clarification of the enzymatic characteristics.

In chapter III, it was attempted to isolate a cDNA encoding tobacco P450 reductase from tobacco plants and determine nucleotide sequence to deduce the primary structure. Tobacco P450 reductase cDNA cloned was expressed in the yeast *Saccharomyces cerevisiae* to analyze the function. Isolation and expression of a cDNA would give molecular information on the structure and function relationship of tobacco P450 reductase. In addition, co-expression of both tobacco P450 reductase and plant P450 cDNAs in the yeast as well as in tobacco plants has a large potential for identification of the function of P450 species as well as genetically engineering of transgenic plants.

In chapter IV, it was attempted to isolate a cDNA encoding 5-*epi*-aristolochene 3-hydroxylase from green pepper, which catalyzes a final oxidation reaction to synthesize capsidiol, which is one of the phytoalexins in Solanaceae plants. The biosynthesis of phytoalexin is important for defense mechanism in green pepper, although molecular information on the biosynthesis mechanism was quite limited. Determination of the primary structure of 5-*epi*-aristolochene 3-hydroxylase from green pepper would give us important information on the mechanism of biosynthesis of capsidiol in green pepper.

In chapter V, it was attempted to isolate P450 cDNAs related to herbicide metabolism from tobacco cultured cells and determine the nucleotide sequences to deduce the primary structure. P450 cDNAs cloned were each expressed in the yeast *Saccharomyces cerevisiae* to examine the enzyme function. it is important to clarify the structure and function of P450 species in order to understand herbicide selectivity and resistance on a molecular level. Elucidation of the herbicide resistance enables to develop genetically engineered plants with enhanced herbicide resistance as well as to develop chemicals stimulating the plant inherent herbicide resistant mechanism.

Molecular cloning and expression of plant P450 and P450 reductase cDNAs may enable to understand the secondary metabolism and xenobiotic metabolism in higher plants and may provide many applications in biotechnology.

CHAPTER II

PURIFICATION AND IMMUNOCHEMICAL CHARACTERISTICS OF NADPH-CYTOCHROME P450 OXIDOREDUCTASE FROM TOBACCO CULTURED CELLS

INTRODUCTION

NADPH cytochrome P450 oxidoreductase (EC1.6.2.4) transfers electrons to cytochrome P450 species in the microsomes of animal and plant cells [30, 31]. The terminal enzyme cytochrome P450 catalyzes oxidative reactions in the biosynthesis of a variety of secondary metabolites including lignin, flavonoids [32], phytoalexins [33] and sterols [34, 35] as well as in the metabolism of xenobiotics [36, 37]. NADPH-cytochrome P450 oxidoreductase contains noncovalently bound FAD and FMN as prosthetic groups [38, 39]. Up to the present, the enzymes have been purified from some plant species including *Catharanthus roseus* [40], sweet potato [41], *Helianthus tuberosus* [39] and mung bean [42]. The amino acid sequence of the mung bean reductase deduced from the nucleotide sequence of cDNA, showed 38% similarity in the amino acid sequence with the mammalian enzymes [42]. Recently, it was reported that multiple forms of NADPH-cytochrome P450 oxidoreductase were present in the microsome of Jerusalem artichoke tuber [43].

Here I report NADPH-cytochrome P450 oxidoreductase activity in tobacco cultured cells and purification of the enzyme from the microsomal fraction of tobacco BY2 cells. The purified tobacco reductase was enzymatically and immunochemically compared with yeast reductase.

MATERIALS AND METHODS

Chemicals

NADPH and $NADP^+$ were purchased from Oriental Yeast (Tokyo, Japan). FMN, FAD, dilauroylphosphatidylcholine (DLPC), 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitroblue tetrazolium (NBT) were obtained from Wako Pure Chemicals (Osaka, Japan). *trans*-Cinnamic acid, *p*-coumaric acid and a Cosmosil C_{18} packed column were purchased from Nacalai Tesque Co. Ltd. (Kyoto, Japan). CHAPS was products of Dojindo (Kumamoto, Japan). Q-Sepharose 4B and 2',5' ADP-Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden). DEAE-Toyopearl gel and TSK $G3000sw_{XL}$ column (7.5 × 30 cm) were obtained from Tosoh (Tokyo, Japan). Ribi Adjuvant System was purchased from Ribi Immunochem Research (MT, USA). PVDF-plus transfer membranes were obtained from Micron Separations (MA, USA). A Micro BCA protein assay reagent was a product of Pierce (Rockford, IL). All other chemicals were of reagent grade.

Analytical Methods

SDS-PAGE was carried out according to methods of Laemmli [44]. FMN and FAD contents were determined by the microfluorimetric method [45]. Protein was determined by the method of Lowry *et al.* [46]. When detergents were present in protein samples, Micro BCA protein assay was used for protein determination using Bovine serum albumin as a standard. Cytochrome P450 was determined spectrophotometrically according to the method of Omura and Sato [47].

Plant Materials

Nicotiana tabacum cultured cell lines BY2 [48], SL and S401 [49] were each cultivated in MS medium with 0.2 mg of 2,4-D per liter. Seven days after, tobacco cultured cells were transferred to fresh medium.

Preparation of Microsomes

All experiments were carried out at 4°C. Seven days after cultivation, tobacco cells were collected by filtration through a filter paper (Advantec, 5A). A 100 g

fresh weight of cells was homogenized in a Potter-Elvehjem type homogenizer with 50 ml of 0.1 M potassium phosphate buffer (pH 7.4), containing 0.5 M mannitol, 50 mM ascorbic acid, 42 mM 2-mercaptoethanol and 5 mM EDTA. A homogenate was centrifuged at 10,000 × *g* for 15 min to obtain a supernatant fraction, which was centrifuged at 100,000 × *g* (Beckman 70Ti) for 60 min. Pellets (1 ml) were then resuspended in 10 ml of 0.1 M sodium phosphate buffer (pH 7.5) containing 30% (w / v) glycerol and 1.4 mM 2-mercaptoethanol (buffer A), and then stored at -80°C until used.

Enzyme Purification

Step 1: Solubilization. A frozen microsomal preparation (450 mg protein in 25 ml of buffer A) was thawed on ice at 4°C and then slowly mixed with an equal volume of 2% (w / v) Emulgen 911 solution containing 10 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 1.5 mM 2-mercaptoethanol and 20% glycerol (buffer B) for 30 min at 4°C. A solubilized microsomal fraction was centrifuged at 100,000 × *g* (Beckman 70Ti) for 60 min to obtain a supernatant fraction.

Step 2: Q-Sepharose 4B column chromatography. A prepared supernatant fraction was diluted with two volumes of buffer A without Emulgen 911 and then loaded onto a Q-Sepharose 4B column (2.2 × 45 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 1.5 mM 2-mercaptoethanol, 0.2% (w / v) Emulgen 911 and 20% glycerol (buffer C). The column was washed with 3 volumes of buffer C. Protein was eluted by a linear gradient 0 to 1 M KCl in buffer C (600 ml) at a flow rate of 3 ml min^{-1}. Fractions containing the enzyme activity were collected.

Step 3: 2',5' ADP-Sepharose 4B column chromatography. A pooled fraction from Q-Sepharose 4B column chromatography was applied onto a 2',5' ADP-Sepharose 4B column (1.5 × 6.8 cm) equilibrated with buffer C. After washing with 5 volumes of 200 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 1.5 mM 2-mercaptoethanol, 0.2% (w / v) Emulgen 911 and 20% glycerol (buffer D), protein was eluted with buffer C containing 0.5 mM $NADP^+$

(50 ml) at a flow rate of 0.5 ml min^{-1}. Fractions containing the enzyme activity were collected.

Step 4: DEAE-Toyopearl column chromatography. A pooled fraction from 2',5' ADP-Sepharose 4B column chromatography was loaded onto a DEAE-Toyopearl column (1.6 × 5 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 1.5 mM 2-mercaptoethanol, 0.6% (w / v) sodium cholate and 20% glycerol (buffer E). To remove Emulgen 911 from the DEAE-Toyopearl column, buffer E was applied onto the column until absorption at 280 nm (due to Emulgen 911) became lower than 0.01. Elution of the reductase was carried out by linear gradient 0 to 1 M KCl in buffer E (100 ml) at a flow rate of 1.0 ml min^{-1}.

Partial Purification of Tobacco Cytochrome P450

A microsomal preparation from BY2 cells was solubilized with CHAPS at a final concentration of 16 mM and then centrifuged at 100,000 × *g* for 60 min to obtain a supernatant, which was loaded onto a DEAE-Toyopearl column (1.6 × 5 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 1.5 mM 2-mercaptoethanol, 0.2% (w / v) CHAPS and 20% glycerol (buffer F). The column was washed with buffer F. Elution of P450 was carried out by a linear gradient 0 to 0.5 M KCl in buffr F. Fractions showing an absorbance at 410 nm were pooled. A major part of the pooled P450-containing fractions was applied to 2',5' ADP-Sepharose 4B column (1.5 × 6.8 cm) equilibrated with buffr C. A pass-through fraction which contained no NADPH-cytochrome c reductase was concentrated 20-fold by using an Amicon ultrafiltration membrane (YM-30) and stored at -20°C.

***In Vitro* Reconstitution Systems for Cytochrome P450 Monooxygenase Assays**

A reconstituted system for cinnnamic acid 4-hydroxylase (CA4H) was prepared by a method similar to those described by Benveniste *et al.* [39]. Cinnnamic acid 4-hydroxylase activity was assayed by the procedure modified by Werck-Reichhart

et al. [50]. A reaction mixture in a final volume of 200 ml, contained 26 pmol of tobacco P450, 97 nmol of tobacco reductase, 100 μM FMN, 250 mg of DLPC, 0.5 mM NADPH, 1 mM *trans*-cinnnamic acid and 0.1 M potassium phosphate buffer (pH 7.4). Reaction was carried out at 25°C for 1 h and then stopped by addition of 10 μl of 4N HCl. Precipitated proteins were removed by centrifugation to obtain a supernatant which was concentrated to 20 μl and then directly applied to a Cosmosil C_{18} packed column. HPLC analysis of *trans*-cinnamic acid and *para*-coumaric acid was carried out according to the method of Blume and Saunders [51].

A reconstitution system for aminopyrine *N*-demethylase contained 17.5 nmol of rabbit P4502C14 [52], 110 nmol tobacco reductase preparation, 100 μM FMN, 10 mg of DLPC, 0.5 mM NADPH, 5 mM aminopyrine and 0.1 M Tris-HCl (pH 7.5) to a final volume of 0.5 ml. Reaction was carried out at 37°C and stopped after 30 min by addition of 250 ml of 20% (w / v) TCA. After mixing and centrifugation at 20,000 × *g* for 10 min, a 0.5 ml supernatant obtained was mixed with 0.75 ml Nash-reagent [53] and incubated at 37°C for 60 min. Amounts of formaldehyde formed were determined by measuring absorbance at 412 nm.

Glycoprotein Analysis

A purified tobacco reductase preparation was analyzed for glycoprotein by the method using concanavalin A and peroxidase [54].

Preparation of Antibodies

About 50 mg of a purified tobacco reductase preparation was mixed with a Ribi Adjuvant System (1 ml) and then administered to a female rabbit (New Zealand white, 2.5 kg) by intramuscular injection. After two weeks, an equal amount of the reductase was administered in the same way. After four weeks, blood samples were collected for preparation of anti-serum.

For enzyme inhibition studies, anti-serum was further purified by ammonium sulfate precipitation. Immunoglobulins from pre-immune rabbit sera were prepared in the same way.

Western Blot Analysis

Western blot analysis using anti-reductase antibodies was carried out according to the procedure of Towbin *et al* [55], with some modifications. Tobacco microsomal and reductase preparations were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto PVDF-plus transfer membranes. Remaining protein-binding sites on PVDF membranes were by Tris-HCl buffer (pH 7.5), containing 3% bovine serum albumin, 150 mM NaCl, 0.05% (w / v) NaN_3 and 0.05% (w / v) Tween 20. After treatment with rabbit anti-tobacco reductase serum diluted 100-times, specific antigen-antibody complexes were detected by goat anti-rabbit immunoglobulins coupled with alkaline phosphatase. BCIP and NBT, the substrate of alkaline phosphatase, were used for detection of protein bands.

Cytochrome c Oxidoreductase Activity

Reconstitution of NADPH-cytochrome c reductase with FMN was carried out according to the method of Benveniste *et al.* [39]. NADPH-dependent reduction of cytochrome c was monitored by measuring absorbance at 550 nm, at 30°C, in the presence of 0.3 M potassium phosphate buffer (pH 7.5), 100 mM cytochrome c, 200 mM NADPH and 90 mM EDTA. KCN (1 mM) was used to inhibit cytochrome c oxidase due to possible mitochondrial contaminations. A molar absorption coefficient of 21 mM^{-1} cm^{-1} for horse heart cytochrome c was used for determination of enzyme activity.

RESULTS

Purification of NADPH-Cytochrome P450 Oxidoreductase from Tobacco Cultured Cells

When NADPH-cytochrome c reductase activities in BY2, SL and S401 were measured, almost equal enzyme activities were observed. Since a growth of tobacco BY2 cells was the firstest of three tobacco cell lines, BY2 was used for a NADPH-cytochrome P450 oxidoreductase purification.

About 450 mg of 100,000 × *g* pellets was obtained from 500 g of 7-day-old cultured tobacco BY2 cells. The NADPH-cytochrome c reductase activity of this microsomal fraction was about 0.03 mmol of cytochrome c min^{-1} mg^{-1} of protein. This microsomal fraction was solubilized in buffer B. the solubilized fraction was loaded onto a Q-Sepharose 4B column equilibrated with buffer c, and the eluted by a linear gradient 0 to 1 M KCl in buffer C. The pooled fraction with enzyme activity was applied onto a 2',5' ADP-Sepharose 4B column equilibrated with buffer C and then eluted with buffer C containing 0.5 mM $NADP^+$. The pooled fraction with enzyme activity was applied onto DEAE-Toyopeal column equilibrated with buffer E and then eluted by a liner gradient 0 to 1 M KCl in buffer E. The pooled fraction with enzyme activity showed a single band with a molecular size of 79 kDa on SDS-PAGE (Fig. 2-1) When the purified enzyme was analyzed by TSK $G3000SW_{XL}$ column chromatography, the molecular mass was approx. 80 kDa (data not shown). Thus, purified reductase seemed to be a monomer enzyme (data not shown). The purification of the NADPH-cytochrome c (P450) reductase from tobacco cultured BY2 cells was summarized in Table 2-1. The reductase was purified 383-fold with a recovery of 16%. The specific activity of the purified enzyme was 11.5 mmol cytochrome c reduced min^{-1} mg^{-1} of protein.

Enzymatic Characteristics of the Purified Tobacco Reductase

The NADPH-cytochrome c oxidoreductase activity of the purified tobacco reductase was assayed at various pH values raging from 6.0 to 9.0. The results showed a bell-shaped pH profile. The optimal pH for the reductase activity was around 7.5.

Fig. 2-1. SDS-PAGE analysis of the purified tobacco NADPH-cytochrome P450 oxidoreductase. Polyacrylamide gel concentration was 7.5%. Lane 1: NADPH-cytochrome P450 oxidoreductase after chromatography on a DEAE-Toyopearl column (0.5 μg). Lane 2: standard molecular weight marker (2.0 μg): myosin (M_r, 205,000); β-galactosidase (M_r, 116,000); phosphorylase b (M_r, 97,400); albumin, bovine (M_r, 66,000); albumin, egg (M_r, 45,000).

Table 2-1

Purification of NADPH-cytochrome P450 oxidoreductase from tobacco BY2 cells

	Protein (mg)	Total activity (mmol / min)	Specific activity (mmol min^{-1} mg^{-1})	Purification factor	Recovery (%)
Microsomes	450.0	14.4	0.03	-	100
Solubilized microsomes	298.0	21.6	0.07	2	150
Q-Sepharose	75.2	12.1	0.16	5	84
2',5'-ADP Sepharose	0.7	6.2	8.86	297	43
DEAE-Toyopearl	0.2	2.3	11.50	383	16

Cytochrome c reduction was measured in the presence of 0.3 M potassium phosphate buffer (pH 7.5), 100 mM cytochrome c, 200 mM NADPH and 90 mM EDTA at 30°C.

Kinetic properties were determined for the purified tobacco reductase. The NADPH-cytochrome c oxidoreductase activity of the purified enzyme was found to follow Michaelis-Menten kinetics. The purified reductase had an apparent K_m for NADPH was 24 μM. The apparent K_m for cytochrome c was 16 μM (data not shown). As shown in Table 2-2, when FMN was added to the purified tobacco reductase, a remarkable enhancement of the NADPH-cytochrome c oxidoreductase activity was found. Particularly, about 10-fold enhancement of the activity was observed in the presence of 100 μM FMN. On the other hand, the addition of FAD to the purified enzyme showed only a small effect. Fig. 2-2 shows the oxidized spectrum of the reconstituted tobacco reductase. Under the oxidized conditions, the absorbance maximum occurred at 452 nm and 372 nm with a shoulder at about 472 nm in the visible-region indicating the presence of FMN and FAD as prosthetic groups. The flavin contents of the reconstituted reductase was spectrofluorimetrically determined to be 0.54 mol of FMN and 0.57 mol of FAD per mol of the purified reductase by the methods of Fader and Siegel [45].

The glycoprotein nature of the purified tobacco reductase was examined. The purified tobacco reductase and Ovalbumin were separated by SDS-PAGE and electrophoretically blotted onto PVDF-membranes. As shown in Fig. 2-3, the purified reductase was not stained with the concanavalin A-peroxidase technique, although Ovalbumin was stained by using concanavalin A and peroxidase. These results suggested that the purified reductase was not glycosylated by α-D mannopyranosyl and α-D-glucopyrosyl residues.

P450 Monooxygenase Activities in an *In Vitro* Reconstituted System

The CA4H activity was assayed in an *in vitro* reconstitution system containing a partially purified P450 fraction from BY2 cells, the purified tobacco reductase, DLPC and NADPH. The specific CA4H activity in the reconstitution system was 14 pmol min^{-1} $nmol^{-1}$ P450. When P450 or reductase was omitted from the complete assay mixture, enzyme activities were not detected. Therefore it was found that the purified reductase transferred electrons from NADPH to a tobacco P450 to exhibit the CA4H activity. When the purified tobacco reductase and rabbit P4502C14 were used for the reconstitution system, as shown in Table 2-3, the

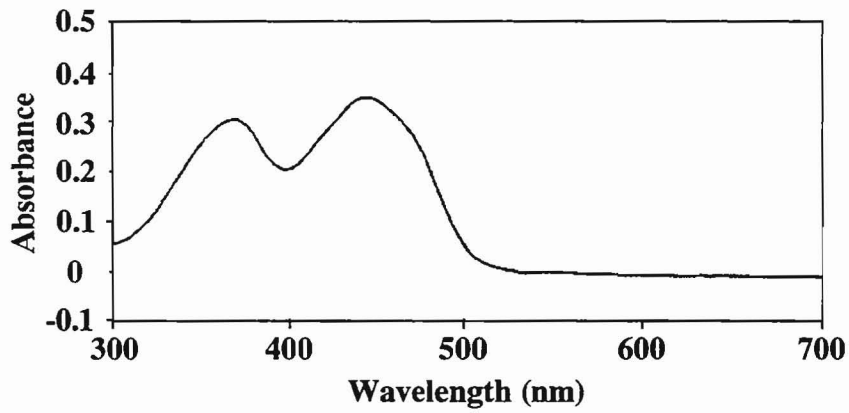

Fig. 2-2. The oxidized spectrum of the purified NADPH-cytochrome P450 oxidoreductase from tobacco cultured cell. The reductase concentration was 2.0 mg / ml.

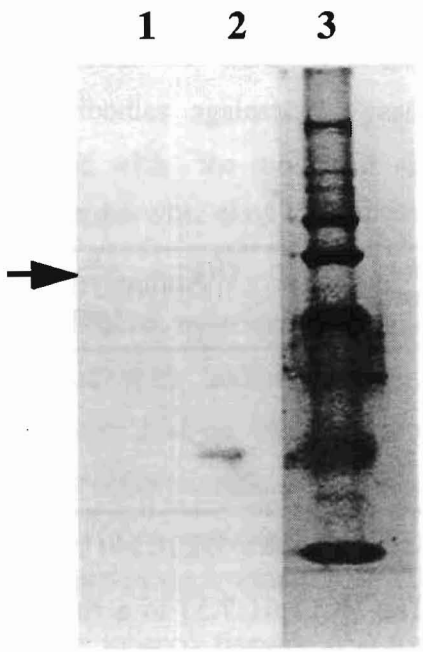

Fig. 2-3. Glycoprotein analysis. The purified tobacco NADPH-cytochrome P450 oxidoreductase (lane 1, 50 ng), and Ovalbumin (lane 2, 50 ng) were analyzed by SDS-PAGE (7.5%) and electrophoretically blotted onto PVDF-membrane. Protein was stained by concanavalin A-peroxidase technique. Lane 3 contains M_r markers. Position of the tobacco reductase was marked by arrow.

Table 2-2

Effect of FMN on the activity of purified tobacco reductase

FMN (μM)	Enzyme activity ($\Delta A_{550\ nm}$ / min)
0	0.01
2	0.03
5	0.05
10	0.09
100	0.10

The enzyme activity of the tobacco NADPH-cytochrome P450 oxidoreductase preparation (5 μg) after chromatography on a DEAE-Toyopearl column was measured in the presence of FMN.

Table 2-3

Aminopyrine *N*-demethylation activity in an *in vitro* reconstitution system

Source of the reductase	Aminopyrine *N*-demethylation activity (pmol / min / nmol P450)
Tobacco BY2 cells	6
Yeast	12
Rat	38

Reaction mixtures contained purified rabbit P4502C14 (17.5 nmol), the purified reductase (110 nmol), 100 mM FMN, 10 mg of dilauroyl phosphatidylcholine, 0.5 mM NADPH, 5 mM aminopyrine and 0.1 M Tris-HCl (pH 7.5) to a final volume of 0.5 ml. Reaction mixtures were incubated for 30 min at 37°C.

aminopyrine *N*-demethylation activity was 6 pmol min^{-1} $nmol^{-1}$ of P450. On the other hand, when yeast reductase was reconstituted with rabbit P4502C14, the specific activity was 12 pmol min^{-1} $nmol^{-1}$ of P450. The reconstituted system between rat reductase and P4502C14 exhibited the specific activity of 38 pmol min^{-1} $nmol^{-1}$ of P450. Thus the tobacco reductase was found to be efficient transfer electrons from NADPH to P4502C14 to exhibit the aminopyrine *N*-demethylase activity, although the efficiency of transfer electron to P4502C14 was higher with yeast and rat reductase than the tobacco reductase.

Immunochemical Characteristics of the Tobacco Reductase

Rabbit polyclonal antibodies raised against the purified tobacco reductase was prepared and used for western blot analysis of the microsome fraction prepared from each of leaves, stems and roots of *Nicotiana tabacum*.

As shown in Fig. 2-4, western blot analysis revealed that the antibodies against tobacco reductase reacted with the protein band of the purified tobacco reductase with the molecular size of about 79 kDa, but did not react with the yeast reductase. In contrast, the antibodies against the yeast reductase reacted with the protein band of yeast reductase with the molecular size of 82 kDa, but not cross react with tobacco plant reductase.

As shown in Table 2-4, the activity of the tobacco reductase was totally inhibited by addition of anti-tobacco reductase antibodies, whereas no inhibition of the yeast reductase was found with addition of an equivalent amount of the antibodies. Pre-immune antibodies had no effect on both reductase activities.

As shown in Fig. 2-5, anti-tobacco reductase antibodies reacted with a protein in the microsomes of leaves, root and shoots of vegetatively growing tobacco plants. From the band intensity, almost an equal amount of the enzyme protein may be present in each of the tobacco tissues.

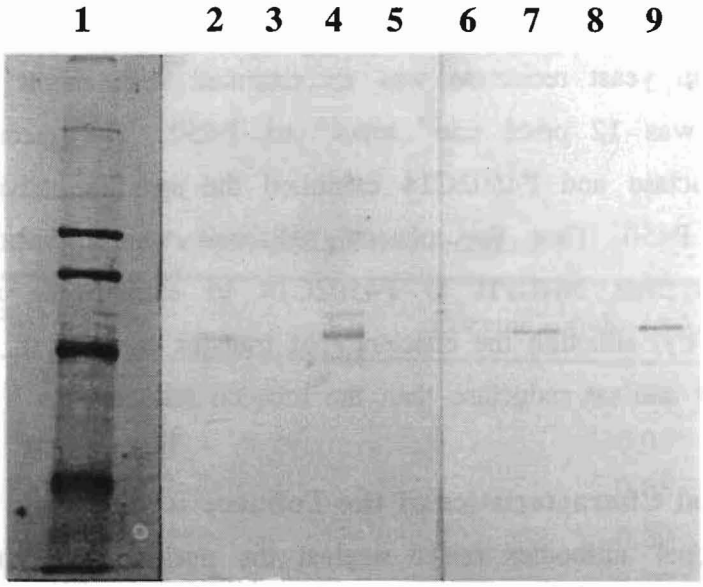

Fig. 2-4. Western blot analysis of NADPH-cytochrome P450 oxido-reductase preparations from tobacco cultured BY2 cells and yeast. Lane 1, standard molecular weight markers ; lane 2 (60 ng), 4 (120 ng), 6 (60 ng), 8 (120 ng), purified NADPH-cytochrome P450 reductase from yeast; lane 3 (60 ng), 5 (120 ng), 7 (60 ng), 9 (120 ng), purified tobacco reductase from tobacco cultured BY2 cells. Lane 2, 3, 4, and 5, Western blot analysis were carried out with an anti-tobacco reductase antibody. Lane 6, 7, 8 and 9, western blot analysis were carried out with an anti-yeast reductase antibody.

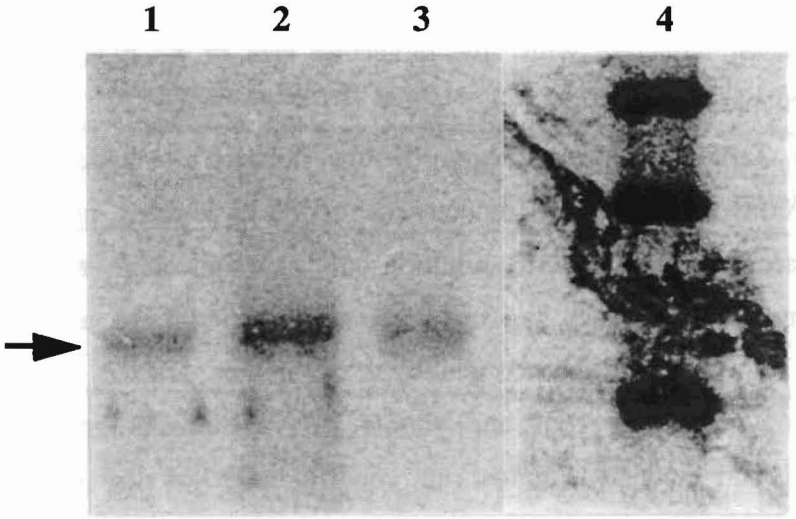

Fig. 2-5. Western blot analysis of the microsomes prepared from leaves, stems and roots of tobacco plants. Western blot analysis was carried out by using antibodies raised against the tobacco reductase. Concentration of polyacryamide gel was 7.5%. Each lane contains 10 mg of the microsomal protein. Lane 1, leaf; lane 2, root; lane 3, shoot; lane 4 shows standard molecular weight markers. Position of the tobacco reductase is marked by arrow. The minor bands of lower M_r probably resulted from protein degradation in some microsomal preparations.

Table 2-4

Immunotitration of the purified NADPH-cytochrome c oxidoreductase activity of tobacco and yeast enzymes.

IgG/Tobacco reductase (mg / ng)	Enzyme activity (% of control)	IgG/Yeast reductase (mg / ng)	Enzyme activity (% of control)
0	100	0	100
10	28	10	101
100	0	100	98

After the purified NADPH-cytochrome P450 reductase (10 mg) from tobacco or yeast was preincubated with an increasing amount of anti-tobacco reductase antibodies for 30 min. The reaction mixtures were centrifuged at 100,000 × *g* for 10 min. Supernatants were assayed with the standard method as described in *Materials and Methods*. The activity in control was measured in the absence of IgG. Pre-immune IgG did not inhibit the tobacco and yeast reductase activities.

DISCUSSION

The present study described the purification of NADPH-cytochrome P450 oxidoreductase from tobacco BY2 cells. The specific reductase activity for cytochrome c per mg of protein in the microsomes of BY2 cells was about 1 / 10 less than that of the liver microsomes of untreated rabbits [56], whereas the apparent total cytochrome P450 concentration was 1 / 20 less than that of rabbit livers [57]. The levels of both NADPH-cytochrome P450 oxidoreductase and total cytochrome P450 were less in the plant microsomes than the mammalian liver microsomes [4, 31]. We examined several detergents for solubilization of tobacco microsomes, about 150% of the reductase activity was recovered from the microsomal membranes by treatment with 2% Emulgen 911. Similar results were also reported with the reductase of *Herianthus tuberosus* L. [39]. It was likely that the treatment with this detergent changes the reductase molecule into a more activated form. The specific activity of the purified tobacco reductase for cytochrome c was 11.5 mmol min^{-1} mg^{-1} of protein. This value was lower than those described for *Catharanthus roseus* (17 mmol min^{-1} mg^{-1} of protein) [40], for *Helianthus tuberosus* L. (33 mmol min^{-1} mg^{-1} of protein) [39] and for pig kidney (60 mmol min^{-1} mg^{-1} of protein) [58]. The apparent molecular weight of the tobacco purified reductase was determined to be 79 kDa on SDS-PAGE. This molecular size was smaller than that of *Helianthus tuberosus* L. (82 kDa) [39], *Vigna radiata* ver. Berken (82 kDa) [42], sweet potato (81 kDa) [41] and the yeast *Saccharomyces cerevisiae* (83 kDa) [59], but nearly the same as that *of Catharanthus roseus* (78 kDa) [40]. The apparent Michaelis-Menten Kinetics with K_m values of this purified enzyme for cytochrome c and NADPH were 16 μM and 24 μM, respectively, these values are similar to the other published values [39].

The specific activity of aminopyrine *N*-demethylatiom in *in vitro* reconstituted system containing purified rabbit liver P4502C14 was 6 pmol min^{-1} $nmol^{-1}$ of P450, which was nearly a half of that of the reconstituted system containing P4502C14 and yeast reductase (12 pmol min^{-1} $nmol^{-1}$ of P450). The previous studies showed that NADPH-cytochrome P450 oxidoreductases reconstituted with

P450 species from different origins to exhibit the monooxygenase activities. For example, trout reductase reconstituted with rat P450 [60]. Likewise, avocado P450 was reconstituted with rat liver reductase [61]. Shet and his co-workers reported that the reconstitution system of porcine P45017A and mung bean reductase showed 17α–hydroxylation activity [42]. Similarly, the tobacco reductase reconstituted with rabbit liver P4502C14 to exhibit the corresponding monooxygenase activity, although tobacco reductase was less efficient than that of the rabbit and yeast reductases in the reactions.

It was reported that microsomal NADPH-cytochrome P450 oxidoreductases in plants, mammals and yeast contains both FAD and FMN in the molecule [30, 31]. The loss of FMN occurred easily during preparation and resulted in formation of an inactive enzyme [43]. But, the activity was restored by addition of FMN to the enzyme preparation. Flavin contents in the purified reductase were 0.54 mol of FMN and 0.57 mol of FAD per mol of the purified reductase. These values were comparable with those of the reductase, *Catharanthus roseus* (0.76 mol of FMN and 0.37 mol of FAD per mol of enzyme) [40], mung bean (0.92 mol of FMN and 0.62 mol of FAD per mol of enzyme) [42] and *Helianthus tuberosus* L. (0.41 mol of FMN and 0.31 mol of of FAD per mol of enzyme) [39], but lower than those of mammalian enzymes [30]. The lower specific activity of tobacco reductase was may be due to the lower content of flavins in the tobacco reductase as compared with the mammalian reductases.

The structural difference between the reductase from higher plants and the enzymes from rat and yeast has been reported. Benveniste and his co-workers reported that the Jerusalem artichoke, avocado, maize, *Vicia fava*, sunflower and blamble reductases cross reacted with anti-Jerusalem artichoke antibodies, while rat liver and yeast reductases did not cross react with anti-Jerusalem artichoke antibodies [62]. On the other hand, from a comparison of the amino-acid sequence of mung bean reductase with the corresponding sequence of *Arabidopsis*, yeast and rat, *Arabidopsis* reductase had 73 % amino acid sequence identity with the mung bean reductase, while yeast and rat reductases have 38.8 % and 32.8 % amino acid sequence identity with the mung bean reductase, respectively [42]. In this study, the antibodies raised against the purified tobacco reductase with a

molecular size of about 79 kDa, but did not cross react the purified yeast reductase. Furthermore, anti-tobacco reductase antibodies totally inhibited the reductase activity of the tobacco enzyme, but not inhibited yeast reductase activity. From these results, it was suggested that there are different antibody binding sites, which certainly participate in enzyme activity, between tobacco and yeast reductase. In *in vitro* reconstituted system, the tobacco reductase transferred electrons from NADPH to the tobacco P450 and rabbit P4502C14. Therefore, the structural similarity required for interaction with various P450 isozymes from different species seemed to be conserved among various reductase species from different origins. Indeed, five functional domains, including an amino-terminal domain that anchors the protein to the membrane and binding regions assigned to the interaction of FAD, FMN, NADPH and cytochrome P450 are well conserved among higher plants, yeast and rat reductases [42].

At the present time, the three dimensional structure of any reductase species in plants, yeasts and mammals is not yet known, so we cannot clarify the details of interaction between P450 and the reductase. Purification of the reductase and preparation of antibodies against it will allow us to further examine its role and regulation.

CHAPTER III

MOLECULAR CLONING AND EXPRESSION OF cDNA FOR TOBACCO NADPH-CYTOCHROME P450 OXIDOREDUCTASE IN *Saccharomyces cerevisiae*

INTRODUCTION

Cytochrome P450 monooxygenases (P450 monooxygenases) in higher plants involve in the biosynthesis and metabolism of sterols, glucosinolates, phenylpropanoids / flavonoids, salicylic acid, jasmonic acid, gibberellic acids, abscisic acid, brassinosteroids, and alkaloids as well as in the metabolism of pesticides [8]. The P450 monooxygenases in the microsomes of plant cells consist of a number of cytochrome P450 (P450) species and a few species of NADPH-cytochrome P450 oxidoreductase (P450 reductase). The P450 reductase is a flavoprotein containing one FAD and FMN as prosthetic groups and transfers electrons from NADPH through FAD and FMN of the reductase to heme of each of P450 species [63]. Molecular oxygen is activated and one oxygen atom is incorporated into a substrate on a P450 enzyme. So, the P450 reductase play an important role in the regulation of P450 dependent-monooxygenase activities.

P450 reductase was purified from tobacco cultured cells BY2 as described in chapter II. The purified tobacco P450 reductase coupled with both tobacco and rabbit P450 species in an *in vitro* reconstitution system, and showed the P450-dependent monooxygenase activities. The tobacco P450 reductase was immunochemically different from yeast P450 reductase and detected in leaves, shoots and stems of tobacco plants. Based on these results, I attempted to isolate cDNA encoding tobacco P450 reductase for clarification of the primary structure and of characteristics of the enzyme expressed in the yeast *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Chemicals and Biochemicals

5'-[γ-^{32}P]ATP (6000 Ci / mmol) and [α-^{32}P]dCTP (3000 Ci / ml) were purchased from Amersham International plc. (Buckinghamshire, UK).

Restriction endonucleases and DNA modifying enzymes were purchased from Takara Shuzo Co., Ltd. (Shiga. Japan). *Pfu* DNA polymerase was purchased from Stratagene Cloning Systems (La Jolla, CA).

Other chemicals and biochemicals were purchased from Wako Pure Chemicals (Osaka, Japan), Nacalai Tesque Co., Ltd. (Kyoto, Japan) and Sigma Chemical Co. (St. Louis, MO)

Bacterial Strains

Escherichia coli K12 strains Y1090 and JM109 were used as host for recombinant pharge λgt 11 and for subcloning, respectively (Clontech Laboratories, Inc. Palo Alto, CA and Takara Shuzo). *Saccharomyces cerevisiae* AH22 [64] was used as host for expression of cloned cDNAs.

Cloning of Tobacco P450 Reductase cDNA

Six oligonucleotides (Oligo-1 to Oligo-6) were designed according to the cDNA sequences of *Arabidopsis* and mung bean P450 reductases deposited in GenBank database (X66016, X66017, L07843) [16, 42]. Four oligonucleotides (Oligo-1 to Oligo-4) and two oligonucleotides (Oligo-5 and Oligo-6) corresponded to the possible FMN and NADPH binding regions of the P450 reductases, respectively. The sense primers Oligo-1, Oligo-3 and Oligo-5 were homologous to *Arabidopsis* P450 reductase (*ATR1*) cDNA sequences [16], 337-359 bp, 511-534 bp, 1714-1733 bp, respectively. The antisense primers Oligo-2, Oligo-4 and Oligo-6 were homologous to the sequences, 514-537 bp, 619-642 bp, 2003-2025 bp, respectively: Oligo-1, 5'-GGT ACG CAG ACT GGA ACA GCT GA-3'; Oligo-2, 5'-ATT GTC AGT AGG CTC TCC ATC TCC-3'; Oligo-3, 5'-TAT GGA GAT GGA GAG CCT ACT GAC-3'; Oligo-4, 5'-ATG TTC ATA TTG GCG ATT ACC AAG-3'; Oligo-5, 5'-CCT GGG ACT GGG CTG GCA CC-3'; Oligo-6, 5'-

CGC CAT GCC CTT AGC ATC ACC GCA-3'. PCR was carried out by the use of a λgt 11 tobacco cDNA library (*Nicotiana tabacum* cv. Petite Havana SR-1; a gift of Dr. Iba, Faculty of Science, Kyusyu University, Japan) as a template. The reaction was carried out through 30 cycles of 0.5 min at 94°C, 1 min at 50°C and 1.5 min at 72°C with *Taq* DNA polymerase (Takara Shuzo). PCR products were separated by polyacrylamide gel electrophoresis (PAGE). PCR fragments were subcloned into *Hin*cII site of pUC18 and then sequenced.

Tobacco cDNA library described above was screened by plaque hybridization using a cloned PCR fragment as a probe DNA. About 1×10^6 plaques were screened by plaque hybridization with the cDNA fragment as the probe which was radiolabeled with 5'-[γ-^{32}P]ATP (6000 Ci / mmol) [65]. Hybridization was performed at 42°C for 20 h in a solution containing 50% (v / v) formamide, 5 × SSC, 5 × Denhardt's solution [65], 100 μg per ml salmon sperm DNA, 0.5% SDS, and a probe DNA (about 1×10^8 cpm / μg of DNA). The membrane was washed in 2 × SSC-0.1% SDS for 5 min at room temperature at once, 30 min at 50°C twice, and then in 0.2 × SSC-0.1% SDS for 30 min at 50°C. An insert DNA in a positive plaque was subcloned into pBluescriptII KS+ (Stratagene) for DNA sequencing.

Inverse PCR

Four oligonucleotides (IFP-1 to IFP-4) were designed based on nucleotide sequence of tobacco P450 reductase cDNA cloned (pCTR1). Two antisense primers (IFP-1 and IFP-2) and two sense primers (IFP-3 and IFP-4) corresponded to the 5' and 3' regions of the cDNA clone (pCTR1), respectively. IFP-1, IFP-2, IFP-3 and IFP-4 corresponded to the nucleotide sequence of tobacco reductase cDNA cloned pCTR1, 18-37 bp, 33-52 bp, 1773-1792 bp and 1799-1818 bp, respectively. IFP-1; 5'-A GTA CCA GTC TGG GTA CCA A-3', IFP-2; 5'-AG CAA AGC CTT CAG CAG TAC-3', IFP-3; 5'-A GGC CTT GGT GAA GAA CTT G-3', IFP-4; 5'-ACT GGA AGA TAT CTG CGT GA-3'.

Tobacco (*Nicotiana tabacum* cv. Petite Havana SR-1) genomic DNA (1 μg) was digested with *Hin*dIII. The digested DNA was subjected to ligation reaction [66]. PCR was carried out with the primers IFP-2 and IFP-3 using the ligated

DNA described above as a template. PCR was repeated with primers IFP-1 and IFP-4 using the PCR mixture as a template DNA. These PCR were carried out through 20 cycles of 1 min at 94°C, 2 min at 50°C and 3 min at 72°C with *Pfu* DNA polymerase. An amplified DNA fragment was subcloned for DNA sequencing.

DNA Sequencing and Sequence Analysis

Plasmid DNA containing a cDNA as well as PCR fragment were purified by precipitation with polyethylene glycol [65]. Both strands were sequenced by the dideoxy-termination method using T7 DNA polymerase with an AutoRead™ Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) in a Shimadzu model DSQ1 DNA sequencer (Shimadzu Co. Ltd., Kyoto, Japan). Sequence analysis was performed with DNASIS-Mac software (Hitachi Software Engineering Co. Ltd., Yokohama, Japan). Multiple sequence alignment was performed using a BLAST computer program [67]. Prediction of protein localization was performed by a PSORT computer program (UTL; http://www.yk.rim.or.jp/~aisoai/molbio-j.html).

Southern and Northern Blot Analysis

Tobacco genomic DNA was prepared as described [68]. Genomic DNA (20 μg) was digested with *Bam*HI, *Eco*RI and *Hin*dIII. Hybridization was performed under the same conditions of plaque hybridization described above.

Total RNA was prepared from frozen tissues of tobacco plants. Frozen tissues were crushed to a fine powder under liquid nitrogen and transferred to extraction buffer (Tris-HCl pH 8.0, 0.01 M EDTA pH 8.0, 1% SDS, 1 mM aurintricarboxylic acid (ATA), 10 mM 2-mercaptoethanol). After dispersing the tissues, 0.1 volume of 3 M KCl was added and mixed. Insoluble materials were removed by centrifugation at 9000 × *g* for 20 min. The supernatant was mixed with 0.2 volume of 8 M LiCl, stored at 4°C overnight and centrifuged at 9000 × *g* for 20 min. The pellets were resuspended in H_2O and extracted with an equal volume of phenol-chloroform. Total RNA was recovered by ethanol precipitation. Northern hybridization was carried out under the same conditions of plaque hybridization described above, although SSPE was used instead of SSC.

Peptide Sequencing

Tobacco P450 reductase was purified as described in chapter II. A purified P450 reductase (10 μg) was concentrated by ultrafiltration (Centricon, YM-30, GRACE company, MA) and then added to 0.01% (w / w) TPCK trypsin (Sigma) in 0.003 N HCl. Digestion was carried out at 37°C for 4 h. Polypeptides were separated by SDS-PAGE (10% gel) and then electroblotted onto a PVDF membrane as described [69]. Peptides were stained with Coomassie brilliant blue R. Amino acid sequence was determined by automated Edman degradation in an Applied Biosystems model 470A protein sequencer (Perkin-Elmer, Foster City, CA).

Constraction of an Expression Plasmid for the Yeast

Two oligonucleotides, FP-1; 5'-AAGCTTCC ATG GAG TCT ACA TCA GAGA-3' and FP-2; 5'-AAGCTT TCA CCA CAC ATC ACG CAG AT-3' were synthesized based on DNA sequences of a cDNA cloned (pCTR1) and a genomic DNA cloned (pGTR1) containing the start and stop translation codons (double underlined), respectively, with *Hin*dIII site (underlined) at 5' ends. Reverse transcriptase (RT)-PCR was carried out using FP-1 and FP-2 with a poly(A) RNA from tobacco leaves. An amplified 2.1 kbp fragment was subcloned into *Hin*dIII site of pUC18, and nucleotide sequence was confirmed. The cloned 2.1 kbp cDNA pFTR was inserted into *Hin*dIII site between ADH promoter and terminator regions of the yeast expression vector pAAH5 [64]. The recombinant plasmid was transformed into the yeast *S. cerevisiae* AH22 cells by the lithium chloride method as described [70].

Culture and Preparation of a Microsomal Fraction of the Yeast

Transformed yeast cells were grown to about 1.5×10^7 cells per ml in SD medium [64] supplement with 160 μg per ml of histidine. Preparation of yeast microsomal fraction was carried out by the procedure of Oeda *et al.* [64].

Protein Analysis

A Bio-Rad Protein Assay kit (BIO-RAD Laboratories, Inc., Hercules, CA) was used for protein determination as bovine serum albumin as a standard. Western blot analysis using anti-tobacco P450 reductase antibodies was carried out as described in chapter II.

Enzyme Assay

Cytochrome c reductase activity was measured as described in chapter II. 7-Ethoxycoumarin *O*-deethylase activity was determined as described previously [64].

RESULTS

Cloning of Tobacco P450 Reductase cDNA

Based on preliminary sequencing of a purified protein sample of tobacco P450 reductase, the amino terminus seemed to be modified. Therefore the protein sample was subjected to limited proteolysis. One major band P1 (43 kDa), and two minor bands P2 (40 kDa) and P3 (38 kDa) were obtained. The amino terminal sequence of P1 was found to be Val-Val-X-His-Glu-Lys-Pro-Asn-Asn-Asp-Leu-X-Asn (X; not determined). This sequence was not found in the protein sequences deposited in protein detabase. Therefore the sequence seemed not to be a conserved region of the P450 reductase sequences reported. Also, the sequence was short and not suitable for designing degenerate primers used as a probe DNA for screening of cDNA library. Therefore six oligonucleotides were designed based on the cDNA sequences of *Arabidopsis* and mung bean P450 reductases reported [5,6]. PCR was carried out with a λgt 11 tobacco cDNA library as a template. With a set of Oligo-1 and Oligo-2 corresponding to FMN binding region, about 200 bp fragment was obtained as a major product. This PCR fragment was subcloned and sequenced. The nucleotide sequence and the deduced amino sequence showed a high similarity with those of *Arabidopsis* and mung bean P450 reductases [16, 42].

Tobacco cDNA library was screened with the PCR fragment as a probe. Two positive clones pCTR1 and pCTR2 contained the same 2.2 kbp insert DNA. The nucleotide sequence and the deduced amino acid sequence of pCTR1 contained the nucleotide sequence of the probe used and the peptide sequence determined by microsequencing of the purified enzyme, respectively. The molecular weight of the polypeptide deduced from pCTR1 was found to be shorter than the molecular mass of the purified tobacco P450 reductase. Therefore, the cDNA pCTR1 seemed not to encode a full length tobacco P450 reductase and lack 5' region of tobacco P450 reductase cDNA. Although the same cDNA library was rescreened, no longer cDNA clones were obtained.

To isolate a DNA fragment containing 5' region of tobacco P450 reductase gene, inverse PCR was carried out with tobacco genomic DNA as a template. A

1.0 kbp fragment was amplified and subcloned. Based on sequencing, 10 clones pGTR1 to pGTR10 were found to have a 0.5 kbp DNA fragment corresponding to 5' region of tobacco P450 reductase gene. Then, RT-PCR was carried out using oligonucleotides FP-1 and FP-2, based on the sequences of 5' region of pGTR1 and 3' region of pCTR1, respectively. The clone pFTR was obtained by cloning of an amplified 2.1 kbp DNA fragment and the nucleotide sequence of pFTR was found to contain the combined sequences both pGTR1 and pCTR1. As a result, the cDNA pFTR was found to encode the entire tobacco P450 reductase.

Sequence Analysis

Figure 3-1 shows the nucleotide sequence and deduced amino acid sequence information on tobacco P450 reductase by the combination of the cDNA clone pCTR1 and genomic DNA clone pGTR1. The nucleotide sequence (AAACATGG) resembled to the plant initiation start consensus sequence [71]. From the first ATG codon, the open reading frame encoded 713 amino acids. The calculated molecular weight of the polypeptide was 78531.7 daltons, which agreed well with the molecular mass of the purified tobacco P450 reductase estimated by SDS-PAGE [72]. The peptide sequence determined by microsequencing of the purified enzyme was found in the deduced amino acid sequence from 285th to 297th. The nucleotide sequences of pGTR1, pCTR1 and pFTR corresponded to the nucleotide sequence from 1st to 481th, from 482th to 2657th and 167th to 2309th, respectively, as shown in Fig. 3-1.

The deduced amino acid sequence of tobacco P450 reductase was compared with those of P450 reductases from the other sources reported (Fig. 3-2). *Arabidopsis* (ATR2) [16], yeast [73] and rat [74] P450 reductases showed 72%, 31% and 35% identity in amino acid sequence with tobacco P450 reductase. The amino-terminal region of tobacco P450 reductase was longer than those of yeast and rat enzymes. A hydropathy profile of tobacco P450 reductase showed the presence of hydrophilic region with Ser and the other hydrophilic amino acid residues preceding to the hydrophobic region, which seemed to be a membrane anchor. The functional domains involved in FMN, FAD and NADPH binding reported previously [75] were well conserved in the sequence of tobacco P450

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↓pGTR1 5'
AAGCTTTTACTTTGTCATTCTTTTTCCCCTCGTTTTCATCGCCTTCTCTCCAGTTTTGTCGCCGGCTGGATCATCAGAGTTAATCTCATC     90
                                                                                ↓pFTR 5'
AACTGAGTAATTAACGCTTGCCCAGTTGAGCAAAATTCACATAGAGTTCAGTTTGAATTCTTGATTACATAGAAAACATGGAGTCTACAT    180
                                                                                M  E  S  T      4

CAGAGAAACTTTCTCCTTTTGATTTTATGGCGGCGATTTTTAAAGGTGGAAAGATATTCGATCAACTGAACTCATCATCAGATTCTGGCG    270
S  E  K  L  S  P  F  D  F  M  A  A  I  F  K  G  G  K  I  F  D  Q  L  N  S  S  S  D  S  G      34

ACTCAAGTTCTCCTGCTTCGTTGGCAGCTCTGCTGATGGAGAACAAAGATTTAATGATGATATTGACAACCTCGGTTGCTGTCTTGATCG    360
D  S  S  S  P  A  S  L  A  A  L  L  M  E  N  K  D  L  M  M  I  L  T  T  S  V  A  V  L  I      64

GATGTGCAGTTGTCTTGATGTGGCGGCGCTCCTCAACTTCTGCTAAGAAGGTGGTAGAGCCGCCCAAGTTGGTGGTTCCTAAGTCGGTTA    450
G  C  A  V  V  L  M  W  R  R  S  S  T  S  A  K  K  V  V  E  P  P  K  L  V  V  P  K  S  V      94
                     pGTR1 3'↓↓pCTR1 5'
TTGAACCTGAAGAGATTGATGATGGCAAGAAGAAAGTTACCATCTTTTTTGGTACCCAGACTGGTACTGCTGAAGGCTTTGCTAAGGCAC    540
I  E  P  E  E  I  D  D  G  K  K  K  V  T  I  F  F  G  T  Q  T  G  T  A  E  G  F  A  K  A     124

TTGCCGAGGAAGCCAAGGCCAGATATGATAAGGCTACCTTTAAAGTGATTGATATGGATGATTATGCGGCTGATGATGATGACTATGAAG    630
L  A  E  E  A  K  A  R  Y  D  K  A  T  F  K  V  I  D  M  D  D  Y  A  A  D  D  D  D  Y  E     154

AGAAATTGAAGAAAGAAACATTGGCATTCTTTTTCTTGGCCACATATGGAGATGGTGAGCCAACTGATAATGCTGCCAGATTCTATAAAT    720
E  K  L  K  K  E  T  L  A  F  F  F  L  A  T  Y  G  D  G  E  P  T  D  N  A  A  R  F  Y  K     184

GGTTTGTCGAGGGAAAAGAGAGGGGTGACTACTTTAAAAATCTTCAGTATGGAGTATTTGGCCTTGGTAACAGACAGTACGAGCATTTCA    810
W  F  V  E  G  K  E  R  G  D  Y  F  K  N  L  Q  Y  G  V  F  G  L  G  N  R  Q  Y  E  H  F     214

ACAAGATTGCAAAAGTGGTGGATGACCTTCTCGCTGAGCAAGGCGGGCAGAGGCTTGTTCCTGTGGGTCTTGGAGATGATGACCAATGCA    900
N  K  I  A  K  V  V  D  D  L  L  A  E  Q  G  G  Q  R  L  V  P  V  G  L  G  D  D  D  Q  C     244

TTGAAGATGATTTTGCTGCATGGCGTGAATTAGTGTGGCCTGAATTGGATAAGTTTCTTCTGGATGGGGATGATGCAACTCGTGCAACTC    990
I  E  D  D  F  A  A  W  R  E  L  V  W  P  E  L  D  K  F  L  L  D  G  D  D  A  T  R  A  T     274

CATATACCGCTGCAGTTTTGGAATATAGGGTTGTTACCCATGAAAAGCCTAACAACGACTTGAGTAACACAAATGGTCATGCAAATGGAC   1080
P  Y  T  A  A  V  L  E  Y  R  V  V  T  H  E  K  P  N  N  D  L  S  N  T  N  G  H  A  N  G     304

ATGCAATCATTGATGCTCAACATCCCTGCATAGCTAATGTTGCTGTGAAGAAGGAGCTTCATACTCCTGCTTCTGATCGTTCTTGCACTC   1170
H  A  I  I  D  A  Q  H  P  C  I  A  N  V  A  V  K  K  E  L  H  T  P  A  S  D  R  S  C  T     334

ATTTGGAGTTTGACATTTCTGGAACTGGAGTTGTTTATGAAACTGGTGATCATGTCGGTGTGTACTGTGAAAATTTGATTGAAACCGTAG   1260
H  L  E  F  D  I  S  G  T  G  V  V  Y  E  T  G  D  H  V  G  V  Y  C  E  N  L  I  E  T  V     364

AGGAAGCTGAAAGGTTACTAAACATATCACCTGACACTTTCTTTTCCATTCACACTGATAAAGAAGATGGCACACCACTGGGCGGAAGCT   1350
E  E  A  E  R  L  L  N  I  S  P  D  T  F  F  S  I  H  T  D  K  E  D  G  T  P  L  G  G  S     394

CATTGCCGTCTCCCTTCCCTCCTTGCACTTTAAGAACAGCATTGACTCTGTATGCTGATCTTTTGAGTTCTCCTAAAAAGTCTGCTTTAC   1440
S  L  P  S  P  F  P  P  C  T  L  R  T  A  L  T  L  Y  A  D  L  L  S  S  P  K  K  S  A  L     424

TTGCTTTAGCGGCATGTGCTTCTGATCCAAATGAAGCTAATCGATTAAGAAATCTTGCATCACCGGCTGGAAAGGAAGAATATGCTCAGT   1530
L  A  L  A  A  C  A  S  D  P  N  E  A  N  R  L  R  N  L  A  S  P  A  G  K  E  E  Y  A  Q     454

GGATGGTTGCAAGTCAGAGAAGCCTTCTTGAAGTCATGGCTGAATTTCCTTCAGCCAAGCCTTCACTTGGGGTTTTCTTTGCTTCTGTTG   1620
W  M  V  A  S  Q  R  S  L  L  E  V  M  A  E  F  P  S  A  K  P  S  L  G  V  F  F  A  S  V     484

CTCCTCGCCTACAACCGAGATTCTACTCTATCTCATCATCTCATAGGATGGCGCCATCTAGAATTCATGTTACTTGTGCACTGGTTTACG   1710
A  P  R  L  Q  P  R  F  Y  S  I  S  S  S  H  R  M  A  P  S  R  I  H  V  T  C  A  L  V  Y     514

ACAAAATGCCAACCGGACGAGTTCACAAGGGTGTCTGCTCAACATGGATGAAGAATGCTGTTCCTCTAGAAGAAAGCCTTTCCTGCAGTA   1800
D  K  M  P  T  G  R  V  H  K  G  V  C  S  T  W  M  K  N  A  V  P  L  E  E  S  L  S  C  S     544

CAGCACCTATTTTTGTTCGGCAATCAAACTTCAAACTGCCAGCTGATAACAAGGTTCCAATCATAATGATCGGCCCTGGTACTGGGTTGG   1890
T  A  P  I  F  V  R  Q  S  N  F  K  L  P  A  D  N  K  V  P  I  I  M  I  G  P  G  T  G  L     574

CACCATTCAGGGGTTTCCTCCAGGAAAGATTAGCTTTTAAGAAAGAAGGAGCTGAGCTTGGTCCTGCAGTGTTATTTTTTGGATGCAGGA   1980
A  P  F  R  G  F  L  Q  E  R  L  A  F  K  K  E  G  A  E  L  G  P  A  V  L  F  F  G  C  R     604

ACCGCCAAATGGACTACATCTATCAAGAAGAGTTGGACAATTTCCTTGAGGCCGGTGCACTTTCTGAGCTAGTAGTTGCTTTCTCTCGTG   2070
N  R  Q  M  D  Y  I  Y  Q  E  E  L  D  N  F  L  E  A  G  A  L  S  E  L  V  V  A  F  S  R     634

AAGGACCTAACAAAGAATACGTGCAACATAAAATGTCAGAGAAGGCTGCGGATATCTGGAACATGATTTCTCAGGGAGGATACGTATATG   2160
E  G  P  N  K  E  Y  V  Q  H  K  M  S  E  K  A  A  D  I  W  N  M  I  S  Q  G  G  Y  V  Y     664

TCTGCGGTGATGCAAAAGGCATGGCTAGGGACGTTCATCGGGCTCTTCACACTATTGCCCAGGATCAGGGATCGCTCGACAGCTCCAAGG   2250
V  C  G  D  A  K  G  M  A  R  D  V  H  R  A  L  H  T  I  A  Q  D  Q  G  S  L  D  S  S  K     694
                                                   pFTR 3'↓
CTGAGGCCTTGGTGAAGAACTTGCAAATAACTGGAAGATATCTGCGTGATGTGTGGTGATCGTCTCTATATTATATTATGCAAGCCAATT   2340
A  E  A  L  V  K  N  L  Q  I  T  G  R  Y  L  R  D  V  W  *                                    713

GTGATTAGCGAAATATATAGGAAGGCAACGTGCAAAGAACAATCAGCCTTTCAACAGGGAAAAGAAGTGTACGAACTGTCGACACAGCTT   2430
TTCCGATTGTATGATTTTTGATGAAGAATTTTTTGATGAAGAATTTTTTGGTTCATGTTAATTTATATACAAGAATGTAAGGCAAATAAT   2520
TACGTTAGTATTAAGTTTAGGCAATGTATATCGAGAGCTTGTAATCTCCCTATTGAACCAGCAAAATGGTTCAGACAACACTGTATGCTG   2610
                                        pCTR1 3'↓
TTGATTAATTGGTTTAAGACCATCTCGTTTTATACAAAAAAAAAAAA                                              2657
```

Fig. 3-1. The nucleotide sequence obtained by the combination of the cDNA pCTR1 and genomic DNA pGTR1 and deduced amino acid sequence of tobacco NADPH-cytochrome P450 reductase. The nucleotide sequences of pGTR1, pCTR1 and pFTR corresponded to the nucleotide sequence from 1st to 481th, from 482th to 2657th and 167th to 2309th, respectively. The 5' and 3' ends of pGTR1, pCTR1 and pFTR were indicated with arrows. The amino acid sequence corresponded to the peptide sequence determined by microsequencing of the purified enzyme was underlined.

```
Tobacco      MESTSEKLSP-FDFMAAIFKGGKIFDQLNSSSDSGDSSSPASLAALLMENKDLMMILTTSVAVLIGCAVVLMWRRSSTSA   79
Arabidopsis  MSSSSSSSTSMIDLMAAIIKGEPVIVS-DPANASAYESVAAELSSMLIENRQFAMIVTTSIAVLIGCIVMLVWRRSGSGN   79
Yeast        MPFGIDNTDF------------TVLAG-LVLAVLLYVK-------------------RNSIKELLM---------SDDGD   39
Rat          MGDSHEDTSA------------TMPEA-VAEEVSLFSTTDMVLFSLIVGVLTYWFIFRKKKEEIPE---------FSKIQ   58
             *

Tobacco      KKVVEPPKLVVPKSVIEPEEIDDGKKKVTIFFGTQTGTAEGFAKALAEEAKARYDKATFKVIDMDDYAADDDDYEEKLKK  159
Arabidopsis  SKRVEPLKPLVIKP--REEEIDDGRKKVTIFFGTQTGTAEGFAKALGEEAKARYEKTRFKIVDLDDYAADDDEYEEKLKK  157
Yeast        ITAV----SSGNRD--IAQVVTENNKNYLVLYASQTGTAEDYAKKFSKELVAKFN-LNVMCADVENYDFESLNDVPVI--  110
Rat          TTAP----PVKESS--FVEKMKKTGRNIIVFYGSQTGTAEEFANRLSKD-AHRYG-MRGMSADPEEYDLADLSSLPEIDK  130
                                                 ******  *                    *   *
                                                      FMN
Tobacco      ETLAFFFLATYGDGEPTDNAARFYKWFVEGKERGDYFKNLQYGVFGLGNRQYEHFNKIAKVVDDLLAEQGGQRLVPVGLG  239
Arabidopsis  EDVAFFFLATYGDGEPTDNAARFYKWFTEGNDRGEWLKNLKYGVFGLGNRQYEHFNKVAKVVDDILVEQGAQRLVQVGLG  237
Yeast        ---VSIFISTYGEGDFPDGAVNFEDFICNAE--AGALSNLRYNMFGLGNSTYEFFNGAAKKAEKHLSAAGAIRLGKLGEA  185
Rat          -SLVVFCMATYGEGDPTDNAQDFYDWLQETD--VD-LTGVKFAVFGLGNKTYEHFNAMGKYVDQRLEQLGAQRIFELGLG  206
                     *** *   * *  *                     *****  ** **   *     *   * *    *

Tobacco      DD-DQCIEDDFAAWRELVWPELDKFLLDGDDATPAT-PYTAAVLEYRVVTHEKPNNDLSNT--------N-GHANGHAII  308
Arabidopsis  DD-DQCIEDDFTAWREALWPELDTILREEGDTAVAT-PYTAAVLEYRVSIHDSEDAKFNDI--------TLANGNGYTVF  307
Yeast        DDGAGTTDEDYMAWKDSILEVLKDELHLDEQEAKFT-SQFQYTVLNEITDSMSLGEPSAHYLPSHQLNRN-ADGTQLGPF  263
Rat          DD-DGNLEEDFITWREQFWPAVCEFFGVEATGEESSIRQYELVVHEDMDVAKVYTGEMGRL--------K-SYENQKPPF  276
             **        *   *
                                 FAD
Tobacco      DAQHPCIANVAVKKELHTPASDRSCTHLEFDISGTGWVYETGDHVGVYCENLIETVEEAERLLNISPDTFFSIHTDKEDG  388
Arabidopsis  DAQHPYKANVAVKRELHTPESDRSCIHLEFDIAGSGLTMKLGDHVGVLCDNLSETVDEALRLLDMSPDTYFSLHAEKEDG  387
Yeast        DLSQPYIAPIVKSRELPS-SNDRNCIHSEFDLSGSNIKYSTGDHLAVWPSNPLEKVEQFLSIFNLDPETIFDLKPLD--P  329
Rat          DAKNPFLAAVTANRKLNQ-GTERHLMHLELDISDSKIRYESGDHVAVYPANDSALVNQIGEILGADLDVIMSLNNLDEES  355
             *   *  *        *      *   * * *          ***  *   *    *

Tobacco      TPLGGSSLPSPFPPCTLRTALTLYADLLSSPKKSALLALAACASDPNEANRLRNLASPAG--KEEYAQWMVASQRSLLEV  466
Arabidopsis  TPI-SSSLPPPFPPCNLRTALTRYACLLSSPKKSALVALAAHASDPTEAERLKHLASPAG--KDEYSKWVVESQRSLLEV  464
Yeast        TVK-VPFPTPT----TIGAAIKHYLEITGPVSRQLFSSLIQFAPNADVKEKLTLLSKDKDQFAVEITSKYFNIADALKYL  415
Rat          NKK-HPFPCPT----TYRTALTYYLDITNPPRTNVLYELAQYASEPSEQEHLHKMASSSGEGKELYLSWVVEARRHILAI  430
                                *   *              *   *        *
                                                          FAD
Tobacco      MAEFPSAKPSLGVFFASVAPRLQPRFYSISSSHRMKPSRYHVTCALVYD---KMPTGRVHKGVCSTWMRNAVPLEES---  540
Arabidopsis  MAEFPSAKPPLGVFFAGVAPRLQPRFYSISSSPKIAETRIHVTCALVYE---KMPTGRIHKGVCSTWMKNAVPYEKS---  538
Yeast        SDGAKWDTVPMQFLVESV-PQMTPRYYSISSSSLSEKQTVHVTSIVENFPNPELPDAPPVVGVTTNLLRNIQLAQNNVNI  494
Rat          LQDYPSLRPPIDHLCELL-PRLQARYYSIASSSKVHPNSVHICAVAVEY---EAKSGRVNKGVATSWLRAKEPAGEN---  503
                                *    * *** **          *                    **

Tobacco      ------------LSCSTA------PIFVRQSNFKLPADNKVPIIMIGPGTGLAPFRGFLQERLAFKKEGAELGPA-----  597
Arabidopsis  ------------EKLFLGR-----PIFVRQSNFKLPSDSKVPIIMIGPGTGLAPFRGFLQERLALVESGVELGPS-----  596
Yeast        AETNLPVHYDLNGPRKLF-ANYKLPVHVRRSNFRLPSNPSTPVIMIGPGTGVAPFRGFIRERVAFLESQKKGGNNVSLGK  573
Rat          ------------GGRALV------PMFVRKSQFRLPFKSTTPVIMVGPGTGIAPFMGFIQERAWLREQGKEVGET-----  560
                                  *  ** * * **     * ** ***** *** **  **          *
                                                      NADPH
Tobacco      -VLFFGCRNRQMDYIYQEELDNFLEAGALS-ELVVAFSR-EGPNKEYVQHKMSEKAADIWNMISQG-GYVYVCGDAKGMA  673
Arabidopsis  -VLFFGCRNRRMDFIYEEELQRFVESGALA-ELSVAFSR-EGPTKEYVQHKMMDKASDIWNMISQG-AYLYVCGDAKGMA  672
Yeast        HILFYGSRNTD-DFLYQDEWPEYAKKLDGSFEMVVAHSRLPNTKKVYVQDKLKDYEDQVFEMINNG-AFIYVCGDAKGMA  651
Rat          -LLYYGCRRSDEDYLYREELARFHKDGALT-QLNVAFSR-EQAHKVYVQHLLKRDREHLWKLIHEGGAHIYVCGDARNMA  637
               *  * *    *  *  *                ** **     * ***              *  *    ******  **

Tobacco      RDVHRALHTIAQDQGSLDSSKAEALVKNLQITGRYLRDVW   713
Arabidopsis  RDVHRSLHTIAQEQGSMDSTKAEGFVKNLQTSGRYLRDVW   712
Yeast        KGVSTALVGILSRGKSITTDEATELIKMLKTSGRYQEDVW   691
Rat          KDVQNTFYDIVAEFGPMEHTQAVDYVKKLMTKGRYSLDVWS  678
               *      *            *    * *   ***  ***
```

Fig. 3-2. Comparison of the deduced amino acid sequence of tobacco P450 reductase with those of *Arabidopsis* (GenBank accession number X66017), yeast (D13788) and rat (M10068). Possible FMN, FAD and NADPH binding domains are shaded. The conserved amino acid residues are marked with an asterisk.

reductase.

P450 Reductase Gene and its Transcript in Tobacco Tissues

To estimate a copy number of tobacco P450 reductase gene, Southern blot analysis was carried out. The tobacco genomic DNA was digested with *Bam*HI, *Eco*RI and *Hin*dIII, and probed with pCTR1. One band was found in *Bam*HI digest, three and two bands were detected in *Eco*RI and *Hin*dIII digests (Fig. 3-3), respectively. One *Eco*RI site was present, but no *Bam*HI and *Hin*dIII sites in pGTR1 and pCTR1. In addition, the results of inverse PCR suggested that *Hin*dIII sites may not be present in the tobacco P450 reductase gene. Therefore, it was suggested that one or two P450 reductase genes may be present in tobacco (*Nicotiana tabacum* cv. Petite Havana SR-1) genome.

Total RNA was isolated from roots, stems, leaves, and flowering buds of tobacco plants, and the cultured BY2 cells. Northern blot analysis was carried out using pCTR1 as a probe DNA (Fig. 3-4). A band with about 2700 nucleotides was found in the tissues examined. A slightly higher level of the transcript was found in flowering buds and cultured BY2 cells than in the other tissues.

Expression of the Tobacco P450 Reductase cDNA in the Yeast

The recombinant plasmid pATR was constructed for expression of the full length cDNA pFTR of tobacco P450 reductase in the yeast. *S. cerevisiae* AH22 cells were transformed with the expression plasmid pATR and the vector pAAH5. The transformed yeast cells were analyzed by northern blotting using pFTR as a probe DNA (Fig. 3-5A). A transcript with about 2500 nucleotides was found in the AH22/pATR cells, but not in the AH22/pAAH5 cells. Western blot analysis with anti-tobacco P450 reductase IgG revealed that about a 78 kDa protein band was found in the microsomes of the AH22/pATR cells but not in the AH22/pAAH5 cells (Fig. 3-5B). The molecular mass of the band agreed well with that of the purified P450 reductase from the microsomes of tobacco BY2 cells [72] and the predicted molecular weight according the deduced amino acid sequence of the cDNA pFTR. Thus, it was found that the cloned cDNA pFTR was expressed in the yeast to produce the corresponding protein in the microsomes.

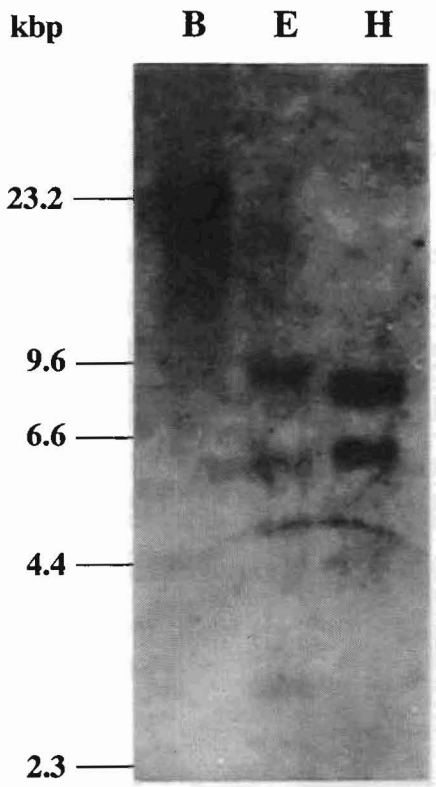

Fig. 3-3. Genomic Southern blot analysis of tobacco P450 reductase gene. A 20 μg of genomic DNA were digested with restriction endonucleases *Bam*HI (B), *Eco*RI (E) and *Hin*dIII (H). The tobacco P450 reductase cDNA clone pCTR1 was used as probe DNA.

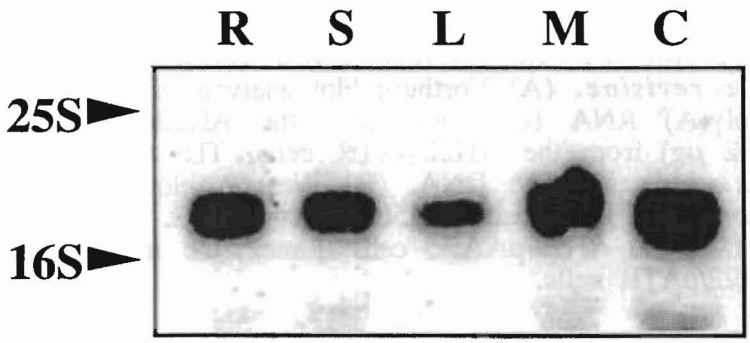

Fig. 3-4. Northern blot analysis of P450 reductase mRNA in tobacco tissues and the cultured BY2 cells. 20 μg of total RNA from roots (R), stems (S), leaves (L), floral buds (F) and the cultured cells (C) were probed with the tobacco P450 reductase cDNA pCTR1.

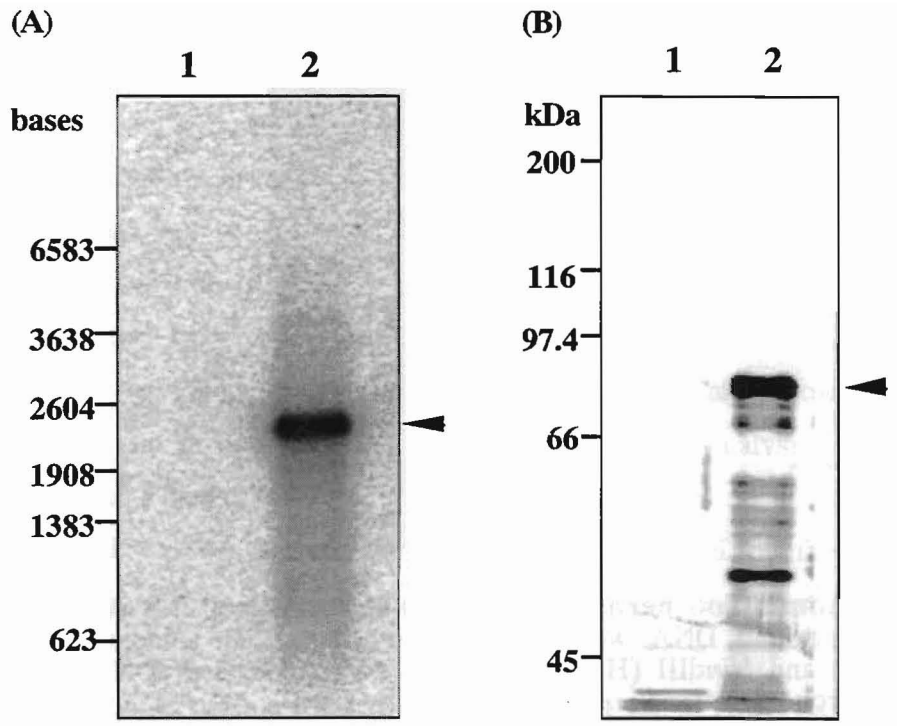

Fig. 3-5. Expression of the tobacco P450 reductase cDNA pFTR in *Saccharomyces cerevisiae*. (A) Northern blot analysis of the recombinant yeast cells. Lane1, Poly(A) RNA (0.2 μg) from the AH22/pAAH5 cells. Lane2, Poly(A) RNA (0.2 μg) from the AH22/pATR cells. The tobacco P450 reductase cDNA pFTR was used as probe DNA. (B) Western blot analysis of the yeast microsomal fractions with anti-tobacco P450 reductase IgG. Lane1, the microsomal fraction (10 μg) from the AH22/pAAH5 cells; Lane2, the microsomal fraction (10 μg) from the AH22/pATR cells.

Cytochrome c reductase activity was measured in the microsomal fraction of the transformed yeast cells. The activity of the microsomes of the AH22/pATR cells was 699 nmol/ min/ mg protein, which was about 8 times higher than that of the control AH22/pAAH5 cells (Table 3-1). Therefore, tobacco P450 reductase encoded on pFTR seemed to be a microsomal enzyme.

An *in vitro* reconstitution systems consisting of the AH22/pATR microsomes expressed the tobacco P450 reductase and the AH22/pAMC microsomes expressed rat P4501A1 [64] was assayed for 7-ethoxycoumarin *O*-deethylase activity in the presence of NADPH. The P4501A1-dependent activity was four times higher than that of AH22/pAMC. These results suggested that the tobacco P450 reductase expressed in the yeast microsomes was enzymatic active and coupled with rat P4501A1, enhancing the P450-dependent monooxygenase activity (Table 3-2).

Table 3-1
Cytochrome c reductase activity in the microsomal fraction of the recombinant yeast cells expressing the tobacco P450 reductase.

Yeast microsomes	Cytochrome c reductase activity (nmol / min / mg protein)
AH22/pAAH5*1	83
AH22/pATR*2	699

*1. *S. cerevisiae* AH22/pAAH5 (control strain)
*2. *S. cerevisiae* AH22/pATR expressed tobacco P450 reductase in the microsomes

Tabale 3-2
7-Ethoxycoumarin *O*-deethylase activities in an *in vitro* reconstitution system consisting of the yeast microsomal fractions containing tobacco P450 reductase and rat P450 1A1

Yeast strain		7-Ethoxycoumarin *O*-deethylase activity
AH22/pAMC*1	AH22/pATR*2	(nmol / min / pmol P450)
-	-	<1
-	+	<1
+	-	4
+	+	15

*1. *S. cerevisiae* AH22/pAMC expressed rat P450 1A1 in the microsomes (20 pmol).
*2. *S. cerevisiae* AH22/pATR expressed tobacco P450 reductase in the microsomes (0.01 units).

DISCUSSION

I obtained a full length information on tobacco P450 reductase by the combination of the cDNA clone pCTR1 and the genomic DNA clone pGTR1. The deduced amino acid sequence of tobacco P450 reductase consisting of 713 amino acid residues was compared with those of *Arabidopsis*, yeast, and rat enzymes. Tobacco P450 reductase was found to contain the membrane anchor from Leu-52 to Trp-72 followed by two Arg residues, and the cytosolic region including the domains for binding of FMN, FAD and NADPH.

The tobacco P450 reductase had a preceding amphipatic sequence of about 50 amino acid residues, which was longer than those of yeast and rat enzymes, as reported with plant P450 reductases [16, 76]. Urban et al. recently reported that the amphipatic region is similar to the chloroplast targeting sequence [16]. In tobacco P450 reductase, Met-14-Ala-15, and Ser rich sequence seemed to be a typical chloroplast leader sequence [77]. The prediction of the protein localization with a PSORT computer program suggested that if tobacco P450 reductase starts from Met-1, it would be an endoplasmic reticulum membrane protein, but if tobacco P450 reductase starts from Met-14, the enzyme would be localized on the thylakoid membranes of chloroplasts. On the expression of the full length cDNA pFTR of tobacco P450 reductase in the yeast, the enzyme was found to be mainly localized on the microsomes. Therefore, tobacco P450 reductase seemed to be a microsomal enzyme.

Recently the three dimensional structure of rat P450 reductase proposed the domains for binding of FMN, FAD and NADPH [75]. In spite of 35% similarity in the total amino acid sequence of rat P450 reductase, tobacco P450 reductase conserved these functional domains. By sequence alignment with rat P450 reductase, in tobacco P450 reductase, the regions for binding of FMN, FAD and NADPH appeared to be from Pro-97 to Pro-258, from Asn-303 to Gln-358 and from Leu-488 to Leu-557, and from Pro-558 to Arg-707, respectively.

The interaction of P450 reductase with cytochrome c and P450 depends on ionic as well as hydrophobic interations [78, 79]. The docking model [75], chemical crosslinking [78] and site-directed mutagenesis [80] suggested that the

peptide loop from Asp-207 to Asn-211 in rat P450 reductase consisting of acidic residues was involved in the binding to cytochrome c and P450. The corresponding region from Asp-240 to Cys-244 was found in tobacco P450 reductase. The hydrophobic interaction between the membrane binding domain of P450 reductase and P450 was also important [79]. It is interesting to study the structure and function relationship of the region, since the amino-terminal region of tobacco reductase was not conserved as described above.

Southern blot analysis suggested that one or two copies of P450 reductase genes may be present in tobacco (*Nicotiana tabacum*) genome although one gene was cloned in the present study. Since *Nicotiana tabacum* is an amphidiploid between *N. tomentosiformis* and *N. sylvestris*, two genes may be derived from two different tobacco species. It was reported that a single copy of P450 reductase gene is present in rat [81], yeast [73], and *Catharanthus roseus* [76], and that a few copies of P450 reductase genes was found in *Arabidopsis* and Jerusalem artichoke [16, 82]. In *Arabidopsis*, two P450 reductase cDNAs *ATR1* and *ATR2* were isolated [16]. ATR1 showed 64% similality in the amino acid sequence with ATR2. The tobacco P450 reductase in the present study showed 72% similarity with ATR2, but 65% with ATR1 [16]. Thus tobacco P450 reductase may belong to the same subfamily as ATR2.

The tobacco P450 reductase gene cloned in the present study was expressed in the whole plants, probably constitutively. On the other hand, most of P450 genes were highly regulated spatially and stage-specifically, and induced by physical, chemical and biological stress. Therefore, P450 monooxygenases appeared to be mainly regulated by the expression of each of P450 species, although the level of P450 reductase expressed in plant cells may be related to interaction with each of many P450 species, leading to the total P450 dependent monooxygenase activity.

The full length cDNA pFTR of tobacco P450 reductase was expressed in the yeast *S. cerevisiae* under the control of ADH promoter and terminator. The recombinant yeast strain AH22/pATR showed about 8 times higher cytochrome c reductase activity than AH22/pAAH5 strain in the microsomes. The specific activity was higher than that of the yeast cells expressed rat P450 reductase [83] but lower than that of the yeast cells overexpressed yeast P450 reductase [84]. In

addition, the activity of the yeast strain expressed tobacco P450 reductase was higher than that of the yeast strains expressed *Arabidopsis* ATR1 and lower than that of ATR2 expressed under the control of *GAL10-CYC1* promoter [16]. The difference in the specific activity may result from the expression level and enzyme stability in the yeast cells.

The *in vitro* reconstitution system of the tobacco P450 reductase and rat P4501A1 expressed in the yeast microsomes showed an enhanced 7-ethoxycoumarin *O*-deethylase activity. The specific activity was lower than that of the yeast strain for co-expression of rat P4501A1 and yeast P450 reductase [84]. Probably, the reaction conditions for the *in vitro* reconstitution system may not be suitable for the plant enzyme.

Estimates from current genome projects imply that the number of P450 genes exceeds 160 in *Arabidopsis thaliana*. Although over 60 P450 sequences from plants are currently known, the function of very few P450 species has been identified [6]. So, co-expression of a certain plant P450 cDNA and tobacco P450 reductase cDNA in the yeast as well as in tobacco plants may have a large potential for identification of the function of P450 species as well as engineering of transgenic plants for an enhanced P450 monooxygenase activity.

CHAPTER IV

MOLECULAR CLONING AND SEQUENCE ANALYSIS OF CATALASE cDNA FROM GREEN PEPPER SEEDLINGS ELICITED WITH ARACHIDONIC ACID

INTRODUCTION

Higher plants have developed broad and complex biochemical systems to defend themselves against wounding, environmental changes, absorption of xenobiotics, attack of pathogens and herbivores. A biosynthesis of phytoalexins is one of the important defense mechanisms in higher plants. Capsidiol is a sesquiterpenoid phytoalexin produced in green pepper [85] and tobacco [86], and has been studied on the biosynthesis and induction mechanisms [87 - 90]. In these plant species, farnesyl pyrophosphate was cyclized to yield the intermediate 5-*epi*-aristolochene, which was oxidized to produce capsidiol. The biosynthesis of capsidiol was induced by the treatment with arachidonic acid in green pepper seedlings [87] and by the treatment of a number of fungal elicitors in tobacco cultured cells [88]. The farnesyl pyrophosphate cyclization was an important reaction for switching the biosynthesis pathways of terpenoids from a healthy state to a stressed state [88]. In addition, the final oxidation from 5-*epi*-aristolochene to capsidiol by 5-*epi*-aristolochene 3-hydroxylase seemed to be an important step in the biosynthesis. Hoshino *et al.* reported that a 59 kDa protein partially purified from the microsomes of the green pepper seedlings treated with arachidonic acid was 5-*epi*-aristolochene 3-hydroxylase [91].

In this report, we attempted to determine amino acid sequences of the purified 59 kDa protein from the green pepper seedlings elicited with arachidonic acid for cDNA cloning. Based on the deduced amino acid sequence of the cDNA isolated, we attempted to identify the protein encoded on the cDNA.

MATERIALS AND METHODS

Plant Materials

Green pepper (*Capsicum annuum*) seeds were purchased from Sapporo Konoen Seed Co. (Sapporo, Japan). Seeds were sterilized in 3% sodium hypochlorite solution for 10 min, and plated on an autoclaved medium prepared from 0.1% (v/v) Hyponex and 0.7% agar, and incubated at 25°C under light and dark conditions for 12 h each. After 10 days, seedlings were collected by removing agar carefully, immersed in water containing 1 mM arachidonic acid solution and then incubated for an appropriate time at 25°C.

PCR Cloning

Green pepper seedlings grown for 10 days were treated with arachidonic acid for 6, 12, 24 h and then subjected to poly(A)$^+$ RNA preparation using a QuickpPrep Micro mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden). First strand cDNA was synthesized from 0.1 μg of poly(A)$^+$ RNA using a first strand cDNA synthesis kit (Pharmacia) and 10% of the mixture was used as a template for PCR, which was carried out in 100 μl of 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 50 pmol of degenerate primers synthesized on the base of amino acid sequences and 1.25 units of AmpliTaq DNA polymerase (Takara Shuzo Co. Ltd., Otsu, Japan). The reaction was performed through 35 cycles of 1 min at 94°C, 2 min at 45°C and 3 min at 72°C. PCR products were separated on 5% polyacrylamide gel electrophoresis. DNA fragment was extracted and subcloned into pBluescript KSII+ (Stratagene Cloning Systems, La Jolla, CA).

Construction and Screening of cDNA Library.

A cDNA library consisting of 2×10^6 recombinants was constructed from 5 μg of the poly(A)$^+$ RNA using a λZAP cDNA synthesis kit (Stratagene) according to the manufacturer's instructions. The cDNA library was screened by plaque hybridization with a random-primed ^{32}P-labeled PCR fragment as a probe. Hybridization was performed at 42°C for 15 h in a solution containing 50% (v /

v) formamide, 5 × SSC (1 × SSC: 15 mM sodium citrate buffer containing 150 mM NaCl), 5 × Denhardt's solution [65], 100 μg per ml salmon sperm DNA, 0.5% SDS, and a probe DNA (about 5 × 10^8 cpm per μg of DNA). Membranes were washed in 2 × SSC-0.1% SDS for 5 min at a room temperature at once, 30 min at 50°C twice, and then in 0.2 × SSC-0.1% SDS for 30 min at 50°C. After autoradiography, positive plaques were isolated and converted to phagemids by *in vivo* excision according to manufacture's instructions.

DNA Sequencing and Sequence Analysis

Insert DNA fragments of positive clones as well as PCR fragment were sequenced with an AutoRead Sequencing Kit (Pharmacia) using a Shimadzu model DSQ1 DNA sequencer (Shimadzu Co. Ltd., Kyoto, Japan). Sequence analysis was performed with a DNASIS-Mac software (Hitachi Software Engineering Co. Ltd., Yokohama, Japan). Multiple sequence alignment was performed using a BLAST computer program [67].

Northern Hybridization

Poly(A)$^+$ RNA preparations were loaded onto 1.0% agarose gel containing 1.8% formaldehyde with running buffer of 20 mM MOPS Good's buffer (pH 7.0) containing 5 mM EDTA and 8 mM sodium acetate. RNA bands were transferred to a Hybond N nylon membrane (Amersham International plc., Buckinghamshire, U.K.). About a 400 bp fragment of pCT01 cDNA and 1.3 kbp fragment from the β-ATPase gene were used as probe DNA [92]. Hybridization was performed at 42°C for 15 h in a solution containing 50% (v / v) formamide, 5 × SSPE (1 × SSPE: sodium phosphate buffer (pH 7.4) containing 180 mM NaCl and 1 mM EDTA), 5 × Denhardt's solution, 100 μg per ml salmon sperm DNA, and 0.5% SDS. A membrane was washed in 2 × SSPE-0.1% SDS for 5 min at room temperature once, 30 min at 50°C twice, and in 0.1 × SSPE-0.1% SDS for 20 min at 50°C twice.

Peptide Sequencing

A 59-kDa protein sample purified from green pepper seedlings treated with arachidonic acid was given by Dr. T. Hoshino (Hokkaido National Industrial Research Institute, Sapporo, Japan). The protein sample of 1 μg with about 90% purity were applied onto 9% SDS polyacrylamide gel electrophoresis according to Laemmli [44], and then electroblotted onto a PVDF membrane [69]. Protein bands were stained with Coomassie Blue and then a major band with 59 kDa was cut out. About 100 pmol of protein on the membrane was soaked in 150 μl of 70% formic acid containing 1.0% CNBr. Cleavage reactions were carried out at room temperature in the dark for overnight. Then peptides were eluted from the membrane with 10 mM Tris-HCl buffer (pH 6.8) containing 2% SDS by shaking for 1.5 h. Then, these peptides were separated by 15% SDS polyacrylamide gel electrophoresis and electroblotted onto a PVDF membrane. Stained bands were subjected to amino acid sequencing using Applied Biosystems model 470A protein sequencer (The Perkin-Elmer Co., Foster City, CA).

RESULTS AND DISCUSSION

Peptide Sequences

We attempted to sequence the amino terminal region of the 59 kDa protein, which was purified from green pepper seedlings treated with arachidonic acid. From the preliminary sequencing, the amino terminus of the purified protein seemed to be modified. Therefore the protein sample was subjected to CNBr cleavage. As shown in Fig. 4-1, three major bands were obtained. The amino terminal amino acid sequences of three peptides with 31.6 kDa (P1), 23.2 kDa (P2) and 14.5 kDa (P3) were found to be Asp-Leu-Ser-Leu-Tyr-X-Pro-Ser-Ser-Ala-Tyr-Asp-Ser-Pro for P1 and P2, and X-X-Asp-Glu-Glu-Val-Asp-Tyr-Leu-Pro-Ser-Ser-Phe for P3 (X : not identified), respectively. The amino-terminal sequence of P1 and P2 was identical. These two amino terminal sequences (P1 / P2 and P3) showed high similarity with those of plant catalases reported. Both P1 / P2 and P3 sequences had 85.7% and 76.9% identity with those of tobacco catalase [93].

cDNA Cloning

Based on the amino acid sequences determined, four degenerate oligonucleotide primers were synthesized. The sense degenerate primer C01S [5'-AACTCGAG CCI (A/T)(C/G)I (A/T)(C/G)I GCI TA(C/T) GA(C/T) (A/T)(C/G)I CC-3'; I = inosine] and the antisense primer C01AS [5'-AACTCGA GGG I(C/G)(A/T) (A/G)TC (A/G)TA IGC I(C/G)(A/T) I(C/G)(A/T) IGG-3'] were based on the peptide sequence Pro-Ser-Ser-Ala-Tyr-Asp-Ser-Pro from P1 / P2 with *Xho*I site at 5' end. The sense degenerate primer C02S [5'-GAAAGCTT GA(A/G) GA(A/G) GTI GA(C/T) TA(C/T) (C/T)TI CC-3'] and antisense primer C02AS [5'-GAAAGCT TGG IA(A/G) (A/G)TA (A/G)TC IAC (C/T)TC (C/T)TC-3'] were based on the peptide sequence Glu-Glu-Val-Asp-Tyr-Leu-Pro from P3 with *Hin*dIII site at 5' end. PCR was carried out with first strand cDNA as a template using these primers. With a set of C01S and C02AS, 1.2 kbp fragment was obtained as a major product (Fig. 4-2). The 1.2 kbp fragment was subcloned and

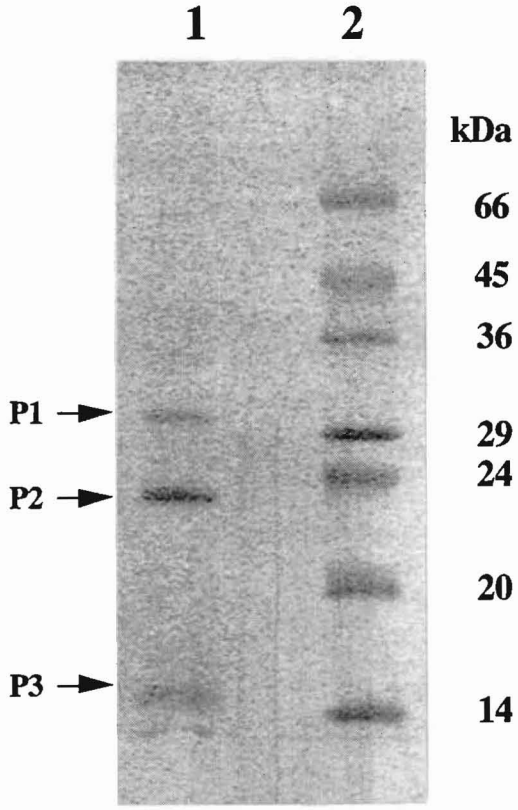

Fig. 4-1. CNBr cleavage of 59 kDa protein purified from the microsomal fraction of green pepper seedlings treated with arachidonic acid. Cleaved peptides were separated by SDS-polyacrylamide gel electrophoresis (15% gel) and electroblotted onto PVDF membrane. Lane 1, cleaved peptides. Lane 2, protein markers. 66 kDa, bovine serum albumin; 45 kDa, egg albumin; 36 kDa, glyceraldehyde-3-phosphate dehydrogenase; 29 kDa, carbonic anhydrase; 24 kDa, trypsinogen; 20 kDa, trypsin inhibitor; 14 kDa, α-lactalbumin. Main bands (P1, P2 and P3) were cut out and were subjected to amino acid sequencing.

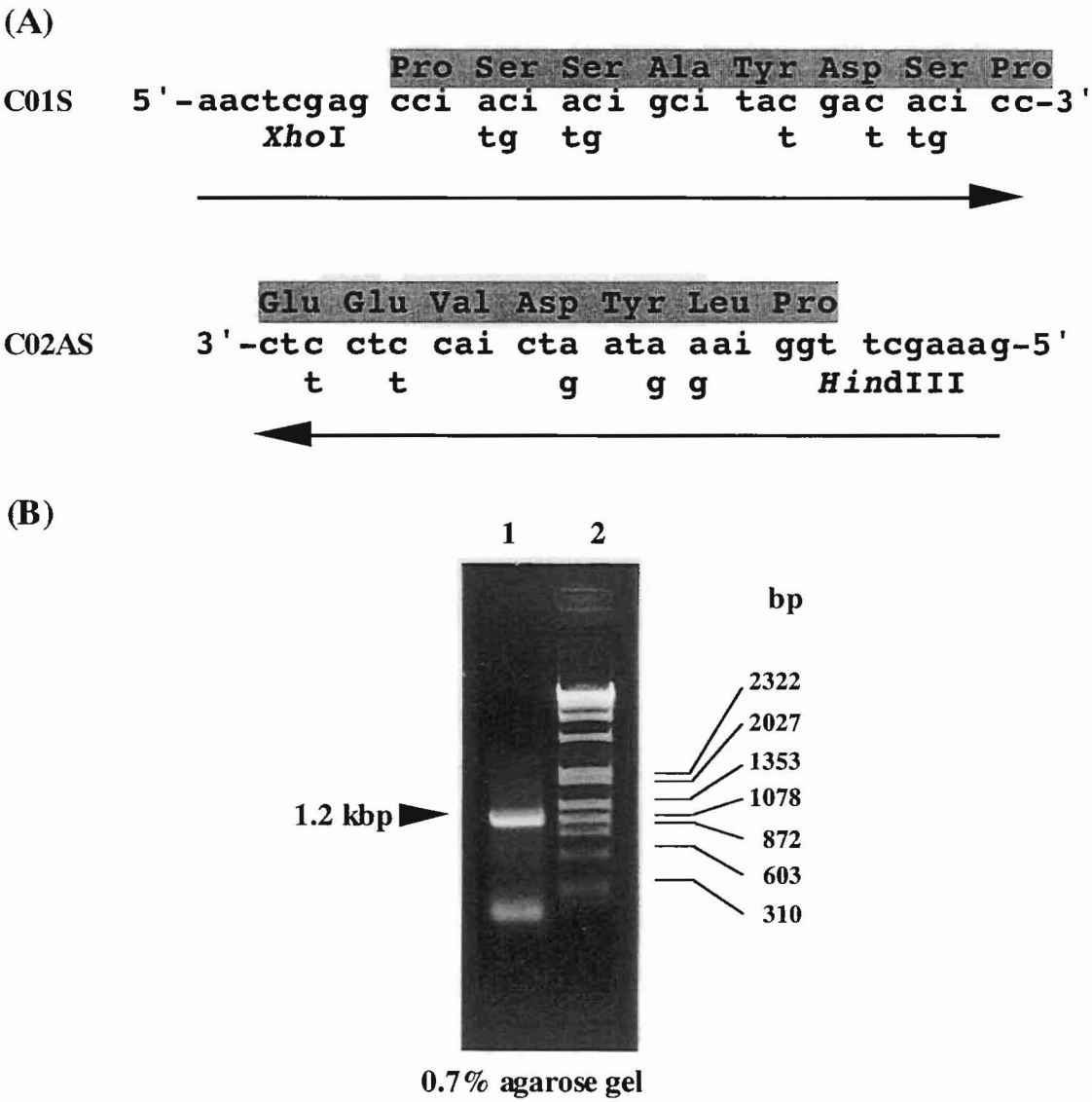

Fig. 4-2. PCR amplification of a DNA fragment with the first strand cDNA from green pepper seedlings treated with arachidonic acid. (A) The DNA sequences of degenerate oligonucleotides C01S and C02AS, aligned with the peptide sequences. (B) PCR products amplified with the first strand cDNA as a template (lane 1). The DNA markers were mixture of λ *Hin*dIII and ϕX174 *Hae*III (lane 2). The 1.2 kbp fragment was subcloned, sequenced and used as a probe for screening of cDNA library.

sequenced. The deduced amino acid sequence contained the peptide sequences as described before, and showed high similarity with that of tobacco catalase [93].

The cloned PCR fragment was used as a probe for screening of green pepper cDNA library which was prepared from a poly(A)$^+$ RNA fraction of the seedlings treated with 1 mM arachidonic acid for 12 h. About 3.0 × 10^5 plaques were screened by plaque hybridization. After screening was repeated three times, over 14 positive clones were isolated. The insert size of these clones were analyzed by PCR with the oligonucleotide primers corresponding to the nucleotide sequence of T3 and T7 promoter in pBluscript SKII+. The clone pCT01 contained the longest insert DNA (1.8 kbp), which was subjected to sequence analysis.

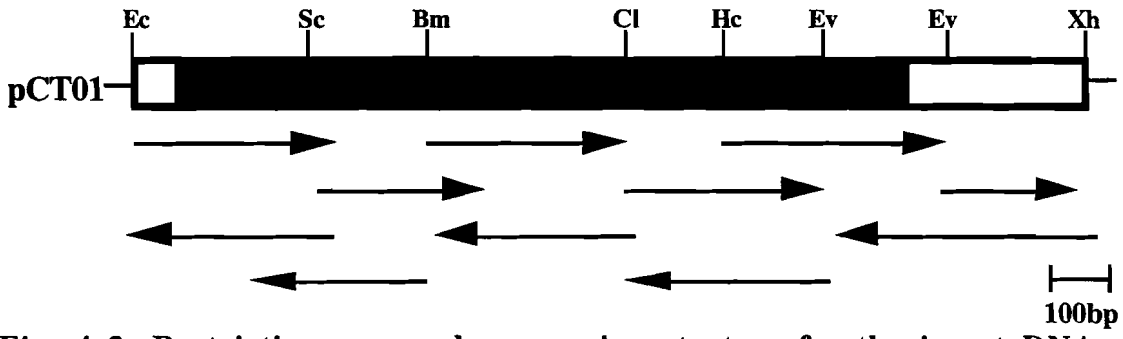

Fig. 4-3. Restriction map and sequencing strategy for the insert DNA of pCT01. The rectangular box shows the cDNA insert of pCT01. The closed box presents the coding region and the open box presents the 5'- and 3'-untranslated regions. Horizontal arrows indicate the direction and extent of sequencing. Ec, *Eco*RI ; Sc, *Sac*I ; Bm, *Bam*HI ; Cl, *Cla*I ; Hc, *Hin*cII ; Ev, *Eco*RV ; Xh, *Xho*I.

Sequence Analysis

Figure 4-3 shows the restriction map and sequencing strategy of the 1.8 kbp insert of pCT01. The cDNA was found to contain a 1476 bp open reading frame from the first ATG codon to TGA stop codon, a 71 bp 5'-untranslated region, a 298 bp 3'-untranslated region as well as a 18 bp poly(A) tail. The 5'-untranslated region was AT-rich, as found with plant cDNA clones [71]. The poly(A) additional signal, AATAA was present 122 bp upstream of poly(A). The sequence (ACTATGGAT) resembled to the initiation start consensus sequence in plant genes [94]. From the first ATG codon, the open reading frame encoded 492 amino acids. The calculated molecular weight of the polypeptide was 56439.0, and

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GGCACGAGCAAAAACCTTATATTAATAAAAAAAACGCCTCTCTCTTCAATTTTCATCTCATTTCAATCACTATGGATCTCTCTAAGTATC     90
                                                                          M  D  L  S  K  Y      6

GCCCATCAAGTGCATATGATTCCCCTTTCTTGACAACAAATGCTGGTGGTCCTGTCTACAACAATGTATCTTCCTTGACTGTTGGACCTA    180
R  P  S  S  A  Y  D  S  P  F  L  T  T  N  A  G  G  P  V  Y  N  N  V  S  S  L  T  V  G  P      36

GAGGGCCTGTTCTTCTTGAGGATTATCACTTAATTGAGAAGCTTGCGACTTTTGATCGTGAGCGGATACCTGAGCGTGTTGTTCATGCTA    270
R  G  P  V  L  L  E  D  Y  H  L  I  E  K  L  A  T  F  D  R  E  R  I  P  E  R  V  V  H  A      66

GAGGTGCCAGTGCCAAGGGCTTCTTTGAAGTCACTCATGATATTTCTCATCTTACCTGTGCTGATTTTCTCCGAGCTCCTGGTGTTCAAA    360
R  G  A  S  A  K  G  F  F  E  V  T  H  D  I  S  H  L  T  C  A  D  F  L  R  A  P  G  V  Q      96

CACCTGTTATTTGTCGCTTCTCTACTGTCGTCCATACGCGTGGAAGCCCCGAATCCATCAGGGATATTCTCGGTTTTGCTGTCAAATTTT    450
T  P  V  I  C  R  F  S  T  V  V  H  T  R  G  S  P  E  S  I  R  D  I  L  G  F  A  V  K  F     126

ACACCAGAGAGGGTAACTTTGATCTTGTTGGAAACAATGTCCCCGTCTTCTTTAATCGTGATACAAAGTCATTCCCTGACACGATTCGTG    540
Y  T  R  E  G  N  F  D  L  V  G  N  N  V  P  V  F  F  N  R  D  T  K  S  F  P  D  T  I  R     156

CATTGAAACCAAATCCAAAGTCACACATTCAGGAAAACTGGAGGATCCTTGATTTCTTCTCTTTCCTCCCGGAGAGTTTGCATACGTTTG    630
A  L  K  P  N  P  K  S  H  I  Q  E  N  W  R  I  L  D  F  F  S  F  L  P  E  S  L  H  T  F     186

CTTTCTTCTACGATGATGTTTGTCTCCCGACAGATTACAGACACATGGAAGGTTTTGGTGTTCACGCTTATCAATTGATCAACAAAGCTG    720
A  F  F  Y  D  D  V  C  L  P  T  D  Y  R  H  M  E  G  F  G  V  H  A  Y  Q  L  I  N  K  A     216

GGAAAGCACATTATGTGAAGTTCCACTGGAAGCCAACTTGCGGTGTCAAGTCCATGACGGAGGAAGAAGCTATTAGGGTCGGAGGTACTA    810
G  K  A  H  Y  V  K  F  H  W  K  P  T  C  G  V  K  S  M  T  E  E  E  A  I  R  V  G  G  T     246

ATCATAGCCACGCCACCAAGGATCTCTACGATTCAATTGCTGCTGGAAACTACCCCGAGTGGAAACTTTTTATCCAAATTATGAACCCTG    900
N  H  S  H  A  T  K  D  L  Y  D  S  I  A  A  G  N  Y  P  E  W  K  L  F  I  Q  I  M  N  P     276

AGGATGTTGACAAATTCGACTTTGACCCTCTTGACGTAACCAAGACCTGGCCTGAGGATATCTTGCCATTGATGCCAGTTGGTCGATTGG    990
E  D  V  D  K  F  D  F  D  P  L  D  V  T  K  T  W  P  E  D  I  L  P  L  M  P  V  G  R  L     306

TATTGAATAGGAACATCGATAACTTCTTTGCAGAGAACGAGCAGCTCGCATTTAACCCTGGACATATTGTCCCTGGTGTCTACTATTCGG   1080
V  L  N  R  N  I  D  N  F  F  A  E  N  E  Q  L  A  F  N  P  G  H  I  V  P  G  V  Y  Y  S     336

AGGATAAGCTTCTCCAGACTAGGATATTTGCATATGCTGATACTCAGAGACACCGTATTGGACCAAACTATATGCAGCTCCCAGTTAACG   1170
E  D  K  L  L  Q  T  R  I  F  A  Y  A  D  T  Q  R  H  R  I  G  P  N  Y  M  Q  L  P  V  N     366

CTCCCAAGTGTGCTCATCACAATAATCACCGTGATGGTGCCATGAACTTCATGCATCGCGATGAGGAGGTGGATTATTTGCCCTCAAGGT   1260
A  P  K  C  A  H  H  N  N  H  R  D  G  A  M  N  F  M  H  R  D  E  E  V  D  Y  L  P  S  R     396

TTGATCCTTGTCGTCCTGCCGAGCAGTACCCAATTCCTTCTTGTGTCTTGACAGGAAGGCGTGAAAAGTGTGTCATTCCGAAAGAGAACA   1350
F  D  P  C  R  P  A  E  Q  Y  P  I  P  S  C  V  L  T  G  R  R  E  K  C  V  I  P  K  E  N     426

ACTTCAAGCAGGCTGGGGAGAGATACAGATCCTGGGCACCTGACAGGCAAGACAGATATATCAACAAATGGGTTGAGTCTTTATCCGATC   1440
N  F  K  Q  A  G  E  R  Y  R  S  W  A  P  D  R  Q  D  R  Y  I  N  K  W  V  E  S  L  S  D     456

CCCGAGTCACTCATGAGATACGCAGTATATGGATATCATACTTGTCTCAGGCTGACAAGTCTTGTGGTCAGAAGGTCGCTTCTCGTCTCA   1530
P  R  V  T  H  E  I  R  S  I  W  I  S  Y  L  S  Q  A  D  K  S  C  G  Q  K  V  A  S  R  L      486

CTGTGAAGCCAACAATGTGAAGATGAAAACAGTTGGAAACGGGAAATGTTTCAAGTTGCAATGCTGAAGGACTAATACAAGAAAACGTTC   1620
T  V  K  P  T  M  *                                                                            492

GCGTGTGGGTGATATAAACTGCACTGTTTTTTCCTGTGATAATGTTGTATTTTGTACCGAACTTTCGATATCTTGTGTTTTATTACGATG   1710

ATGTTGGAAACTGAATAAGTTTAAAGTTGTACTTGTATGTTCAAGTATTGTAATCATCTTCTTTGTTTCACTTTTTTCAAGTCATGTTGG   1800

GTACTTGTTCACTTTGGTGCTTGAAGTAAACACTTTCAAGTCAATAAAAAAAAAAAAAAAAAA                              1863
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Fig. 4-4. Nucleotide sequence and the deduced amino acid sequence of the insert DNA of pCT01. The deduced amino acid sequence was shown below the nucleotide sequence. The first ATG codon at position 72 is presumed to be translation initiation start codon. Shaded box indicate the amino acid sequence determined by peptide sequencing. Open box shows putative targeting sequence to peroxisome. Asterisk represents a stop codon. Putative poly(A) additional signal is underlined.

```
pepper     MDLSKYRP-S----------SAYDSPFLTTNAGGPVYNNVSSLTVGPRGPVLLEDYHLIE   49
eggplant   MDLSKYRP-S----------SAYDTPFLTTNAGGPVYNNVSSLTVGPRGPVLLEDYHLIE   49
tobacco    ---SKFRP-S----------SAYDSPFLTTNAGGPVYNNVSSLTVGPRGPVLLEDYHLIE   46
bovine     --ADNRDPASDQMKHWKEQRAAQKPDVLTTGGGNPVGDKLNSLTVGPRGPLLVQDVVFTD   58
                 * *            *     ***  * **     ********* *  *
                      ¶Δ                                        ¶ Δ¶
pepper     KLATFDRERIPERVVHARGASAKGFFEVTHDISHLTCADFLRAPGVQTPVICRFSTVVHT  109
eggplant   KLATFDRERIPERVVHARGASAKGFFEVTHDVSHLTCADFLRAPGVQTPVICRFSTVVHE  109
tobacco    KLATFDRERIPERVVHARGASAKGFFEVTHDISHLTCADFLRAPGVQTPVICRFSTVVHE  106
bovine     EMAHFDRERIPERVVHAKGAGAFGYFEVTHDITRYSKAKVFEHIGKRTPIAVRFSTVAGE  118
             * ************* ** * * ******      *      *  **   *****
                                        Δ     ¶       ¶
pepper     RGSPESIRDILGFAVKFYTREGNFDLVGNNVPVFFNRDTKSFPDTIRALKPNPKSHIQEN  169
eggplant   RGSPESIRDIRGFAVKFYTREGNFDLVGNNVPVFFNRDAKSFPDTIRALKPNPKSHIQEN  169
tobacco    RGSPESLRDIRGFAVKFYTREGNFDLVGNNVPVFFNRDAKSFPDTIRALKPNPKSHIQEY  166
bovine     SGSADTVRDPRGFAVKFYTEDGNWDLVGNNTPIFFIRDALLFPSFIHSQKRNPQTHLKDP  178
            **    **  ********  ** ****** * ** **   **  *   * **  *

pepper     WRILDFFSFLPESLHTFAFFYDDVCLPTDYRHMEGFGVHAYQLINKAGKAHYVKFHWKPT  229
eggplant   WRILDFFSFLPESLHTFAFFYDDVCLPINYRHMEGFGVHAYQLINKAGKAHYVKFHWKPT  229
tobacco    WRILDFFSFLPESLHTFAWFFDDVCLPTDYRHMEGYGVHAYQLINKAGKAHYVKFHWKPT  226
bovine     DMVWDFWSLRPESLHQVSFLFSDRGIPDGHRHMDGYGSHTFKLVNADGEAVYCKFHYKTD  238
               ** *  *****       *   *   *** * * *   * *  * * * *** *

pepper     CGVKSMTEEEAIRVGGTNHSHATKDLYDSIAAGNYPEWKLFIQIMNPEDVDKFDFDPLDV  289
eggplant   CGVKSMTEEEAIRVGGTNHSHATKDLYDSIAAGNYPEWKLFIQIMDPEDVDKFDFDPLDV  289
tobacco    CGVKCMSEEEAIRVGGTNHSHATKDLYDSIAAGNYPEWKLFIQIMDTEDVDKFDFDPLDV  286
bovine     QGIKNLSVEDAARLAHEDPDYGLRDLFNAIATGNYPSWTLYIQVMTFSEAEIFPFNPFDL  298
            * *    * * *            **   ** **** * * ** *        * * * *
                                              †                     †   †
pepper     TKTWPEDILPLMPVGRLVLNRNIDNFFAENEQLAFNPGHIVPGVYYSEDKLLQTRIFAYA  349
eggplant   TKTWPEDILPLMPVGRLVLNRNIDNFFAENEQLAFNPGHIVPGVYYSEDKLLQTRIFAYA  349
tobacco    TKTWPEDILPLMPVGRLVLNRNIDNFFAENEQLAFNPGHIVPGLYYSEDKLLQTRIFAYA  346
bovine     TKVWPHGDYPLIPVGKLVLNRNPVNYFAEVEQLAFDPSNMPPGIEPSPDKMLQGRLFAYP  358
           ** **    ** *** ******  * *** ***** *    **   * ** ** * ***

pepper     DTQRHRIGPNYMQLPVNAPKCAH-HNNHRDGAMNFM-HRDEEVDYLPSRFDPCRPAEQYP  407
eggplant   DTQRHRIGPNYMQLPVNAPKCAH-HNNHRDGAMNFM-HRDEEVDYLPSRFDPCRPAEQYP  407
tobacco    DTQRHRIGPNYMQLPVNAPKCAH-HNNHRDGAMNFM-HRDEEVDYLPSRFDPCRHAEQYP  404
bovine     DTHRHRLGPNYLQIPVNCPYRARVANYQRDGPMCMMDNQGGAPNYYPNSFSAPEHQPSAL  418
           ** *** **** * *** *  *   *  *** *  *        * *  *

pepper     IPSCVLTGRREKCVIPKENNFKQAGERY-RSWAPDRQDRYINKWVESLSDPRVTHEIRSI  466
eggplant   IPSCVLTGRREKCVIPKENNFKQAGERY-RTWEPDRQDRYINKWVESLSDPRVTHEIRSI  466
tobacco    IPSRVLTGRREMCVIEKENNFKQAGERY-RSWEPDRQDRYVSKWVEHLSDPRVTYEIRSI  463
bovine     EHRTHFSGDVQRFNSANDDNVTQVRTFYLKVLNEEQRKRLCENIAGHLKDAQLFIQKKAV  478
                 *           *  *    *           *        * *

pepper     WISYLSQADKSCGQKV--ASRLTVKPTM  492
eggplant   WISYLSQADKSCGQKV--ASRLLVKPTM  492
tobacco    WICSLSQADKSCGQKV--ASRLTLKPTM  489
bovine     KNFSDVHPEYGSRIQALLDKYNEEKPKN  506
                                 **
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Fig. 4-5. Comparison of the deduced amino acid sequence of pCT01 with the corresponding sequences from eggplant, tobacco [93] and bovine [95] catalases. Asterisk shows the location of identical amino acid. ¶ and † indicate amino acid residues involved in distal and proximal heme binding, respectively. Δ presents amino acid residues related to catalytic activity.

agreed well with the molecular mass of the purified protein estimated on SDS polyacrylamide gel electrophoresis. The peptide sequences of P1 / P2 and P3 were also found in the deduced amino acid sequence of pCT01 from 2nd to 15th and 387th to 397th residues, respectively, although both sequences contained one different residue of Lys at 5th position instead of Leu and Arg at 396th position instead of Ser. In addition, putative CNBr cleavage sites were found in the deduced amino acid sequence. The deduced amino acid sequence of pCT01 showed high similarity to those of plant catalases reported. Figure 4-5 shows a comparison of the deduced amino acid sequence of pCT01 with those of three catalases reported. Eggplant, tobacco [93] and bovine [95] catalases showed 97.8%, 94.1% and 38.8% amino acid sequence identity with that of pCT01. The primary structures of plant catalases were also determined with sweet potato [96], maize [97], caster bean [98], tobacco [99], barley [100], tomato [101] and rice [102]. These sequences were found to be highly conserved among them. Based on the three dimensional structure of bovine catalase determined [103], the amino acid residues involved in the catalytic site (His-65, Ser-104, and Asn-138), and distal (Val-64, Arg-102, Thr-105, Phe-143, and Phe-151) and proximal (Pro-326, Arg-344, and Tyr-348) heme binding sites were also conserved in pCT01. A putative targeting sequence (Ser-Arg-Leu) to peroxisomes was also conserved in pCT01 [98]. Actually, Gould *et al.* reported that Ser (Cys or Ala)-Lys (His or Arg)-Leu is a signal sequence for targeting to peroxisomes [104]. Therefore, the cloned cDNA pCT01 was found to encode green pepper catalase.

We could not confirm whether green pepper catalase in the present study shows 5-*epi*-aristolochene 3-hydroxylase activity because of no availability of the substrate, 5-*epi*-aristolochene. However it is possible to postulate that 5-*epi*-aristolochene is oxidized to produce capsidiol in the presence of O_2 produced by catalase, since the compound is easily autooxidized (Hoshino, personal communication).

Expression of Green Pepper Catalase mRNA

The expression of green pepper catalase gene was analyzed by northern blotting using pCT01 cDNA as a probe DNA. When green pepper seedlings were treated

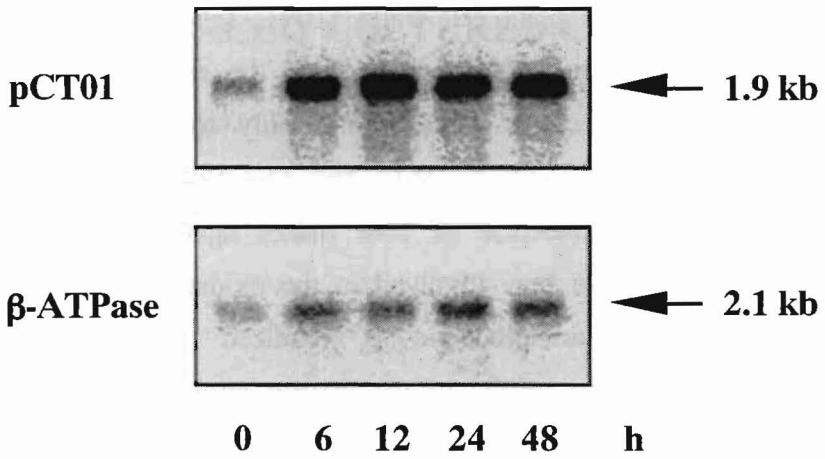


Fig. 4-6. Induction of pCT01 mRNA in green pepper seedlings treated with arachidonic acid. Green pepper seedlings were elicited with 1 mM arachidonic acid for the time indicated. Poly(A)$^+$ RNA was isolated from the seedlings at various time after addition of arachidonic acid (6, 12, 24 and 48 h) and 0.3 μg of poly(A)$^+$ RNA was probed with green pepper catalase cDNA (pCT01). β-ATPase signal is an internal standard.

was also induced by the same treatment in green pepper seedlings [91]. Thus, arachidonic acid seemed to induce catalase gene in green pepper seedling.

Arachidonic acid, which is an unsaturated fatty acid, is not present in higher plants, but present in lower plants and fungi. It was reported that arachidonic acid was released from the cell walls of incompatible fungi and elicited fungitoxic sesquiterpenes in potato [105]. Arachidonic acid or its hydroperoxide produced by oxygen attack may be a signal molecule, which induce defense related genes, resulting in hypersensitive responses. In plant-pathogen interactions, reactive oxygen species (ROS) are generated at the infection site and the produced ROS seemed to play an important role in plant defense [106]. H_2O_2 contributes to the structural reinforcement of plant cell walls [107], salicylic acid (SA) accumulation [108], as well as alteration of redox balance in the reacting cells. [106] However, H_2O_2 is also toxic and cause damages not only to pathogen but also to host plants. Catalase converts H_2O_2 to H_2O and O_2. So, induction of catalase gene may be important for protection of host plants against the damage caused by H_2O_2. In addition, catalase may involved in the oxidation of 5-*epi*-aristolochene to capsidiol because 5-*epi*-aristolochene is oxidized immediately under aerobic conditions. Based on these results of the present study, it is possible to presume that catalase inducible with arachidonic acid may play an important role in plant defense mechanisms in green pepper.

CHAPTER V

MOLECULAR CLONING AND CHARACTERIZATION OF 2,4-D INDUCIBLE P450 SPECIES IN TOBACCO CULTURED CELLS

INTRODUCTION

Cytochrome P450 monooxygenases (P450 monooxygenases) catalyze oxidative reactions in the biosynthesis of secondary metabolites as well as in the metabolism of xenobiotics in higher plants [8]. Particularly, herbicide metabolism is important for herbicide selectivity and resistance. However, molecular information on plant P450 species related to herbicide metabolism is quite limited.

The phenylurea herbicide chlortoluron has been used for selective weed control in wheat and barley, which can detoxify chlortoluron through ring-methyl hydroxylation and *N*-demethylation [37, 109, 110]. Both reactions appeared to be mediated by P450 species. Since the ring-methyl hydroxylation produces a nonphytotoxic metabolites and the *N*-demethylation gives a partially phytotoxic metabolite, metabolism through ring-methyl hydroxylation is primarily responsible for chlortoluron resistance, whereas *N*-demethylation makes a relatively minor contribution towards chlortoluron resistance. On the other hand, in susceptible plants such as cotton and tobacco, chlortoluron was metabolized through *N*-demethylation to produce *N*-demethylated chlortoluron, which is partially phytotoxic. Thus, it was suggested that chlortoluron metabolism with P450 monooxygenases is important for the herbicide selectivity and resistance.

In the tobacco cultured cells S401, chlortoluron was found to be metabolized through both ring-methyl hydroxylation and *N*-demethylation, and both activities were induced by the treatment with 2,4-D. However, purification of the P450 enzymes were difficult because of low contents and instability. In order to clarify the structure and function of P450 species related to metabolism of chlortoluron, I attempted to clone cDNAs of novel P450 species from 2,4-D treated S401 cells, to sequence cDNA clones and to express each of them in the yeast *Saccharomyces cerevisiae* to identify these functions.

MATERIALS AND METHODS

Chemicals and Biochemicals

[α-^{32}P]dCTP (3000 Ci / ml) was purchased from Amersham International plc. (Buckinghamshire, UK). [Phenyl-U-^{14}C]Chlortoluron (specific activity, 2.99 MBq mg^{-1}; radiochemical purity, >98%) and nonlabeled reference standards were provided by Novartis Co., Ltd. (Basel, Switzerland).

Restriction endonucleases and DNA modifying enzymes were purchased from Takara Shuzo Co., Ltd. (Shiga. Japan). *Pfu* DNA polymerase was purchased from Stratagene Cloning Systems (La Jolla, CA).

Other chemicals and biochemicals were purchased from Wako Pure Chemicals (Osaka, Japan), Nacalai Tesque Co., Ltd. (Kyoto, Japan) and Sigma Chemical Co. (St. Louis, MO)

Plant Materials

The tobacco cultured cell line S401 resistant to 2,4-D was derived from *Nicotiana tabacum* L. cv. Samsun [111]. The cultured cells were grown in a modified MS medium containing 3% sucrose and 1.5 μM 2,4-D at 25°C with shaking. About 1 g of the cells were subcultured into 150 ml of the medium every seven days.

PCR Cloning

The tobacco S401 cells grown for 7 days were treated with 150 μM 2,4-D for 2, 6, 12, 24 h and then subjected to poly(A)$^+$ RNA preparation using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden). Poly(A)$^+$ RNA fractions prepared were mixed and then 0.1 μg of the mixture was subjected to first strand cDNA synthesis. About 10% of the first strand cDNA prepared was used as a template for PCR. PCR reaction was performed using *Taq* polymerase (Takara Shuzo) through 30 cycles of 0.5 min at 94°C, 1 min at 40°C and 1.5 min at 72°C. PCR products were separated on 5% polyacrylamide gel electrophoresis. DNA fragments were extracted and subcloned into pBluescript KSII+ (Stratagene) for DNA sequencing.

Construction and Screening of cDNA Library

A cDNA library consisting of 2×10^6 recombinants was constructed from 5 μg of the mixture of poly(A)$^+$ RNA fractions described above using a λZAP cDNA synthesis kit (Stratagene) according to the manufacturer's instructions. The cDNA library was screened by plaque hybridization as described in Chapter IV.

Inverse PCR

Four oligonucleotides (ITCP1-1 to ITCP1-4) were designed based on the nucleotide sequence of the cDNA clone TCP1 from the cDNA library. Two antisense primers (ITCP-1 and ITCP-2) and two sense primers (ITCP1-3 and ITCP1-4) corresponded to the 5' and 3' regions of TCP1; 71-90 bp, 93-112 bp, 126-145 bp and 149-168 bp, respectively. ITCP-1; 5'-AGT AAG AAA TGG AGC TGG TG -3', ITCP1-2; 5'-A AAG ATG GCC AAT AAA GGG G-3', ITCP1-3; 5'-A CCA CTT CAA CGT ACC TTA G -3', ITCP1-4; 5'-AA ATC TCC GAA CGT TAT GGC -3'.

Genomic DNA (1 μg) prepared from the tobacco S401 cells was digested with *Pst*I. The digested DNA was subjected to ligation reaction [66]. PCR was carried out with the primers ITCP1-2 and ITCP1-3 using the ligated DNA described above as a template. PCR was repeated with primers ITCP1-1 and ITCP1-4 using the PCR mixture as a template DNA. These PCR were carried out through 20 cycles of 1 min at 94°C, 2 min at 50°C and 3 min at 72°C with *Pfu* DNA polymerase. An amplified DNA fragment was subcloned for DNA sequencing.

DNA Sequencing and Sequence Analysis

Insert DNA fragments of positive clones as well as PCR fragments were sequenced by the use of a Thermo Sequenase™ premixed cycle sequencing kit (Amersham) and a BigDye Terminator cycle sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA) in a Hitachi SQ-5500 DNA sequencer (Hitachi Co. Ltd., Tokyo, Japan) and an ABIPRISM™ 310 Genetic Analyzer (Perkin-Elmer). Sequence analysis was performed with a DNASIS-Mac software (Hitachi Software Engineering Co. Ltd., Yokohama, Japan). Homology search was carried

out by the use of a BLAST computer program [67]. A phylogenic tree was calculated by a DDBJ computer program "malign" (http://www.ddbj.nig.ac.jp/htmls/E-mail/malwel-e.html.)

Northern Hybridization

Poly(A)$^+$ RNA was prepared from the tobacco S401 cells treated with chemical compounds using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech). Hybridization was performed by the use of an ECL direct nucleic acid labeling and detection systems (Amersham).

Expression of P450 cDNAs in the Yeast

5' and 3' Ends of the coding region of cloned cDNAs were modified by flanking *Hin*dIII sites using PCR. Expression plasmids pATCP1 and pATCP2 were constructed by insertion of the modified DNA fragments at *Hin*dIII site between ADH promoter and terminator of pAAH5N [112]. The expression plasmid pATRN was constructed by replacement of *Bam*HI site at the end of ADH terminator with *Not*I site on pATR, which was described in Chapter III. The co-expression plasmids pATCP1TR and pATCP2TR were constructed by insertion of 3.6 kbp *Not*I fragment of pATCP1 and pATCP2 into *Not*I site of pATRN. The co-expression plasmids pATCP1YR and pATCP2YR were constructed by insertion of 3.6 kbp *Not*I fragment of pATCP1 and pATCP2 into *Not*I site of pARR3N [84].

Saccharomyces cerevisiae AH22 cells were transformed with each of the expression plasmids by lithium chloride method as described [70].

Measurement of P450 Hemoprotein

Microsomal fractions were prepared from recombinant yeast cells described previously [64]. Reduced CO-deference spectra of the microsomal fractions were measured as described [64].

Monooxygenase Activities in Recombinant Yeast Strains

Metabolism of [^{14}C]chlortoluron in the microsomal fraction of recombinant yeast strains was measured as follows. A reaction mixture in 200 μl of 100 mM potassium phosphate buffer (pH 7.4) containing 20 % glycerol, 100 μM [^{14}C]chlortoluron (1.5 × 10^9 dpm per mmol), 0.5 mM NADPH was incubated at 30°C for 15 h with shaking. Metabolism of [^{14}C]chlortoluron in whole yeast cells was measured under the following condition; 25 nmol of [^{14}C]chlortoluron (1.5 × 10^9 dpm per mmol) was added to 2.5 ml of a culture of recombinant yeast cells (2 × 10^7 cells per ml). The yeast culture was incubated at 30°C with shaking and aliquots (0.5 ml) of the mixture were sampled at 0, 3, 12 and 24 h. Chlortoluron and its metabolites were extracted with 0.1 volume of 4N HCl and two volume of ethyl acetate. Extracts were concentrated and dissolved in 50 μl of 90% methanol (v / v). A half volume of the mixture was spotted on a thin layer plate (0.25 mm silica gel 60F254 plates Merck A.G., Darmstadt, Germany). The plate was developed by the solvent system of hexane / ethyl acetate 2 / 1 (v/ v). Metabolites from [^{14}C]chlortoluron was detected in a BAS2000 Bioimaging Analyzer (Fuji Film Co. Ltd., Tokyo, Japan).

7-Ethoxycoumarin *O*-deethylase activity was measured as described [83].

RESULTS

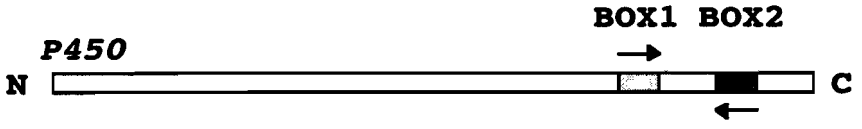
cDNA Cloning of 2,4-D Inducible P450 Species from Tobacco S401 Cells

Since the tobacco cultured cell line S401 was enhanced in the metabolism of the herbicide chlortoluron with 2,4-D treatment, it was attempted to isolate cDNA clones of P450 species from tobacco S401 cells treated with 2,4-D. Ten oligonucleotides were designed based on the amino acid sequences of two regions BOX1 and BOX2 conserved among plant P450 species reported as shown in Fig. 5-1. PCR was carried out with the first strand cDNA from a mRNA fraction of 2,4-D treated S401 cells as a template. Amplified DNA fragments with about 260 bp in length was subcloned, and randomly sequenced for 80 clones, of which 41 clones were found to encode partial sequences related to P450 species by BLAST analysis, and classified into 11 distinct species : A (2 clones), B (9), C (2), D (1), E (13), F (3), G (3), H (4), I (2), J (1) and K (1). Northern blot analysis was performed for 2,4-D treated S401 cells with each of these 11 clones (A-1, B-2, C-20, D-30, E-34, F-39, G-44, H-48, I-54, J-64 and K-70) as a probe DNA. Four clones A-1, B-2, H-48 and I-54 were selected as 2,4-D inducible clones. Then, a cDNA library prepared from 2,4-D treated S401 cells was screened with A-1, B-2, H-48 and I-54 as probe DNAs. As a result, 8 positive clones were isolated and divided to 4 classes : TCP1 (one clone), TCP2 (3), TCP3 (2) and TCP4 (2), since TCP1, TCP2, TCP3 and TCP4 cDNAs were hybridized with A-1, B-2, I-54 and H-48, respectively. Based on the sequence analysis, TCP1 cDNA seemed to lack the 5' region. Therefore, inverse PCR was carried out to obtain the information on the amino terminal region of TCP1 protein. As a result, GTCP1 of about 0.5 kbp fragment containing the 5' region of TCP1 gene was isolated.

Sequence Analysis of P450 cDNAs

Figures 5-2, -3, -4 and -5 showed the nucleotide sequences and the deduced amino acid sequences of the combination of both TCP1 and GTCP1, TCP2, TCP3 and TCP4 cDNA clones, respectively. The open reading flames of TCP1, TCP2, TCP3 and TCP4 encoded 511, 500, 522 and 496 amino acid residues,

(A)



	BOX1		BOX2
CYP75A1	AICKETFRKHP	CYP75A1	PEGAGRRIC
CYP73A2	AVVKETLRLRM	CYP92A2	PFGAGRRMC
CYP92A2	AIVKETMRLHP	CYP71C1	PFGAGRRIC
CYP85	AVILETSRLAT	CYP84	PFGSGRRSC
CYP71B7	LVLKEIFRLHP	CYP73A1	PFGVGRRSC
Conserved	* *** **	Conserved	*** *** *

(B)

Sense primers

F1 5′-ATGAATTC AAA GAA ACA TTT CGA AAG CAC CC-3′

F2 5′-ATGAATTC GCA GTG GTG AAG GAA ACC CTT CG-3′

F3 5′-ATGAATTC GCA ATT GTT AAA GAG ACT ATG CG-3′

F4 5′-ATGAATTC GCT GTG ATT TTA GAG ACC TCC AG-3′

F5 5′-ATGAATTC CTT GTG GTC AAG GAG ATA TTT AG-3′

Antisense primers

R1 5′-ATAAGCTTA AAT TCT TCG TCC AGC ACC AAA TGG-3′

R2 5′-ATAAGCTTA CAT CCT TCT CCC AGC TCC AAA TGG-3′

R3 5′-ATAAGCTTA GAT CCT GCG CCC AGC CCC GAA CGG-3′

R4 5′-ATAAGCTTA CGA TCT ACG ACC CGA CCC GAA CGG-3′

R5 5′-ATAAGCTTA GCT TCT TCT CCC AAC ACC AAA GGG-3′

Fig. 5-1. Design of PCR primers for cloning of novel P450 cDNAs from tobacco S401 cells. (A) Conserved regions among the primary structures of plant P450 species reported. BOX1 and BOX2 indicate conserved regions. Underlined sequences were used to design the PCR primers. (B) DNA sequences of PCR primers. F1, F2, F3, F4 and F5 correspond to the nucleotide sequences of BOX1 region of CYP75A1, CYP73A2, CYP92A2, CYP85 and CYP71B7, respectively. R1, R2, R3, R4 and R5 correspond to BOX2 region of CYP75A1, CYP92A2, CYP71C1, CYP84 and CYP73A1, respectively. Dotted line indicates the restriction sites of *Eco*RI (F1-F5) and *Hin*dIII (R1-R5), respectively.

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↓GTCP1
AAAGAAATAAAATTACTCCATTAGTCTATTAATTCCAAAAACGTGATCTAGACGTACGGCTTGAACACACTT   72
TGCTTTAAAATCATMGTAAATCAACAAGTTTAGACAAAGCAAGTAAGTTGCTTTCCTTTAAAAAAGGAAAAT  144
CAGCGTATTATTAAACAATATACTACACTTCAAATTTCTAGAACCCAAAGAAAAATCTAAGACATTGGTAAA  216
            SBF-1
AAGAACTGACCCCATTATTCACGACATTGACCCCCATCACAAGTCTTCAAATTAAACCCCAATATAAATACA  288

AACAATAACCTAATATGCCAACCATTACAAAAACACATTCATTTCCCTCTTTATCCCAAAAACAAAATTCCA  360
                    GTCP1↓↓TCP1
ATGGTTAACATGTTCACTCCAATTATATACGCTCCTCTCCTTTTAGCTTTTTACATTATCACAAAACATTTC  432
 M  V  N  M  F  T  P  I  I  Y  A  P  L  L  L  A  F  Y  I  I  T  K  H  F   24

TTACGCAAACTCAGAAATAATCCACCAGCTCCATTTCTTACTTTCCCCTTTATTGGCCATCTTTATCTCTTC  504
 L  R  K  L  R  N  N  P  P  A  P  F  L  T  F  P  F  I  G  H  L  Y  L  F   48

AAAAAACCACTTCAACGTACCTTAGCCAAAATCTCCGAACGTTATGGCTCTGTTCTTCTACTCGAATTCGGT  576
 K  K  P  L  Q  R  T  L  A  K  I  S  E  R  Y  G  S  V  L  L  L  E  F  G   72

TCACGAAAAGTACTTTTGGTTTCTTCACCATCTGCAGCTGAAGAATGCTTAACAAAAAACGATATTATTTTC  648
 S  R  K  V  L  L  V  S  S  P  S  A  A  E  E  C  L  T  K  N  D  I  I  F   96

GCGAATCGTCCTCTTTTGATGGCTGGAAAACATCTTGGATATAATTTTACTTCTTTGGCTTGGAGTTCGTAC  720
 A  N  R  P  L  L  M  A  G  K  H  L  G  Y  N  F  T  S  L  A  W  S  S  Y  120

GGAGATCACTGGAGAAATCTTCGTAGGATTACTTCAGTTGAGATGTTTTCGACTCATCGTCTTCAAATGCTA  792
 G  D  H  W  R  N  L  R  R  I  T  S  V  E  M  F  S  T  H  R  L  Q  M  L  144

CATGGAATTCGTATTGATGAAGTGAAATCTATGGTTAAGAGGCTCAATTCCTCTGCCATAGCTGAAAAATCT  864
 H  G  I  R  I  D  E  V  K  S  M  V  K  R  L  N  S  S  A  I  A  E  K  S  168

GTGGATATGAAGTCTATGTTTTTTGAGCTGATGCTCAATGTTATGATGAGGACAATTGCTGGAAAAAGATAT  936
 V  D  M  K  S  M  F  F  E  L  M  L  N  V  M  M  R  T  I  A  G  K  R  Y  192

TACGGTGAGAATGTGGAGGACATTGAGGAAGCTACGAGATTCAAAGGTTTGGTGCAAGAGACTTTCAGGATT 1008
 Y  G  E  N  V  E  D  I  E  E  A  T  R  F  K  G  L  V  Q  E  T  F  R  I  216

GGCGGGGCGACGAATATTGGCGACTTTTTGCCGGCGTTGAAGTTATTGGTGAGGAAATTGGAGAAAAGTTTA 1080
 G  G  A  T  N  I  G  D  F  L  P  A  L  K  L  L  V  R  K  L  E  K  S  L  240

ATTGTGTTGCAAGAGAACAGAGATGAGTTTATGCAGGAATTAATTAAAGATTGCAGAAAAAGAATGGAGAAA 1152
 I  V  L  Q  E  N  R  D  E  F  M  Q  E  L  I  K  D  C  R  K  R  M  E  K  264

GAAGGTACTGTTACTGATTCAGAAATTGAAGGGAACAAGAAATGTTTAATTGAAGTTTTGTTAACACTACAA 1224
 E  G  T  V  T  D  S  E  I  E  G  N  K  K  C  L  I  E  V  L  L  T  L  Q  288

GAAAATGAACCGGAATACTACAAAGATGAAATCATCAGAAGCCTTATGCTTGTTCTATTATCAGCTGGTACA 1296
 E  N  E  P  E  Y  Y  K  D  E  I  I  R  S  L  M  L  V  L  L  S  A  G  T  312

GATACTTCAGTTGGGACAATGGAATGGGCTTTATCATTAATGTTAAACCACCCTGAAACTCTGAAGAAAGCA 1368
 D  T  S  V  G  T  M  E  W  A  L  S  L  M  L  N  H  P  E  T  L  K  K  A  336

CAAGCTGAAATTGATGAACATATAGGACATGAACGTTTAGTGGACGAGTCGGACATCAACAACCTACCTTAC 1440
 Q  A  E  I  D  E  H  I  G  H  E  R  L  V  D  E  S  D  I  N  N  L  P  Y  360

CTACGTTGTATAATCAACGAGACATTCCGAATGTACCCTGCAGGACCACTACTAGTCCCACACGAGTCGTCA 1512
 L  R  C  I  I  N  E  T  F  R  M  Y  P  A  G  P  L  L  V  P  H  E  S  S  384

GAGGAAACCACCGTAGGAGGCTACCGTGTACCCGGAGGAACCATGTTACTTGTGAATTTGTGGGCTATTCAC 1584
 E  E  T  T  V  G  G  Y  R  V  P  G  G  T  M  L  L  V  N  L  W  A  I  H  408

AATGATCCAAAGCTATGGGATGAACCAAGAAAGTTTAAGCCAGAAAGATTTGAAGGACTAGAAGGTGTTAGA 1656
 N  D  P  K  L  W  D  E  P  R  K  F  K  P  E  R  F  E  G  L  E  G  V  R  432

GATGGTTACAAAATGATGCCTTTTGGTTCTGGACGAAGGAGTTGTCCTGGAGAAGGATTGGCTATTCGAATG 1732
 D  G  Y  K  M  M  P  F  G  S  G  R  R  S  C  P  G  E  G  L  A  I  R  M  456

GTTGCATTGTCATTGGGATGTATTATTCAATGCTTTGATTGGCAACGACTTGGGGAAGGATTGGTTGATAAG 1804
 V  A  L  S  L  G  C  I  I  Q  C  F  D  W  Q  R  L  G  E  G  L  V  D  K  480

ACTGAAGGAACTGGACTTACTTTGCCTAAAGCTCAACCTTTAGTGGCCAAGTGTAGCCCACGACCTATAATG 1876
 T  E  G  T  G  L  T  L  P  K  A  Q  P  L  V  A  K  C  S  P  R  P  I  M  504

GCTAATCTTCTTTCTCAGATTTGAACATAATTGGTTTCTACCAAACATCCCCAAACTAGAATATTATTATTG 1948
 A  N  L  L  S  Q  I  *                                                   511

GTTACATATACAATGTAATCAATTTTGAACCATATTATATCTCAATGTATTCCTTTTTTATTTATTTAAAAA 2020
          TCP1↓
AAAAAAAAAAAAAA                                                            2034
```

Fig. 5-2. Nucleotide sequence and the deduced amino acid sequence of tobacco P450 TCP1 clone and GTCP1 clone (CYP81B2). The deduced amino acid sequence is indicated below the nucleotide sequence. Highly conserved amino acid residues and motifs are shown in boldface type. The cysteine residue corresponding to the heme binding is underlined. Asterisk indicates stop codon. Box shows putative TATA box. Underlined DNA sequences SBF-1 is homologous to sequence motifs found in silencer region of a soybean chalcone synthase promoter [113].

```
GGCACGAGAAATGAAGTTCCTTTTAGTGGTAGCTTCTCTCTTTCTCTTTGTTTTCCTAATATTATCGGCCAC   72
            M  K  F  L  L  V  V  A  S  L  F  L  F  V  F  L  I  L  S  A  T   21

AAAGAGGAAAAGCAAAGCAAAAAAGCTCCCTCCAGGTCCAAGGAAACTGCCCGTAATAGGAAACCTTCTTCA  144
 K  R  K  S  K  A  K  K  L  P  P  G  P  R  K  L  P  V  I  G  N  L  L  Q   45

AATTGGAAAATTACCTCATCGTTCACTTCAAAAACTTTCTAATGAATATGGGGATTTCATTTTCTTGCAATT  216
 I  G  K  L  P  H  R  S  L  Q  K  L  S  N  E  Y  G  D  F  I  F  L  Q  L   69

AGGTTCTGTCCCAACTGTGGTTGTCTTTTCAGCTGGCATTGCCCGAGAGATCTTTAGAACTCAAGACCTTGT  288
 G  S  V  P  T  V  V  V  F  S  A  G  I  A  R  E  I  F  R  T  Q  D  L  V   93

TTTCTCAGGCCGTCCTGCTTTGTATGCTGGCAAAAGGTTTAGTTACAATTGTTGTAACGTGTCTTTTGCACC  360
 F  S  G  R  P  A  L  Y  A  G  K  R  F  S  Y  N  C  C  N  V  S  F  A  P  117

CTACGGTAATTACTGGAGAGAGGCTAGGAAAATTCTAGTCTTGGAGTTGCTAAGTACAAAGAGAGTACAAAG  432
 Y  G  N  Y  W  R  E  A  R  K  I  L  V  L  E  L  L  S  T  K  R  V  Q  S  141

TTTCGAGGCAATTCGAGACGAGGAAGTAAGTAGTTTGGTTCAAATTATTTGTAGTTCCTTGAGCTCACCTGT  504
 F  E  A  I  R  D  E  E  V  S  S  L  V  Q  I  I  C  S  S  L  S  S  P  V  165

TAACATAAGCACATTAGCACTATCCTTGGCAAATAACGTTGTTTGTCGAGTGGCTTTTGGGAAAGGGAGTGA  576
 N  I  S  T  L  A  L  S  L  A  N  N  V  V  C  R  V  A  F  G  K  G  S  D  189

TGAAGGAGGAAATGATTATGGGGAGAGGAAGTTTCATGAAATTCTTTTTGAGACACAAGAATTATTGGGTGA  648
 E  G  G  N  D  Y  G  E  R  K  F  H  E  I  L  F  E  T  Q  E  L  L  G  E  213

GTTTAATGTTGCTGATTATTTTCCTGGGATGGCATGGATTAACAAAATAAATGGGCTAGATGAAAGATTGGA  720
 F  N  V  A  D  Y  F  P  G  M  A  W  I  N  K  I  N  G  L  D  E  R  L  E  237

AAAGAATTTTAGGGAATTGGATAAGTTTTATGACAAAATAATAGAAGATCATCTTAATTCAAGTAGCTGGAT  792
 K  N  F  R  E  L  D  K  F  Y  D  K  I  I  E  D  H  L  N  S  S  S  W  M  261

GAAACAAAGGGATGATGAAGACGTTATTGATGTATTGCTTCGAATCCAAAAGGACCCAAACCAAGAAATTCC  864
 K  Q  R  D  D  E  D  V  I  D  V  L  L  R  I  Q  K  D  P  N  Q  E  I  P  285

TCTCAAAGATGATCATATTAAGGGCCTTCTTGCGGATATATTCATAGCTGGAACTGATACATCATCAACAAC  936
 L  K  D  D  H  I  K  G  L  L  A  D  I  F  I  A  G  T  D  T  S  S  T  T  309

CATAGAATGGGCAATGTCAGAACTCATAAAAAATCCAAGAGTCTTGAGAAAAGCTCAAGAGGAAGTTAGAGA 1008
 I  E  W  A  M  S  E  L  I  K  N  P  R  V  L  R  K  A  Q  E  E  V  R  E  333

AGTTGCCAAGGGAAAACAAAAGGTCCAAGAAAGCGATCTTTGCAAACTCGAATACTTGAAATTGGTCATCAA 1080
 V  A  K  G  K  Q  K  V  Q  E  S  D  L  C  K  L  E  Y  L  K  L  V  I  K  357

AGAAACCCTTAGATTACACCCACCAGCCCCATTACTAGTCCCTCGAGTAACAACAGCCAGCTGCAAAATAAT 1152
 E  T  L  R  L  H  P  P  A  P  L  L  V  P  R  V  T  T  A  S  C  K  I  M  381

GGAATACGAAATTCCAGCAGATACAAGAGTCCTTATCAACTCGACAGCAATTGGGACGGATCCAAAATATTG 1224
 E  Y  E  I  P  A  D  T  R  V  L  I  N  S  T  A  I  G  T  D  P  K  Y  W  405

GGAAAATCCATTGACATTCTTGCCAGAGAGATTCTTGGATAAGGAGATTGATTACAGAGGCAAAAATTTTGA 1296
 E  N  P  L  T  F  L  P  E  R  F  L  D  K  E  I  D  Y  R  G  K  N  F  E  429

GTTGTTGCCATTTGGGGCAGGGAGAAGAGGGTGTCCAGGAATTAATTTTTCAATACCACTTGTTGAGCTTGC 1368
 L  L  P  F  G  A  G  R  R  G  C  P  G  I  N  F  S  I  P  L  V  E  L  A  453

ACTTGCTAATCTATTGTTTCATTATAATTGGTCACTTCCTGAGGGGATGCTACCTAAGGATGTTGATATGGA 1440
 L  A  N  L  L  F  H  Y  N  W  S  L  P  E  G  M  L  P  K  D  V  D  M  E  477

AGAAGCTTTGGGGATTACCATGCACAAGAAATCTCCCCTTTGCTTAGTAGCTTCTCATTATAACTTGTTGTG 1512
 E  A  L  G  I  T  M  H  K  K  S  P  L  C  L  V  A  S  H  Y  N  L  L  *  500

ATCTTAAAAGATTTTAACATACTATACTTTGAGGTGTCATTTGTAACATATATGTATCTACTTCTTAATTTT 1584
AATATTATTATGCAAAACTTTCCTCAAAAAAAAAAAAAAAAAAAAAA                          1631
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Fig. 5-3. Nucleotide sequence and the deduced amino acid sequence of tobacco P450 TCP2 (CYP71A11). The deduced amino acid sequence is indicated below the nucleotide sequence. Highly conserved amino acid residues and motifs are shown in boldface type. The cysteine residue corresponding to the heme binding is underlined. Asterisk indicates stop codon.

```
GAATTCGGCACGAGATAGAAGGGACATCTGTTTTATAAACTACACCTACCCTGAGTTCTAAGTTCTATAATT   72
CTCTATTGTTTCAAGTTTTCGCCTATGGAGAATCTGAACTCTAGCTACTACTACTACTACTACTACTTAGCT  144
                          M  E  N  L  N  S  S  Y  Y  Y  Y  Y  Y  Y  L  A    16

ATCTTGCTATTTTTCTTTGTCATTTTATTCAAATACTTGCTTCCTTCCGGAAAACGTTTACCACCTAGTCCT  216
 I  L  L  F  F  F  V  I  L  F  K  Y  L  L  P  S  G  K  R  L  P  P  S  P    40

CTGTCTCTTCCGATAATTGGTCATCTTTACCTTATTAAGAATTCCCTACACGAGACACTAACTTCCCTATCT  288
 L  S  L  P  I  I  G  H  L  Y  L  I  K  N  S  L  H  E  T  L  T  S  L  S    64

ACAAAATATGGCCCTGTTTTGTATCTCCGATTCGGCTGTAGAAATTTGCTTGTTGTGTCTTCTCCATCTGCG  360
 T  K  Y  G  P  V  L  Y  L  R  F  G  C  R  N  L  L  V  V  S  S  P  S  A    88

ATGGAAGAATGCTTCACCAAAAACGATATCATATTTGCAAATCGTCCTCAGAGCATGGCTGGAGATCAGTTT  432
 M  E  E  C  F  T  K  N  D  I  I  F  A  N  R  P  Q  S  M  A  G  D  Q  F   112

TCCTTCAACTATAAGGCTGTTGTCTGGGCTCCTTACGGCTATCTTTGGAGAGCTCTCCGCCGTCTAACTGTT  504
 S  F  N  Y  K  A  V  V  W  A  P  Y  G  Y  L  W  R  A  L  R  R  L  T  V   136

ATTGAGATCTTCTCTTCCAATAGCTTGCAGAAGTCTTCTGCACTGCGGAATGAAGAAATTGGAATTCTTATT  576
 I  E  I  F  S  S  N  S  L  Q  K  S  S  A  L  R  N  E  E  I  G  I  L  I   160

CGCTCTTTGTTTAAAGCCAGCACTAATAATGGCAGTAGTGGTGCAAGAGTTAACTTGAGTCATTGGGTTTTT  648
 R  S  L  F  K  A  S  T  N  N  G  S  S  G  A  R  V  N  L  S  H  W  V  F   184

ACTTTTGCGGTCAATGTTATGATGAGAACTGGCACTGGAAAACGTTGCGTAAGTGAAGAAGACATGGAAACA  720
 T  F  A  V  N  V  M  M  R  T  G  T  G  K  R  C  V  S  E  E  D  M  E  T   208

GAGAAGGGGAAACAAATCATTGAAGAGATAAGAGGATTTTTCTTCGCGACCTTGGTAGTTTTGAACGTGTGT  792
 E  K  G  K  Q  I  I  E  E  I  R  G  F  F  F  A  T  L  V  V  L  N  V  C   232

GATTTCATGCCAGTTTTGAAATGGTTTGGGTACAAAGGGCTAGAGAAAAGGATGGTCTTAGCGCACCAAAAG  864
 D  F  M  P  V  L  K  W  F  G  Y  K  G  L  E  K  R  M  V  L  A  H  Q  K   256

AGAAATGAATTCTTGAACAACTTACTGGACGAATTTCGACAGAAAAAAATAGCTGGTATTTCAGAATCCAGT  936
 R  N  E  F  L  N  N  L  L  D  E  F  R  Q  K  K  I  A  G  I  S  E  S  S   280

ACTGATAGTATCAATGCTAAGAAGACCACGCTGGTCGAAACTCTCTTGTCACTACAAGAATCTGAACCTGAA 1008
 T  D  S  I  N  A  K  K  T  T  L  V  E  T  L  L  S  L  Q  E  S  E  P  E   304

TTTTACACAGATGATCTAATTAAAAGTGTTTTACTGGTTTTATTTATTGCTGGAACAGAGACAACATCAATG 1080
 F  Y  T  D  D  L  I  K  S  V  L  L  V  L  F  I  A  G  T  E  T  T  S  M   328

ACCATTCAATGGGCGATGCGACTTCTTTTAGCTCATCCTAAGGCATTTACAAAACTGAGAGCTGAGATTGAT 1152
 T  I  Q  W  A  M  R  L  L  L  A  H  P  K  A  F  T  K  L  R  A  E  I  D   352

AGCAAAGTGGGGAACGATGGCTTGCTAAATGAATCAGACATTCCCAAGCTTCCTTATTTACATCGTGTTATA 1224
 S  K  V  G  N  D  G  L  L  N  E  S  D  I  P  K  L  P  Y  L  H  R  V  I   376

AACGAGACGCTAAGATTGTACCCTCCAGTACCACTTTTGTTGCCTCACTACTCATTAGAAGATTGTACTGTT 1296
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 G  G  Y  E  V  P  K  H  T  I  L  M  V  N  A  W  A  I  H  R  D  P  K  L   424

TGGGACGAACCTGAAAAGTTCAAACCAGAGCGATTTGAGGCCATGGAAGGAGAAAAAGAAGGATTCAACTAT 1440
 W  D  E  P  E  K  F  K  P  E  R  F  E  A  M  E  G  E  K  E  G  F  N  Y   448

AAATTAGTACCATTTGGAATGGGGAGAAGAGCATGCCCTGGAGCTGCTATGGGCTTGCGCACTGTTTCACTG 1512
 K  L  V  P  F  G  M  G  R  R  A  C  P  G  A  A  M  G  L  R  T  V  S  L   472

GTATTGGGTTCCCTGATTCAGTCGTTCGATTGGAAAAGTGTGGAAGAAGAAAAGTTGGACGCGTGCTATAAT 1584
 V  L  G  S  L  I  Q  S  F  D  W  K  S  V  E  E  E  K  L  D  A  C  Y  N   496

TCTAGAATCACTTTGAACAAAGATAAACCTTTGGAGGCTGTTTGTATTCCACGACAAAATTGGCGTGGTTTC 1656
 S  R  I  T  L  N  K  D  K  P  L  E  A  V  C  I  P  R  Q  N  W  R  G  F   520

CTTTCTTGAGCCGAGGTCTCTCTATCTTCCTAAAGTAGGGGATCTCACTGGGTTTGTACTGTTGTTGTATGT 1728
 L  S  *                                                                  522

ATGTATAGTGTAAAAATTTACCTTTGTGCTCTAAGGTAATGTGACTAAATAAAGTTGACTATGTAGAACAAG 1800
AGATATTAAATAAAGCTTAAGCTATTATTTGGTGAATGTGAAAAAAAAAAAAAAAAAAAAA            1860
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Fig. 5-4. Nucleotide sequence and the deduced amino acid sequence of tobacco P450 TCP3 (CYP81C1). The deduced amino acid sequence is indicated below the nucleotide sequence. Highly conserved amino acid residues and motifs are shown in boldface type. The cysteine residue corresponding to the heme binding is underlined. Asterisk indicates stop codon.

```
AGGTAGACGGTATCGATAAGCTTGATATCGAATTCGGCACGAGGTTTCAAATGGAGAATCTGAATTTCAACT   72
                                                M  E  N  L  N  F  N      7

ACTACTACTACTTAGCTGTCTTGCTATGTTTCTTTGTCATTTTATTCAAATACTTGCTTCATCCCAGAAAAC  144
Y  Y  Y  Y  L  A  V  L  L  C  F  F  V  I  L  F  K  Y  L  L  H  P  R  K     31

GTTTAGCACCTAGTCCTCTGTCTCTTCCAATAATTGGCCACCTTTACCTTATCAAGAATTCCCTACACGAGA  216
R  L  A  P  S  P  L  S  L  P  I  I  G  H  L  Y  L  I  K  N  S  L  H  E     55

CCTTAACTTCCTTATCTACAAAATATGGCCCTGTTTTGTATCTCCGGTTCGGCTGTAGAAATTTGCTTGTTG  288
T  L  T  S  L  S  T  K  Y  G  P  V  L  Y  L  R  F  G  C  R  N  L  L  V     79

TGTCTTCTCCATCTGCGGTAGAAGAATGCTTCACCAAAAACGATATCATATTTGCAAATCGCCCTCAGAGCA  360
V  S  S  P  S  A  V  E  E  C  F  T  K  N  D  I  I  F  A  N  R  P  Q  S    103

TGGCTGGAGATCAGTTTTCCTTCAACTATAAGGCTGTTGTCTGGGCTCCTTACGGCTATCTTTGGAGGGCTC  432
M  A  G  D  Q  F  S  F  N  Y  K  A  V  V  W  A  P  Y  G  Y  L  W  R  A    127

TCCGCCGTCTAACTGTGATCGAGATCTTCTCTTCCAATAGCCTTCAAAAATCTTCTGCACTGCGGAATGAAG  504
L  R  R  L  T  V  I  E  I  F  S  S  N  S  L  Q  K  S  S  A  L  R  N  E    151

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ATTGGGTTTTTACTTTTGCGGTCAATGTTATGATGAGAACTGGTACTGGAAAACGTTGTGTAAGTGAAGAAG  648
H  W  V  F  T  F  A  V  N  V  M  M  R  T  G  T  G  K  R  C  V  S  E  E    199

ACATGGGAACAGAAAAGGGGAAACAAATCATTGAAGAGATAAAGGGATTTTTCTTCGCGACCTTGGTAGTTT  720
D  M  G  T  E  K  G  K  Q  I  I  E  E  I  K  G  F  F  F  A  T  L  V  V    223

TGAACGTGTGTGATTTCATGCCAGTTTTGAAATGGTTTGGGTACAAAGGGCTAGAGAAAAGGATGGTCTTAG  792
L  N  V  C  D  F  M  P  V  L  K  W  F  G  Y  K  G  L  E  K  R  M  V  L    247

CGCACCAAAAGAGAAATGAATTCTTGAACAGCTTACTGGACGAATTTCGACAGAAAAAAAATAGCTGGTATT  864
A  H  Q  K  R  N  E  F  L  N  S  L  L  D  E  F  R  Q  K  K  N  S  W  Y    271

CAGAATCCAGTACTGATAGTATCAATGCGAAGAAGACCACGCTGGTTGAAACTCTCTTGTCTCTGCAGGAAT  936
S  E  S  S  T  D  S  I  N  A  K  K  T  T  L  V  E  T  L  L  S  L  Q  E    295

CAGAACCCGAATTCTATACAGATGATCTTATTAAAAGTGTTTTACTGGTTTTATTTATTGCTGGAACAGAGA 1008
S  E  P  E  F  Y  T  D  D  L  I  K  S  V  L  L  V  L  F  I  A  G  T  E    319

CAACATCAATGACCATTCAATGGGCGATGCGACTTCTTTTAGCTCATCCTAAGGCATTTACAAAACTGAGAG 1080
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ATTGTGTTATAAACGAGACGCTAAGATTGTACCCTCCAGTACCACTTTTGTTGCCTCACTACTCATTAGAAG 1224
Y  C  V  I  N  E  T  L  R  L  Y  P  P  V  P  L  L  L  P  H  Y  S  L  E    391

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D  C  T  V  G  G  Y  E  V  P  K  H  T  I  L  M  I  N  A  W  A  I  H  R    415

ATCCCAAGTTATGGGACGAGCCTGAAAAGTTCAAACCAGAGCGATTTGAGGCAATGGACTTGGGGGAAAAAG 1368
D  P  K  L  W  D  E  P  E  K  F  K  P  E  R  F  E  A  M  D  L  G  E  K    439

AAGGATTCAATTATAAATTTGTACCATTTGGAATGGGGAGAAGAGCATGCCCTGGAGCCACTATGGGCTTGC 1440
E  G  F  N  Y  K  F  V  P  F  G  M  G  R  R  A  C  P  G  A  T  M  G  L    463

GCACTGTTTCACTGGTATTGGGTTCTCTGATTCAGTGGTTCGATTGGGAAAGTGTGGAAAAGAAAAGTTGGA 1512
R  T  V  S  L  V  L  G  S  L  I  Q  W  F  D  W  E  S  V  E  K  K  S  W    487

CGCGTGCTATAATTCTAGAATCACTTTGAACAAAGATAAACCTTTGGAGGCTGTTTGTATTCCCGACAAAAT 1584
T  R  A  I  I  L  E  S  L  *                                              496

TGTCGTGGTTTCCTTGCCCAACTATGAGACTATTCGTACAAATTTGTTCCTTTTGTAGGTATGTATAGTGTT 1656
TTTTTTGAGCCAAGGGTCAGAAACAGTTTTCCTATCTTTCCACAGTAGAGGTAATATATAAAGTCTGCGTGC 1728
ACACTTCCCTCCTCATACCCTACTTGTAAGATCTCACTGGGTTTGTTATTGTTGTATGTATGTATGTATAGT 1800
GTAAAAATTTACCTTTGTGCTCTAAGGTAATGTGACTAAATAAAGTTGACTATGTAGAACAAGAGACATTAA 1872
ATAAAGCTTAAGCTATTATTTGGTGAAAAAAAAAAAAAAAAAA                              1915
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Fig. 5-5. Nucleotide sequence and the deduced amino acid sequence of tobacco P450 TCP4 (CYP81C2). The deduced amino acid sequence is indicated below the nucleotide sequence. Highly conserved amino acid residues and motifs are shown in boldface type. The cysteine residue corresponding to the heme binding is underlined. Asterisk indicates stop codon.

respectively. The calculated molecular weights of the polypeptides were 58178.9, 56670.7, 59705.1 and 57091.4, respectively. The hydrophobic transmembrane region was found in the amino-terminal region of each of TCP1, TCP2, TCP3 and TCP4. The cysteine residue which appeared to be involved in the heme binding was found at 447th, 440th, 460th and 456th in the amino acid sequences of TCP1, TCP2, TCP3 and TCP4, respectively. Based on the sequences, TCP1, TCP2, TCP3 and TCP4 were novel P450 species and they were named as CYP81B2, CYP71A11, CYP81C1 and CYP81C2, respectively, by the P450 nomenclature committee.

Figure 5-6 showed the phylogenic tree of CYP81B2, CYP71A11, CYP81C1 and CYP81C2 as well as plant P450 species reported previously. While plant P450 species were divided into an type A and non-type A groups [116], these four P450 species isolated in the present study appeared to belong to type A group according to the phylogenic tree.

Transcripts of CYP81B2, CYP71A11, CYP81C1 and CYP81C2 in 2,4-D Treated Tobacco S401 Cells

A poly(A)$^+$ RNA fraction was isolated from 2,4-D treated S401 cells for 2, 6, 12, 24 and 48 h after treatment. Northern blot analysis was performed by the use of the cDNAs of CYP81B2, CYP71A11, CYP81C1 and CYP81C2 as probes (Fig. 5-7). CYP81B2 mRNA reached the maximal level at 6 h, and then gradually decreased. CYP71A11 mRNA was induced at 2 h and maintained the level by 48 h. CYP81C1 and CYP81C2 mRNAs were also found to be induced at 2 h, but both mRNA levels were lower than those of CYP81B2 and CYP71A11. Thus, CYP81B2, CYP71A11, CYP81C1 and CYP81C2 genes were found to be induced by the treatment of S401 cells with 2,4-D, although induction patterns were different each other.

Induction of CYP81B2 and CYP71A11 Genes by Several Chemicals

The induction of CYP81B2 and CYP71A11 genes in S401 cells was examined for several chemicals. Northern blot analysis was carried out for S401 cells treated with each of several chemicals : naphthalic anhydride (NA), a herbicide safener

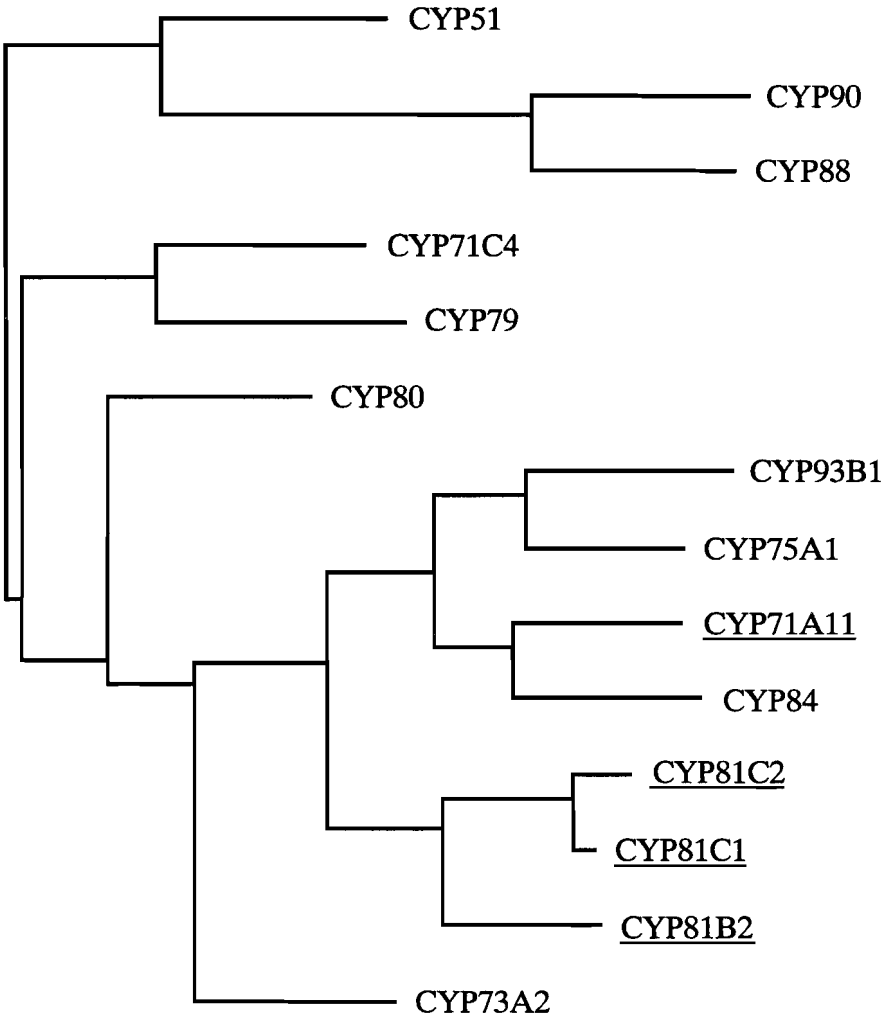

Fig. 5-6. A dendrogram showing the relatedness of tobacco CYP81B1, CYP71A11, CYP81C1 and CYP81C2 with those of the other plant P450 species reported. The dendrogram was generated by the use of the DDBJ program *malign*. CYP51, obtusifoliol 14α-demethylase from *Sorghum bicolor* [9]; CYP71C4, a P450 involved in a cyclic hydroxamic acid biosynthesis from maize [11]; CYP73A2, cinnamic acid 4-hydroxylase from mung bean [13]; CYP75A1, flavonoid 3', 5'-hydroxylase from petunia [22]; CYP79, P450tyr with multifunctional *N*-hydroxylase activity from *Sorghum bicolor* [24]; CYP80, berbamunine synthase from barberry [25]; CYP84, ferulate-5-hydroxylase from *Arabidopsis* [26]; CYP88, a P450 involved in gibberellin biosynthesis from maize [27]; CYP90, a P450 involved in brassinosteroid biosynthesis from *Arabidopsis* [28]; CYP93B1, (2*S*)-flavanone 2-hydroxylase from licorice [29].

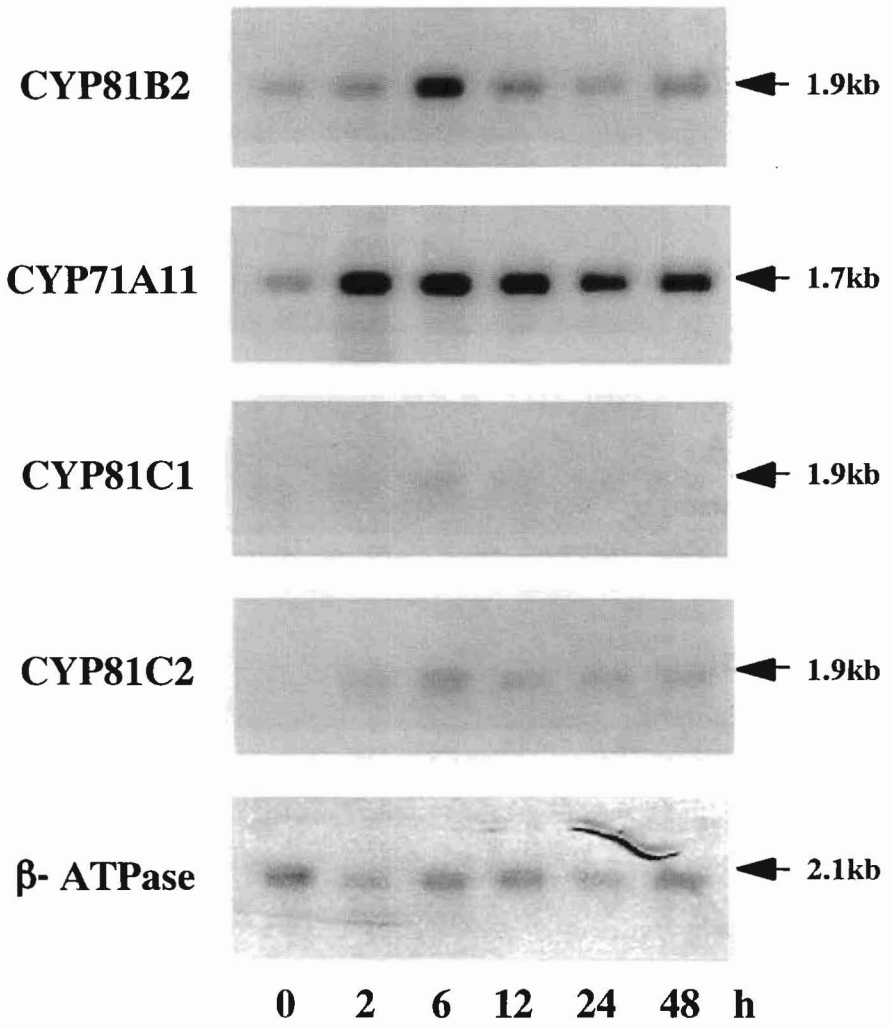

Fig. 5-7. Transcripts of tobacco CYP81B2, CYP71A11, CYP81C1 and CYP81C2 in 2,4-D treated S401 cells. A poly(A) RNA fraction was prepared from the S401 cells treated with 150 μM 2,4-D for indicated time. A poly(A) RNA of 2 μg was loaded on each lane. β-ATPase signal is an internal standard [92].

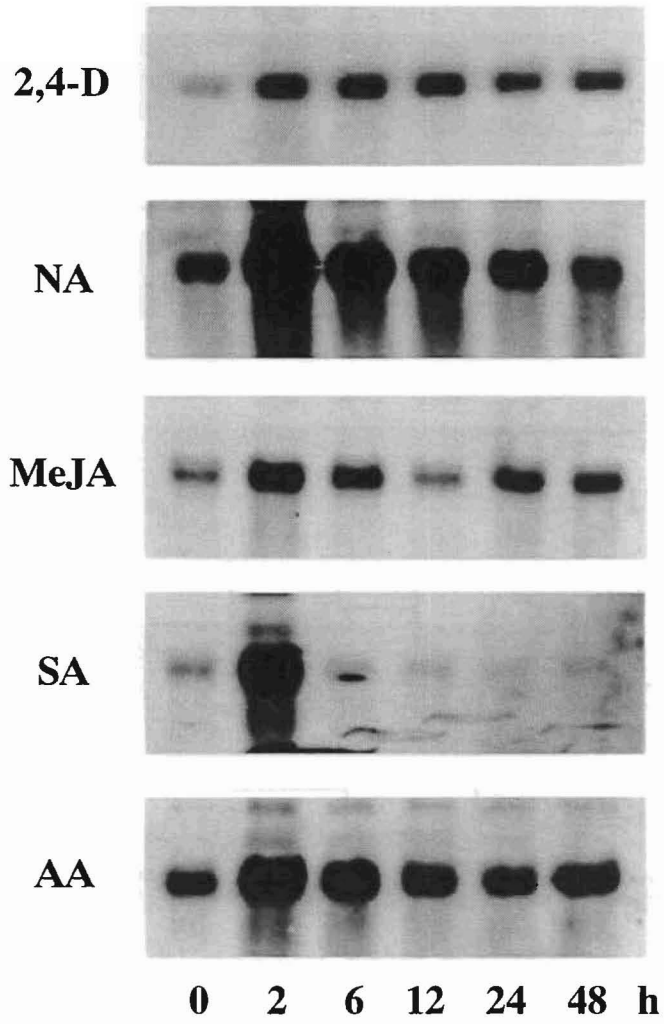

Fig. 5-8. Transcript of CYP71A11 in tobacco S401 cells treated with chemicals and plant signaling compounds. Tobacco S401 cells were treated with 150 μM 2,4-D, 20 μM naphthalic anhydride (NA), 30 μM methyl jasmonate (MeJA), 200 μM salicylic acid (SA), 100 μM arachidonic acid. Poly(A) RNA was each prepared from the S401 cells treated with chemicals for indicated time. A poly(A) RNA of 2 μg was loaded on each lane.

[37]. methyl jasmonate (MeJA) and salicylic acid (SA), signaling compounds [115, 116], arachidonic acid (AA), an elicitor of Solanaceae plants [105]. CYP81B2 mRNA was found to be accumulated by the treatment of 150 μM 2,4-D, but not with 200 μM SA, 20 μM NA, 100 μM AA and 30 μM MeJA (data not shown). On the other hand, CYP71A11 mRNA was induced by the treatment of all five chemicals examined, particularly with NA and SA (Fig. 5-8). The mRNA level was extremely high 2h after treatment with NA and SA. These results suggested that CYP71A11 was inducible by treatment with not only xenobiotics but also signaling compounds.

Expression of CYP81B2 and CYP71A11 cDNAs in *Saccharomyces cerevisiae*

Since CYP81B2 and CYP71A11 which were induced by 2,4-D treatment in S401 cells, both cDNA clones were each examined for expression in the yeast. Six recombinant plasmids were constructed for the expression of CYP81B2 and CYP71A11 cDNAs as shown in Fig. 5-9. pATCP1 and pATCP2 carried the coding regions of CYP81B2 and CYP71A11 cDNA clones, respectively, between ADH promoter and terminator of the expression vector pAAH5N. pATCP1TR and pATCP2TR, and pATCP1YR and pATCP2YR were constructed for simultaneous expression of both the corresponding P450 species and P450 reductase by the introduction of the expression unit of tobacco P450 reductase cDNA described in Chapter III and of yeast P450 reductase gene [84], respectively. *S. cerevisiae* AH22 cells were transformed with each of the expression plasmids constructed.

Figure 5-10 showed the reduced CO difference spectra of the microsomal fractions of AH22/pATCP1 and AH22/pATCP2. Both showed the peak at 449 nm and 448 nm, respectively. The P450 content in AH22/pATCP1 and AH22/pATCP2 was 188 and 22 pmol per mg protein, respectively. The P450 content in AH22/pATCP1TR and AH22/pATCP1YR was 25 and 55 pmol per mg protein, respectively. The P450 content in AH22/pATCP2TR and AH22/pATCP2YR was 12 and 9 pmol per mg protein, respectively (Table 5-1). These results suggested that CYP81B2 and CYP71A11 were each localized in the yeast microsomes. The content of CYP81B2 was higher than that of CYP71A11. Since in CYP71A11,

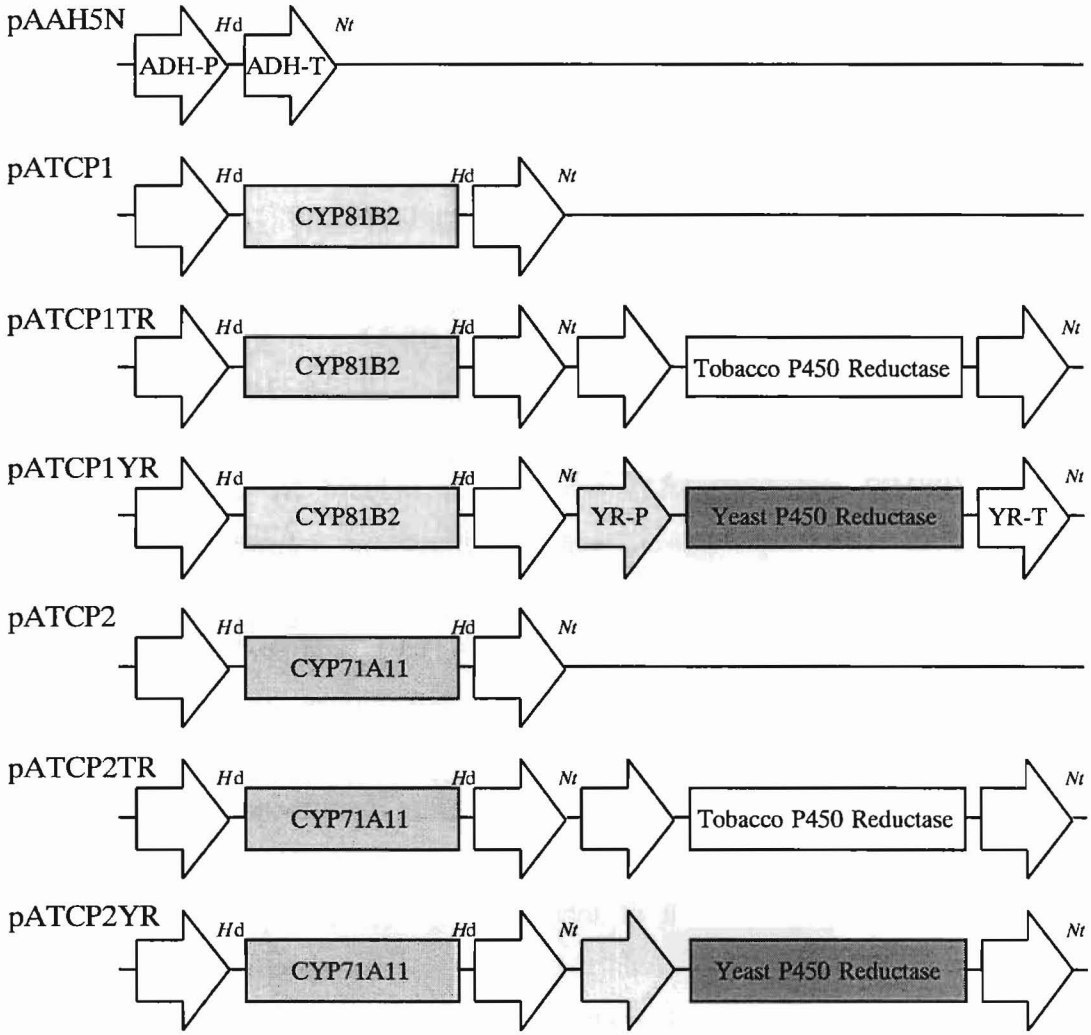

Fig. 5-9. Structures of the recombinant plasmids for expression of CYP81B2 and CYP71A11 in *Saccharomyces cerevisiae* AH22 cells. pAAH5N contains alcohol dehydrogenase I promoter (ADH-P) and the terminator (ADH-T). pATCP1 and pATCP2 had each of CYP81B2 and CYP71A11 coding regions between the promoter and terminator, respectively. pTCP1TR and pTCP2TR were constructed for co-expression by introducing the expression unit of tobacco P450 reductase cDNA. pATCP1YR and pATCP2YR were constructed for co-expression by introducing the expression unit of yeast P450 reductase gene. *Hd* and *Nt* indicate *Hin*dIII and *Not*I sites, respectively.

the peak at 420 nm was also found with 448 nm, the peak at 448 nm may be changed to 420 nm, immediately during preparation of the microsomes. Therefore, CYP71A11 enzyme expressed in the yeast may be less stable in the microsomes as compared with CYP81B2. Table 5-2 showed the activity of 7-ethoxycoumarin *O*-deethylase (ECOD) in the microsomal fractions of the recombinant yeast strains. The activity in AH22/pATCP2 was lower than the detection limit. On the other hand, the ECOD activity was enhanced in the strains of AH22/pATCP1TR, AH22/pATCP1YR, AH22/pATCP2TR and AH22/pATCP2YR. These results suggested that both CYP81B2 and CYP71A11 expressed in the yeast microsomes showed the P450 dependent monooxygenase activity. In addition, co-expression of the corresponding P450 species and yeast or tobacco P450 reductase enhanced the monooxygenase activity.

Chlortoluron Metabolism in Recombinant Yeast Strains

Metabolism of [^{14}C]chlortoluron was examined in the recombinant yeast strains. After incubation of [^{14}C]chlortoluron with each of the microsome preparations, the parent compound and its metabolites were extracted and subjected to TLC analysis. Ring-methyl hydroxylated chlortoluron was found to be increased in the microsomal fraction of AH22/pATCP1TR (Fig. 5-11 (A)) as compared with the control strain AH22/pAAH5N. The production of ring-methyl hydroxylated chlortoluron was dependent on incubation time in the the whole cells (Fig. 5-11 (B)). Therefore, it was suggested that CYP81B2 expressed in the yeast metabolized chlortoluron through ring-methyl hydroxylation. In the microsomal fraction of AH22/pATCP2YR, there were no significant differences between incubated and no incubated samples (Fig. 5-11 (A)). However, in the whole cells, *N*-demethylated and ring-methyl hydroxylated chlortolurons were found to be increased dependent on incubation time, although *N*-demethylated chlortoluron was produced predominantly (Fig. 5-11 (B)). These results suggested that CYP71A11 expressed in the yeast metabolized chlortoluron through *N*-demethylation and ring-methyl hydroxylation *in vivo,* but the metabolites were not detected in the microsomes because CYP71A11 enzyme was unstable during the preparation of the microsomes as shown in Fig.5-10.

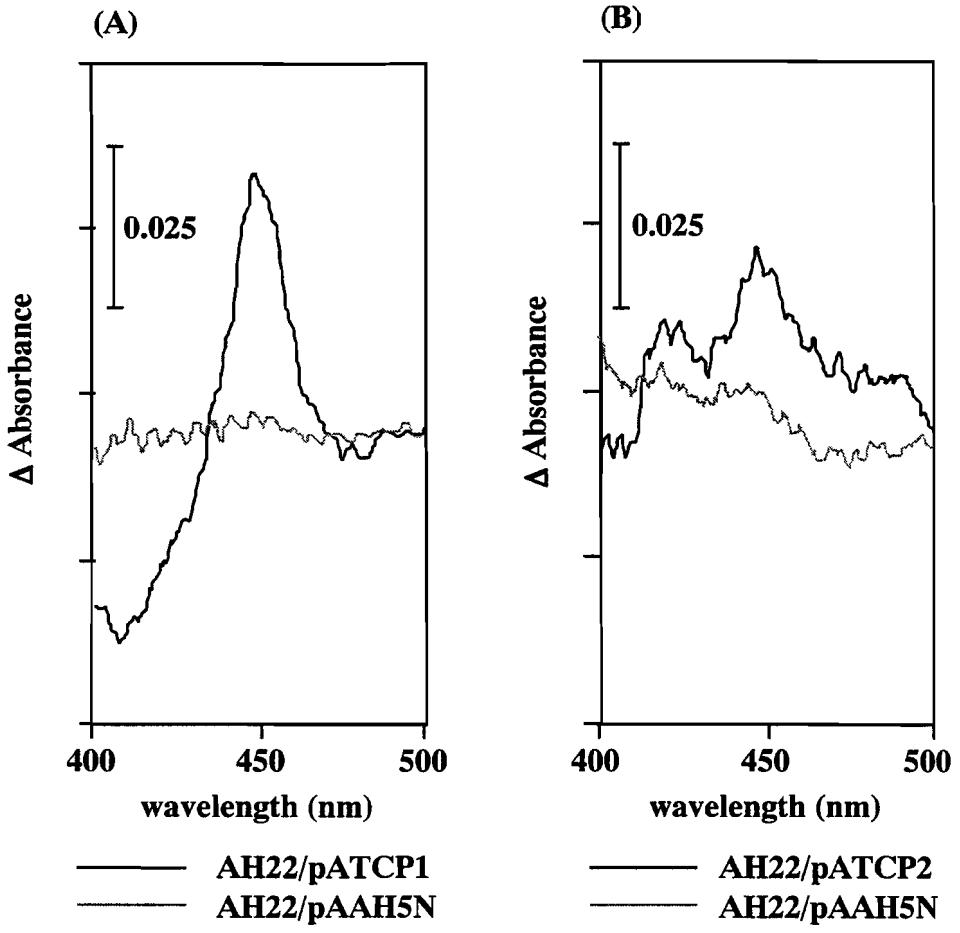

Fig. 5-10. The reduced CO-difference spectra of the microsomal fractions prepared from AH22/pATCP1 (A), AH22/pATCP2 (B) and AH22/pAAH5N (A and B). The microsomal fractions of 2 mg (A) and 6 mg (B) was suspended in 100 mM potassium phosphate buffer (pH 7.4) and bubbled with CO for 30 sec. After addition of solid $Na_2S_3O_4$, spectra were traced.

Table 5-1 P450 content and cytochrome c reductase activity in the microsomal fractions of the recombinant yeast strains

Yeast strain	P450 content (pmol/min/mg protein)	Cytochrome c reductase activity (nmol/min/mg protein)
AH22/pAAH5N	<1	83
AH22/pATCP1	188	60
AH22/pATCP1TR	25	378
AH22/pATCP1YR	55	295
AH22/pATCP2	22	51
AH22/pATCP2TR	12	187
AH22pATCP2YR	9	121

Table 5-2 7-Ethoxycoumarin *O*-deethylase activities in the microsomal fractions of the recombinant yeast strains

Yeast strain	7-Ethoxycoumarin *O*-deethylase activity (pmol/min/nmol P450)
AH22/pAAH5N	<1
AH22/pATCP1	24
AH22/pATCP1TR	113
AH22/pATCP1YR	92
AH22/pATCP2	<1
AH22/pATCP2TR	25
AH22/pATCP2YR	97

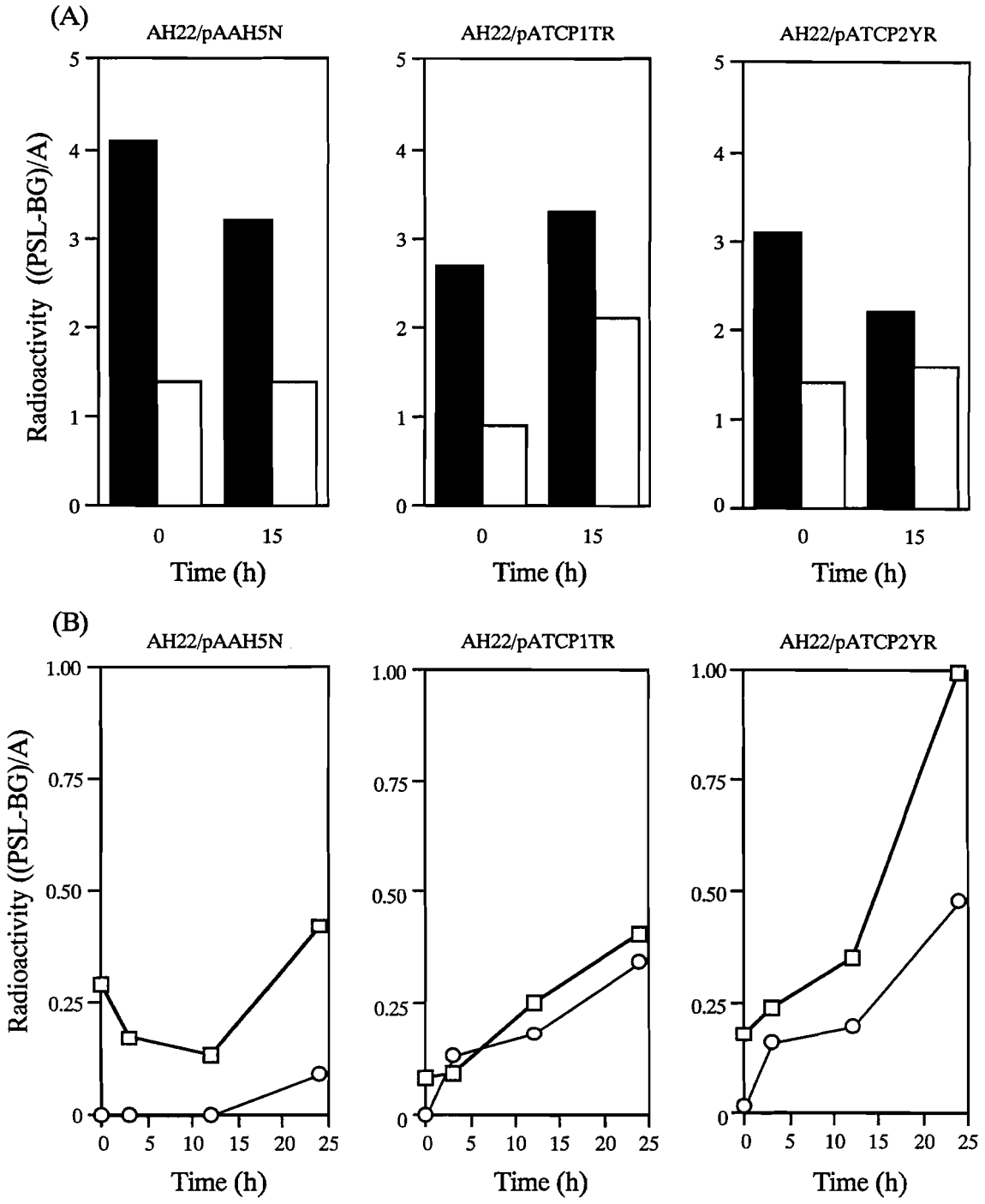

Fig. 5-11. Chlortoluron metabolism in the microsomal fractions (A) and in the cells (B) of recombinant yeast strains AH22/pAAH5N, AH22/pATCP1TR and AH22/pATCP2YR. Metabolites of [^{14}C]chlortoluron were applied to silica gel thin layer plates, and developed by the solvent system of hexane / ethylacetate (2 / 1). Closed box and open box in (A), and square and circle in (B) indicate *N*-demethylated chlortoluron and ring-methyl hydroxylated chlortoluron, respectively.

DISCUSSION

It was known that herbicide chlortoluron was metabolized through *N*-demethylation in tobacco plants [117]. However, chlortoluron was also metabolized through ring-methyl hydroxylation and *N*-demethylation in the microsomes of tobacco S401 cells treated with 2,4-D. The present study revealed that two P450 species related to chlortoluron metabolism were identified as CYP81B2 and CYP71A11. CYP81B2 expressed in the yeast metabolized chlortoluron through ring-methyl hydroxylation. On the other hand, CYP71A11 expressed in the yeast metabolized chlortoluron through ring-methyl hydroxylation and *N*-demethylation. The activities in chlortoluron metabolism of CYP81B2 and CYP71A11 were lower than those of rat CYP1A1 (data not shown). Recently, it was reported that Jerusalem artichoke CYP76B1, which is strongly induced by various chemical treatments, metabolized chlortoluron through ring-methyl hydroxylation [118]. In addition, Jerusalem artichoke CYP73A1 expressed in the yeast showed chlortoluron ring-methyl hydroxylation activity slightly [119]. Therefore, tobacco P450 species CYP81B2 and CYP71A11 appeared to be homologous to CYP76B1 and CYP73A1 in the metabolism of chlortoluron.

The monocot crop plants wheat and barley were highly active in ring-methyl hydroxylase activity of chlortoluron [110]. It is important to know whether P450 species with a higher specific activity are present or the P450 enzymes such as CYP81B2 and CYP71A11 are highly expressed in the monocot plants as compared with the dicot plants. Characterization of P450 species involved in chlortoluron metabolism in monocot plants and comparison of the characteristics of P450 species in dicot plants appeared to be important for understanding the mechanism of herbicide selectivity and resistance.

In the nucleotide sequence of 5' region of CYP81B2 gene, was cis-element homologous to that found in flavonoid biosynthesis genes were present within 0.4 kbp from the putative translation start sites. The primary structure of CYP81B2 was similar to those of P450 species related to phenylpropanoids, lignins and flavonoids. Therefore, it is possible to suppose that CYP81B2 may be involved in the biosynthesis of phenylpropanoids, lignins or flavonoids. It was reported that

more than 15 plant P450 species were known to be involved in the biosynthetic pathways [120]. CYP71A11 gene was induced within 2 h by NA, MeJA, SA and AA. Therefore, CYP71A11 would be related to the P450 species in the biosynthesis of a signal molecule which induce defense related genes or in the biosynthesis of an antimicrobial compound in early step.

P450 species in higher plants are supposed to have evolved to generate biosynthetic pathways to produce a variety of secondary metabolites through co-evolution between plants and animals, or plants and pathogens [7]. Plant P450 species have been thought to have a narrow substrate specificity as compared with mammalian P450 species related to xenobiotic metabolism. However, CYP81B2 and CYP71A11 metabolized both 7-ethoxycoumarin and chlortoluron. Such wide specificity of the P450 species may contribute to self defense against xenobiotics including herbicides and to adaptation to environmental pollution.

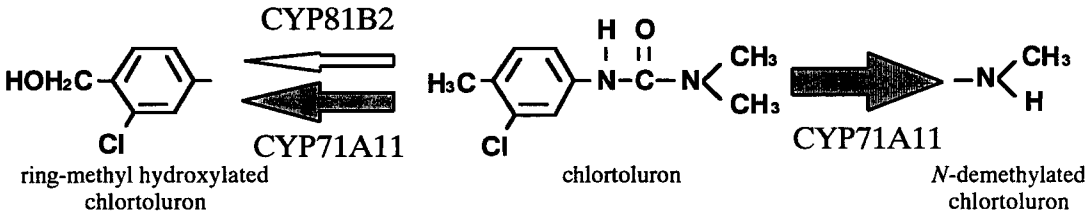

Fig. 5-12. Proposed metabolism of chlortoluron by CYP81B2 and CYP71A11 in tobacco S401 cells. Chlortoluron was metabolized through ring-methyl hydroxylation by CYP81B2 and through ring-methyl hydroxylation and *N*-demethylation by CYP71A11, respectively.

CHAPTER VI

CONCLUDING REMARKS

Cytochrome P450 monooxygenases participate in oxidative reactions related to secondary metabolism as well as xenobiotic metabolism in higher plants. This enzyme system is localized in the microsomes of plant cells, and consists of many cytochrome P450 (P450) species and a few species of NADPH-cytochrome P450 oxidoreductase (P450 reductase). Biochemical studies on plant P450 species are difficult because of low content, instability as well as interference with pigments and phenolic compounds. Therefore, I attempted to isolate cDNAs encoding P450 reductase as well as novel P450 species related to secondary metabolism and xenobiotic metabolism, to determine the nucleotide sequences and to express heterologously for characterization of enzyme functions as described in chapter I.

In chapter II, P450 reductase was purified from tobacco cultured cells BY2 and characterized the enzyme function. An apparent molecular mass of the protein of purified tobacco P450 reductase was about 79 kDa. The purified enzyme showed a typical flavoprotein redox spectrum. An *in vitro* reconstitution system of the purified tobacco P450 reductase with a partially purified tobacco P450 preparations showed the activity of cinnamic acid 4-hydroxylase. Polyclonal antibodies raised against the purified tobacco P450 reductase reacted with tobacco P450 reductase but not with yeast P450 reductase. The anti-tobacco P450 reductase antibody totally inhibited the activity of tobacco P450 reductase but not that of yeast P450 reductase. Based on these results, tobacco P450 reductase was identified biochemically and immunochemically.

In chapter III, cDNA encoding tobacco P450 reductase was isolated and the primary structure was deduced from the nucleotide sequence. The conserved sequences corresponding to FMN, FAD and NADPH binding domains were found in tobacco P450 reductase. Tobacco P450 reductase cDNA was expressed in the yeast *Saccharomyces cerevisiae*. The protein band of tobacco P450 reductase was found and cytochrome c reductase activity was enhanced in the microsomal fraction of the recombinant yeast cells. The *in vitro* reconstitution system with the microsomal fraction expressing rat P4501A1 showed an enhanced activity of 7-ethoxycoumarin *O*-deethylase. Based on these results, the primary structure and enzymatic function of tobacco P450 reductase were clarified.

In chapter IV, cDNA for 5-*epi*-aristolochene 3-hydroxylase which was involved in the biosynthesis of capsidiol was isolated from green pepper. The deduced amino acid sequence showed high similarity with those of plant catalases reported. It is possible to postulate that 5-*epi*-aristolochene is oxidized to produce capsidiol in the presence of O_2 produced by catalase. Based on these results, the primary structure of the enzyme which was supposed to participate in the biosynthesis of capsidiol was determined.

In chapter V, 2,4-D inducible P450 species in tobacco S401 cells were characterized. Four P450 cDNAs were isolated from the cDNA library of 2,4-D treated tobacco S401 cells, which were named as CYP71A11, CYP81B2, CYP81C1 and CYP81C2, respectively. The cDNAs of CYP71A11 and CYP81B2, which were strongly induced by 2,4-D were each expressed in the yeast for characterization of the enzyme functions. It was found that CYP81B2 and CYP71A11 expressed in the yeast metabolized the herbicide chlortoluron through ring-methyl hydroxylation and through both ring-methyl hydroxylation and *N*-demethylation, respectively. Based on these results, novel P450 species involved in xenobiotic metabolism in higher plants were identified.

In the present study, the structures and functions of novel cytochrome P450 species and P450 reductase in tobacco plants were clarified by genetic engineering approach. These results may contribute to understand molecular mechanisms on plant defense mechanism as well as herbicide selectivity and resistance. In addition, the cDNA clones for novel P450 species and P450 reductase are useful for genetic engineering of crop plants on herbicide resistance as well as phytoremediation.

SUMMARY

Cytochrome P450 monooxygenases consisting of a number of cytochrome P450 species and generic NADPH-cytochrome P450 oxidoreductase (P450 reductase) (EC 1.6.2.4) play important role in oxidative reactions for the biosynthesis of secondary metabolism as well as metabolism of xenobiotics in higher plants. Tobacco (*Nicotiana tabacum*) BY2 cells were used for the purification of P450 reductase which localized in the microsomal fraction. The microsomes were solubilized and subjected to chromatography on two anion-exchange columns and 2',5'-ADP-Sepharose 4B column. The purified enzyme showed a single protein band with a molecular weight of 79 kDa on SDS-PAGE and exhibited a typical flavoprotein redox spectrum, indicating the presence of an equimolar quantity of FAD and FMN. The enzyme followed Michaelis-Menten Kinetics with K_m values of 24 μM for NADPH and 16 μM for cytochrome c. An *in vitro* reconstituted system of the purified reductase with a partially purified tobacco cytochrome P450 preparation showed the cinnamic acid 4-hydroxylase activity at the rate of 14 pmol min^{-1} $nmol^{-1}$ P450 protein and with a purified rabbit P4502C14 catalyzed *N*-demethylation of aminopyrine at the rate of 6 pmol min^{-1} $nmol^{-1}$ P450 protein. Polyclonal antibodies raised against the purified reductase reacted with tobacco reductase but not with yeast reductase on Western blot analysis. Anti-yeast reductase antibodies did not react with the tobacco reductase. This result indicate that the tobacco reductase was immunochemically different from the yeast reductase. The anti-tobacco reductase antibodies totally inhibited the tobacco reductase activity, but not the yeast reductase. Also, Western blot analysis using the anti-tobacco reductase antibodies revealed that leaves, roots and shoots of *Nicotiana tabacum* plants contained an equal amount of the reductase protein. From these results, it was suggested that there are different antibody binding sites, which certainly participate in enzyme activity, between tobacco and yeast reductase.

Tobacco P450 reductase cDNA was isolated from a cDNA library of tobacco plants by the use of a DNA fragment corresponding to the FMN binding region of tobacco P450 reductase as a probe DNA. A full length information on tobacco P450 reductase cDNA was clarified by the combination of the cDNA clone pCTR1 and the genomic DNA clone pGTR1. The nucleotide sequence combined

pCTR1 with pGTR1 contained an open reading frame of 2142 bp encoding a protein of 713 amino acid residues with the molecular weight of 78531.7 daltons. The deduced amino acid sequence contained the sequence determined with the purified P450 reductase from tobacco BY2 cells. Also, the primary structure contained the sequence corresponding to FMN, FAD and NADPH binding regions. Based on the information, I also obtained the tobacco P450 reductase cDNA pFTR by RT-PCR. The cDNA was expressed in the yeast *Saccharomyces cerevisiae* under the control of yeast alcohol dehydrogenase I (ADH) promoter and terminator. The transformed yeast cells carrying pFTR produced the corresponding mRNA and protein, and showed an enhanced cytochrome c reductase activity in the microsomes. An *in vitro* reconstitution system of the yeast microsomal fractions expressed tobacco P450 reductase and rat P4501A1 showed an enhanced 7-ethoxycoumarin *O*-deethylase activity. These results suggested that tobacco P450 reductase expressed in the yeast microsomes coupled with rat P4501A1 in the yeast microsomes, enhancing the monooxygenase activity.

A catalase cDNA was isolated from green pepper seedlings elicited with arachidonic acid based on the amino acid sequences of the purified protein. The nucleotide sequence of the isolated cDNA contained a single open reading frame predicted to encode 492 amino acid residues with a calculated molecular weight of 56439.0 daltons. The deduced amino acid sequence contained the amino acid sequences determined by sequencing of the peptides. The total deduced amino acid sequence showed high similarity with those of the other plant catalases reported so far, and was found to possess the peroxisomal targeting sequence conserved among plant catalases. Transcription of the catalase gene in green pepper seedlings was found to be induced by the treatment of arachidonic acid.

Novel P450 species related to herbicide metabolism in higher plants were clarified by genetic engineering. P450 cDNAs were isolated from tobacco cultured S401 cells. The herbicide chlortoluron was metabolized through *N*-demethylation and ring-methyl hydroxylation by P450 monooxygenases in 2,4-D treated tobacco S401 cells. Four 2,4-D inducible P450 cDNAs were isolated by the use of RT-PCR and screening of cDNA library prepared from 2,4-D treated tobacco S401 cells. Isolated P450 species were sequenced. Based on the deduced amino acid

sequences, four clones were named as CYP71A11, CYP81B2, CYP81C1 and CYP81C2, respectively. The coding regions of CYP81B2 and CYP71A11 were each expressed in the yeast *Saccharomyces cerevisiae*. CYP81B2 expressed in the yeast showed chlortoluron ring-methyl hydroxylase activity. CYP71A11 metabolized chlortoluron through ring-methyl hydroxylation and *N*-demethylation. These results suggested that CYP81B2 and CYP71A11 participate in chlortoluron metabolism in tobacco S401 cells. Based on these results, cytochrome P450 monooxygenases consisting of P450 species and P450 reductase in tobacco plants were clarified. These information are useful for understanding molecular mechanisms of plant defense mechanism as well as herbicide selectivity and resistance.

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