Expression of Human Cytochromes P450 1A1 and P450 1A2 as Fused Enzymes with Yeast NADPH-cytochrome P450 Oxidoreductase in Transgenic Tobacco Plants

Noriaki SHIOTA,¹ Susumu KODAMA, Hideyuki INUI, and Hideo OHKAWA²

Department of Biological and Environmental Science, Faculty of Agriculture, Kobe University, Rokkodai-cho 1-1, Nada, Kobe 657-8501, Japan

Received August 16, 1999; Accepted June 15, 2000

Among 11 isoforms of the human cytochrome P450 enzymes metabolizing xenobiotics, CYP 1A1 and CYP 1A2 were major P450 species in the metabolism of the herbicides chlortoluron and atrazine in a yeast expression system. CYP1A2 was more active in the metabolism of both herbicides than CYP1A1. The fused enzymes of CYP1A1 and CYP1A2 with yeast NADPH-cytochrome P450 oxidoreductase were functionally active in the microsomal fraction of the yeast Saccharomyces cerevisiae and showed increased specific activity towards 7-ethoxyresorufin as compared to CYP1A1 and CYP1A2 alone. Then, both fused enzymes were each expressed in the microsomes of tobacco (Nicotiana tabacum cv. Samsun NN) plants. The transgenic plants expressing the CYP1A2 fusion enzyme had higher resistance to the herbicide chlortoluron than the plants expressing the CYP1A1 fusion enzyme did. The transgenic plants expressing the CYP1A2 fusion enzyme metabolized chlortoluron to a larger extent to its non-phytotoxic metabolites through N-demethylation and ring-methyl hydroxylation as compared to the plants expressing the CYP1A1 fused enzyme. Thus, the possibility of increasing the herbicide resistance in the transgenic plants by the selection of P450 species and the fusion with P450 reductase is discussed.

Key words: human cytochrome P450; P450 oxidoreductase; transgenic tobacco plants; herbicide resistance

Cytochrome P450 monooxygenases are recognized as the most versatile enzymes that catalyze the oxidation of a wide variety of endogenous and exogenous lipophilic compounds.¹ They are important in the metabolism of many chemicals including xenobiotics, drugs, and environmental pollutants. The catalytic versatility of cytochrome P450 enzymes is due to the presence of a heme cofactor and a number of reversible monooxygenases involved in the oxidation of a variety of substrates.

Proteins such as NADPH-cytochrome P450 reductase provide electrons for the catalytic sites of cytochrome P450 enzymes. The specificity of cytochrome P450 enzymes in the metabolism of xenobiotics is determined by the enzyme's ability to recognize a specific substrate and catalyze the specific oxidative reaction.

The use of transgenic plants as model systems for studying the role of cytochrome P450 enzymes in herbicide metabolism has been reported. The introduction of cytochrome P450 enzymes into plants can lead to increased resistance to certain herbicides. The use of transgenic plants as a model system for studying the role of cytochrome P450 enzymes in herbicide metabolism has been reported.

In this study, we have shown that the introduction of cytochrome P450 enzymes into transgenic plants can lead to increased resistance to certain herbicides. This suggests that the use of transgenic plants as a model system for studying the role of cytochrome P450 enzymes in herbicide metabolism has potential for application in the development of herbicide resistance in crops.
herbicides with different structures, and CYP1A2 was more active than CYP1A1 towards the herbicide chlortoluuron and atrazine. Therefore, we expressed both CYP1A1 and CYP1A2 as fused enzymes with yeast P450 reductase in the yeast *Saccharomyces cerevisiae* and transgenic tobacco plants, and examined the metabolism of chlortoluuron as well as resistance to the herbicide.

**Materials and Methods**

*Chemicals and biochemicals.* DNA modifying enzymes and a *XhoI* linker DNA, d(CCTCGAGG), were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). 7-Ethoxyresorufin was purchased from Sigma Co. (St. Louis, MO). [phenyl-U-14C]Chlortoluuron (sp. act. 2.99 M bq/mg) as well as its reference metabolite compounds, 3-(3-chloro-p-tolyl)-1-methylurea (designated as DM), 3-chloro-p-tolurea (DDM), 3-[(3-chloro-4-hydroxymethylphenyl)-1,1-dimethylurea (OH), 3-[(3-chloro-4-hydroxymethylphenyl)-1-methylurea (DMOH), 3-[(3-chloro-4-carboxyphenyl)-1,1-dimethylurea (COOH), and [triazinyl-14C]atrazine were gifts from Novartis International Inc. (Basel, Switzerland).

*Construction of expression plasmids.* Recombinant DNA procedures were done by the standard methods.26 PCR was done with *Pfu* polymerase (Stratagene, La Jolla, CA) under the conditions recommended by the supplier. Human CYP1A1 cDNA was obtained by PCR amplification from a human liver cDNA library (Clontech, Palo Alto, CA) with the oligonucleotides, AAGCTTTCGCAACTGCTTTTCCCACCATGGG, designated as a 5' primer and GGCTCTCAAGCAATTCGCTTC, designated as a 3' primer. The primers were used essentially based on the sequences reported previously.17 Those primers contained some modifications in the sequence of 5' and 3' non-coding regions to incorporate not only appropriate restriction sites but also the consensus sequence of plant mRNAs surrounding the AUG initiation codon to increase translational efficiency.18 The cDNA fragment was sequenced in a dideoxy method to confirm its identity with that of the native CYP1A1 cDNA.19 The C-terminal region of CYP1A1 cDNA was modified by insertion of an *XhoI* linker into a *PvuII* site just before the TAG stop codon to optimize for fusion with yeast P450 reductase. Following digestion with *HindIII* and *XhoI*, the HindIII-XhoI fragment encoding a CYP1A1 domain was inserted simultaneously with the XhoI-HindIII fragment encoding a yeast reductase domain excised from pAFCR130 into the unique HindIII site of the yeast expression vector pAAHSN31 to yield the plasmid pYHFA.

Professor R. H. Tukey of the University of California, San Diego,22 kindly provided the human CYP1A2 cDNA subcloned into a pBluescript vector (clone λHP4A). The cDNA clone was mutated with PCR with the oligonucleotides GCTTCGATAAACATGAC as a 5' primer and TGGTCTTCCTGAGTATGGAG as a 3' primer, in which its C-terminal one amino acid residue was deleted and a *XhoI* site was incorporated. The expression plasmid pAF1A2 for the CYP1A2/P450 reductase fused enzyme was constructed in the same way as with the CYP1A1 plasmid.

The plant expression vector pUTRI121H originated from pBI121, in which the cDNA sequence of a 5' untranslated leader sequence from the coat protein mRNA of alfalfa mosaic virus (AMV RNA4)23 was just downstream of the CaMV 35S promoter. Finally, the plant expression plasmids pHF1A1 for CYP1A1/P450 reductase fused enzyme and pHF1A2 for CYP1A2/P450 reductase fused enzyme were constructed by insertion of each of the genes between the AMV RNA4 leader sequence and the nopaline synthase gene terminator. The structure of these expression plasmids thus constructed was confirmed by restriction enzyme mapping.

The resulting expression plasmids were used for the transformation of the yeast *Saccharomyces cerevisiae* strain AH224 and the tobacco strain *Nicotiana tabacum* cv. Samsun NN.25 Primary transformed tobacco plants (R0 plants) were used for further analyses.

*Preparation of microsomal fractions.* Recombinant yeast strains expressing each of 11 human P450 species (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) were obtained from Sumitomo Chemical Co., Ltd. Osaka, Japan, and were cultivated in a concentrated SD medium.24 A microsomal fraction of the yeast was prepared as described previously.8 A microsomal fraction of tobacco plants was prepared as described previously2 with slight modifications. Potassium phosphate buffer (0.1 M, pH 7.4) containing 20% (v/v) glycerol, 0.5 M mannitol, 5 mM EDTA, 2 mM EGTA, 50 mM ascorbate, 2.5 mM dithiothreitol, and 0.5 mM PMSF was used as the extraction buffer. After centrifugation at 10,000 g, the resulting supernatant was mixed with MgCl2 to a final concentration of 7 mM and then stirred for 30 min at 4°C. After centrifugation for 20 min at 4,000 g, the resulting pellet was resuspended in a starting volume of extraction buffer and then ultracentrifuged for 60 min at 100,000 g. The pellet fraction was stored as described previously.12

*Enzyme assay.* 7-Ethoxyresorufin O-deethylation activity was measured at 37°C in a cuvette by monitoring the formation of resorufin essentially as described by Burke and Mayer.29 The activity was evaluated from the initial increase in fluorescence (ex-
citation 550 nm, emission 586 nm) with a Hitachi F-3010 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

Other methods and assays. Protein concentrations were measured with a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) with bovine gamma globulin as the standard. The concentration of P450 hemoproteins was measured by a reduced CO-difference spectrum with a Hitachi U-3000P spectrophotometer.\(^{20}\) Immunoblotting was done as described previously.\(^ {12}\) Metabolism of \([\text{phenyl-U-}\)\(^{14}\)C]\ch{Cl}\ch{H}lortoluron and \([\text{ triazinyl-14}^{14}\)C]\atrazine\ was examined in the yeast microsomal fractions expressing either human CYP1A1 or CYP1A2 using the method with tobacco microsomes.\(^ {10}\) Phenotyping test of the herbicide \ch{Cl}\ch{H}lortoluron and metabolism of \([\text{phenyl-U-}\)\(^{14}\)C]\ch{Cl}\ch{H}lortoluron in intact tobacco plants were examined as described previously.\(^ {14}\)

Results

Herbicide metabolism in the microsome of the recombinant yeast expressing human P450 species

The microsomes of the recombinant yeast strains expressing each of 11 human P450 species including CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4 were used for the metabolism of more than 30 different herbicides. Among these P450 species, both CYP1A1 and CYP1A2 were found to metabolize \ch{Cl}\ch{H}lortoluron and \atrazine (Table 1). Particularly, CYP1A2 was more active than CYP1A1 towards both herbicides. The other herbicides examined were slightly or not metabolized by both P450 species. CYP1A1 predominantly catalyzed ring-methyl hydroxylation of \ch{Cl}\ch{H}lortoluron and additionally its N-demethylation. CYP1A2 showed similar activities of both ring-methyl hydroxylation and N-demethylation. As results, both CYP1A1 and CYP1A2 converted more than 90% of the \ch{Cl}\ch{H}lortoluron added to its oxidation products. CYP1A1 and CYP1A2 also metabolized \atrazine, both catalyzing its N-dealkylation. About 60% of the \atrazine added were converted to its metabolites by CYP1A2, while the activity of CYP1A1 was low. These findings indicated that human CYP1A1 and CYP1A2 metabolized the herbicides \ch{Cl}\ch{H}lortoluron and \atrazine, although CYP1A2 was more active than CYP1A1 towards both herbicides.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (nmol/min/nmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chlorotoluron</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>3.1</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Activity was expressed as the rate of substrate consumed. The activity was measured in a 0.1 M sodium phosphate buffer (pH 7.4) containing 50 \(\mu\)M either of the herbicides, yeast microsome (50 pmol equivalent of P450 per 100 \(\mu\)l) and 0.5 mM NADPH. Data are the means of three individual measurements.

Construction of the expression plasmids for the chimeric fused enzymes of CYP1A1/P450 reductase and CYP1A2/P450 reductase

The expression plasmids for the chimeric fused enzymes of human CYP1A1/yeast P450 reductase and human CYP1A2/yeast P450 reductase were constructed as shown in Fig. 1. Each of the fused enzyme genes was produced by engineering the coding sequence for the corresponding P450 species on the placement at their 5' end, being fused in-frame to that for the N-terminal truncated yeast P450 reductase gene according to the rules summarized previously.\(^ {20}\) The linker sequence encoded Pro-Arg-Ala at the junction between the CYP1A1 domain lacking the C-terminal three amino acid residues and the yeast P450 reductase domain lacking the N-terminal 41 amino acid residues. The CYP1A2 construct encoded Thr-Arg at the junction between the CYP1A2 domain lacking the C-terminal one amino acid.

Fig. 1. The Structure of the Expression Plasmids for Human CYP1A1 and CYP1A2 Each Fused with Yeast P450 Reductase.
(A) The plasmids to be expressed in the recombinant yeast are represented. CYP1A1, CYP1A2, and YR represent the coding regions for human CYP1A1, human CYP1A2, and yeast P450 reductase, respectively. AP and AT represent yeast ADH I promoter and its terminator, respectively, P, R, A, and T indicate amino acid residues derived from the synthesized DNA linker. Numbers below the coding regions indicate the number of amino acid residues counted from the amino terminus of the corresponding enzymes. (B) The plasmids to be expressed in the transgenic tobacco plants were represented. NPT II represents the coding region for neomycin phosphotransferase II. NP, NT, 35SP and UTR indicate nopaline synthase gene promoter, its terminator, CaMV 35S promoter and untranslated leader sequence of AMV RNA4, respectively.
residue and the P450 reductase domain. Finally, the yeast expression plasmids pYHFAR and pAF1A2 were constructed by the use of the pAAH5N vector (Fig. 1A). The plant expression plasmids pHF1A1 and pHF1A2 were also constructed using the pUTR12I1H vector (Fig. 1B).

**Enzyme assay for the fused enzymes expressed in the recombinant yeast**

Both CYP1A1/yeast P450 reductase and CYP1A2/yeast P450 reductase fused enzymes were expressed in the yeast to confirm their enzymatic activities. Immunoblotting of the microsomal fractions prepared from the recombinant yeast cells indicated that both were mainly produced in the microsomes, and the fusion proteins migrating points agreed well with the molecular mass (about 130 kDa) deduced from their cDNA structures (data not shown). The reduced CO-difference spectra of the yeast microsomes showed a strong differential absorption peak at 447 for CYP1A1/P450 reductase fusion or at 448 nm for CYP1A2/P450 reductase fusion, indicating the presence of a hemoprotein in the microsomes. The spectrally detectable P450 content was approximately 14 and 48 pmol/mg protein for CYP1A1/P450 reductase and CYP1A2/P450 reductase fusion proteins, respectively (Table 2). The expression level of CYP1A2/P450 reductase was higher than that of CYP1A2 alone, although the expression level of CYP1A1 was faintly higher than that of CYP1A2. The enzymatic activity of the fused enzymes was also examined in the microsomes of each of the yeast strains. 7-Ethoxyresorufin is known to be a specific substrate for both CYP1A1 and CYP1A2. The microsome of CYP1A2/P450 reductase fusion catalyzed its O-deethylation at a 3-fold higher turnover number than that of CYP1A2 alone (Table 2). A slight increase of the activity was also observed in the CYP1A1/reductase fusion as compared with that of CYP1A1. No detectable activity was found in the microsomes of the yeast transformed with the null vector pAAH5N. The results indicated that the enzyme activity of human CYP1A1 and CYP1A2 was considerably increased by the fusion with yeast P450 reductase, although the CYP1A2 fusion was slightly higher in the specific activity than that of the CYP1A1 fusion.

**Expression of the fused enzymes in the transgenic tobacco plants**

The expression of CYP1A1/P450 reductase and CYP1A2/P450 reductase fusions was examined in transgenic tobacco plants. Transformation was done by infection of leaf discs with Agrobacterium tumefaciens harboring each of the expression plasmids for the fused enzymes. Out of approximately 200 kanamycin-resistant plants transformed with each of the constructs, 18 independent plants with the CYP1A1/P450 reductase construct (designated as la1 plants) and 67 independent plants with the CYP1A2/P450 reductase construct (designated as la2 plants) were selected according to their resistance to the herbicide chlortoluron. Finally, each of two independent plants resistant to chlortoluron (la1-3 and la1-4 transformed with CYP1A1/P450 reductase construct, and la2-82 and la2-101 transformed with the CYP1A2/P450 reductase construct) were further analyzed.

Southern blot analysis demonstrated that the corresponding genes were each integrated into the genome of the transgenic tobacco plants (data not shown). The results also indicated that one copy of the corresponding gene was found in each la1-3 and la2-82 plant, while multiple copies of it were found in la1-4 and la2-101 plants. Northern blot analysis showed that a mRNA band was found in both la1 and la2 plants (data not shown). The size of the hybridized band in both plants was nearly the same as that of the mature transcript of the fused enzyme. The amount of the transcript was comparable among four of the transgenic plants.

Microsomal fractions prepared from both la1 and la2 plants as well as the control plants were analyzed by immunoblotting to detect the corresponding fusion proteins. A low but significant amount of the corresponding protein band at 130 kDa was detected in the microsomal fraction of both la1 and la2 plants, both of which were reactive with either anti-yeast reductase or anti-human P450 antibodies (Fig. 2). These protein bands detected in the transgenic plants each migrated to nearly the same position as the fused enzymes expressed in the recombi-

---

**Table 2. P450 Content and Monooxygenase Activity in the Recombinant Yeast Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme expressed</th>
<th>P450 content (pmol/mg protein)</th>
<th>Monooxygenase activity (nmol/min/nmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH22/pAAH5N</td>
<td>None</td>
<td>&lt;1.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AH22/pAAIA1R</td>
<td>CYP1A1</td>
<td>119.2</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>AH22/pAAIA2</td>
<td>CYP1A2</td>
<td>14.2</td>
<td>0.4 ± 0</td>
</tr>
<tr>
<td>AH22/pYHFAR</td>
<td>CYP1A1/YR</td>
<td>70.7</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>AH22/pAF1A2</td>
<td>CYP1A2/YR</td>
<td>47.5</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

7-Ethoxyresorufin O-deethylation activity was measured in a 0.1 M sodium phosphate buffer (pH 7.4) containing 10 μM 7-ethoxyresorufin, yeast microsome (50 pmol equivalent of P450 per 500 μL) and 0.5 mM NADPH. Data are the means of three individual measurements ± SE. YR, yeast P450 oxidoreductase.
Human Cytochrome P450 Expressed in Transgenic Tobacco Plants

Fig. 2. Immunoblot Analysis of the Microsomal Fractions Prepared from the Transgenic Tobacco Plants.
(A) Microsomal fractions from the transgenic plants of 1a1-3, 1a1-4, 1a2-82, 1a2-101 (lane 1-4, respectively), and the control plants (lane 5) as well as those from the recombinant yeast AH22/pAF1A2 (lane 6) and AH22/pYHFAR (lane 7) were analyzed using rabbit anti-yeast P450 reductase antibody. A hundred μg of the tobacco protein and yeast protein equivalent to 0.5 pmol of each P450 were put on per lane. (B) Microsomal fractions from the transgenic plants of 1a1-3, 1a1-4 (lane 1 and 2, respectively) and the control plants (lane 3) as well as those from the recombinant yeast AH22/pYHFAR (lane 4) were analyzed using rabbit anti-human CYP1A1 antibody. (C) Microsomal fractions from the transgenic plants of 1a2-82, 1a2-101 (lane 1 and 2, respectively), and the control plants (lane 3) as well as those from the recombinant yeast AH22/pAF1A2 (lane 4) were analyzed using rabbit anti-human CYP1A2 antibody.

Fig. 3. Monoxygenase Activity against 7-Ethoxyresorufin in the Microsomes of the Transgenic Tobacco Plants.
Reaction mixture (1 ml) contained 500 μM of NADPH, 1 mg of microsomal proteins in 0.1 M sodium phosphate buffer (pH 7.4). Reaction was started by addition of 5 μM 7-ethoxyresorufin to the cuvette. The initial rate of resorufin formation was expressed as turnover number/min/mg protein (TN).

binant yeast strains. No reactive bands around the same migrating point were detected in those of the control plants. Thus, the fused enzyme proteins produced seemed to be mainly localized in the microsomal fraction of the transgenic plants. Some of the bands with lower molecular masses were probably derived from either their degradation products or endogenous P450 reductase.

Monoxygenase activity was measured in the microsomal fractions of both transgenic and control plants. Since subcellular fractions prepared from higher plants might contain endogenous coumarin and other related compounds, 7-ethoxyresorufin rather than 7-ethoxycoumarin was used as a substrate for enzyme assay of the fused enzymes in the transgenic tobacco plants. When the microsomal fractions were incubated with the substrate 7-ethoxyresorufin in the presence of NADPH, increasing amounts of resorufin were produced in both of the transgenic plants (Fig. 3). On the other hand, only a low level of the product was found in the control plant. The microsomes of the 1a1-4 and the 1a2-82 plants showed approximately 7- and 9-fold higher activity than that of the control plant, respectively.

Herbicide resistance and metabolism in the transgenic tobacco plants
In a preliminary experiment, the tobacco plants transformed with each of the fused enzyme genes showed resistance to the herbicide chlortoluron at concentrations from 30 to 50 μM. We further examined the herbicide resistance of both 1a1 and 1a2...
plants grown on MS medium containing various concentrations of chlortoluron (Fig. 4). In nontransformed tobacco plants as well as the plants transformed with the vector pBI121, extensive growth inhibition with a slight chlorosis of the leaves was observed even at 10 μM. Strong inhibition on its growth was found at more than 20 μM of the herbicide. On the other hand, 1a1-4 and 1a2-101 plants showed higher resistance to chlortoluron at concentrations ranging from 10 to 50 μM. The 1a2-101 plant still survived with greenish leaves even at 100 μM although its growth was considerably inhibited. Thus, the 1a2-101 expressing the CYP1A2/yeast P450 reductase fusion was found to tolerate the herbicide better than the 1a1-4 expressing the CYP1A1 yeast reductase fusion.

Metabolism of [14C]chlortoluron was examined in whole plants of the control, 1a1-4, and 1a2-101 plants. TLC analysis showed that several oxidative metabolites of chlortoluron were formed in both transgenic and control plants (Fig. 5A).

Each of the plant metabolites was identified by the chromatography with the reference standards. Based on the results, chlortoluron was likely to be metabolized through both N-demethylation and ringmethyl hydroxylation in the transgenic plants, although it was done mainly through N-demethylation in the control plants. The transgenic plants showed higher metabolic activity against chlortoluron than the control plant did. Particularly, the 1a2-101 was more active than the 1a1-4, since the 1a2-101 produced more metabolites than the 1a1-4. In the control plant, approximately 30% of chlortoluron remained unchanged, with an equal amount of N-demethylated chlortoluron (DM) (Fig. 5B). The N-demethylated metabolite of chlortoluron was reported to be partially phytotoxic.27 On the other hand, the 1a1-4 plant contained less chlortoluron and the N-demethylated metabolite, and a larger the metabolites retained at the origin, which were likely to be conjugated metabolites derived from ringmethyl hydroxylated forms of chlortoluron, as compared with that of the control. The 1a1-4 plant accumulated the largest amounts of the conjugates among the three plants. Of interest was the observation that chlortoluron was extensively converted to its polar metabolites, including the 4-carboxyphenyl metabolite of chlortoluron (COOH) and the conjugates in the 1a2-101 plant. These findings clearly indicated that the transgenic tobacco plants expressing the fused enzymes of CYP1A1 and CYP1A2 showed resistance to chlortoluron in due to their increased ability to metabolize the herbicide. In addition, the plant expressing the CYP1A2/yeast P450 reductase fused enzyme was slightly more active in the metabolism of the herbicide than the plant expressing the CYP1A1 fusion and produced more metabolites at a different rate than that of the CYP1A1 fusion.

**Discussion**

In human livers, P450 species belonging to the gene families from CYP1 to CYP4 are generally thought to be responsible for the metabolism of xenobiotics. The expression of each of these P450 species in heterologous host cells ensures metabolic reactions of the P450 species towards various chemi-
cals including mutagens and carcinogens.\textsuperscript{28,29} We have tested the metabolism of structurally different herbicides in the recombinant yeast expressing each of 11 human P450 species. In a preliminary experiment, CYP1A1 and CYP1A2 showed much higher activity in the metabolism of chlortoluuron and atrazine than the other P450 species did (Table 1). The metabolism of the other chemicals including herbicides in 11 human P450 species will be reported elsewhere.\textsuperscript{30} Both enzymes catalyzed both ring-methyl hydroxylation and N-demethylation of chlortoluuron, and N-dealkylation of atrazine. Therefore, both CYP1A1 and CYP1A2 seemed to be important for the metabolism of these herbicides in human liver.

In the monoxygenase reactions catalyzed by P450 enzymes, electrons from reduced pyridine nucleotides are generally transferred to the P450 enzymes via electron-transfer proteins (\textit{i.e.} P450 reductase). The electron transfer to P450 is known to be one of the rate-limiting steps for the monoxygenase reaction. The construction of the chimeric fused enzyme between rat CYP1A1 and rat P450 reductase was first reported by Murakami \textit{et al.}\textsuperscript{31} The fusion enzyme showed a higher specific activity than the combination of the P450 and the P450 reductase \textit{in vitro}, probably due to a higher efficiency in the molecular coupling between the hemoprotein and the flavoprotein. A series of the fused enzymes reported so far had higher specific activities than those of the original ones. We have reported the expression of the rat P4501A1/yeast reductase fused enzyme in transgenic tobacco and potato plants. Both plants were resistant to the herbicide chlortoluuron. In this study, the fused enzymes of CYP1A1 and CYP1A2 with yeast P450 reductase were each functionally expressed in the recombinant yeast cells. The specific activity of CYP1A2 was increased by the fusion with yeast P450 reductase. On the other hand, only a small increase was found in its specific activity of the CYP1A1/P450 reductase fused enzyme. Sakaki \textit{et al.}\textsuperscript{20} reported that the fusion of the P450 domain with P450 reductase domain did not change its substrate-binding affinity when compared with that of P450 alone. Therefore, the increase in specific activity of the fused enzymes of CYP1A1 and CYP1A2 with yeast P450 reductase might be related to difference in conformation between the P450 domain and the P450 reductase domain, due to different primary and secondary structures in their hinge regions.

The expression level of the fused enzymes of CYP1A1 and CYP1A2 with yeast P450 reductase in the transgenic tobacco plants was estimated to be approximately two picomoles per mg microsomal protein as judging from immunoblotting analysis. This was at a similar level for the transgenic tobacco plants expressing rat CYP1A1/yeast P450 reductase fused enzyme.\textsuperscript{19} It remains unclear if such a low-level expression was due to inefficient translation of their mRNAs or instability of the fusion proteins arising from proteolytic degradation. It was reported that the rat CYP1A1/P450 reductase fused enzyme expressed in transgenic potato plants was less stable along with many of its degradation bands in transgenic potato plants, although CYP1A1 alone was highly expressed.\textsuperscript{19} The monoxygenase activity toward 7-ethoxyresorufin in the transgenic plants expressing the CYP1A1 and CYP1A2 fusion enzymes was significantly higher than that of the control plant.

In our present and previous studies, \textit{O}-deethylation against 7-ethoxycoumarin and 7-ethoxyresorufin was found in the control tobacco plants.\textsuperscript{13} It was reported that CYP71A11 in tobacco plants catalyzed \textit{O}-deethylation of 7-ethoxycoumarin.\textsuperscript{20} Batard \textit{et al.} also reported that CYP76Bl in Jerusalem artichoke catalyzed the same reaction.\textsuperscript{20} Therefore, these substrates seemed to be good for the assay of not only mammalian P450 species but also plant species.

Metabolism studies demonstrated that the transgenic tobacco plants expressing the CYP1A1 and CYP1A2 fusion enzymes metabolized chlortoluuron to yield a large amount of metabolites. As was observed in the tolerant species of wheat and barley, chlortoluuron was preferentially metabolized through metabolized ring-methyl hydroxylation, which was also found in the transgenic plants and is probably related to the herbicide resistance (reviewed in ref. 34). The CYP1A2 plants showed higher resistance to chlortoluuron while the CYP1A1 plants showed moderate resistance, which seemed to be due to a higher activity of the CYP1A2/P450 reductase fused enzyme in the metabolism of the herbicide than in the CYP1A1/P450 reductase fusion. Indeed, the transgenic plants of the CYP1A2/P450 reductase fused enzyme produced a larger amount of the metabolites than the plants with the CYP1A1 fusion enzyme. Therefore, CYP1A1 and CYP1A2 fusion enzymes were differently contributed in the metabolism of the herbicide in the transgenic tobacco plants, and the CYP1A2 fusion more actively metabolized the herbicide, resulting in a higher resistance to the herbicide as compared to the CYP1A1 fusion.

Recently, it was reported that the certain plant P450 species metabolized the phenylurea herbicides.\textsuperscript{6,7,32,35} According to Werck-Reichhart and her co-workers, CYP76Bl from Jerusalem artichoke efficiently catalyzed \textit{N}-demethylation of chlortoluuron, but did not mediate ring-methyl hydroxylation.\textsuperscript{6} Simonszky \textit{et al.} identified soybean CYP71A10 metabolizing chlortoluuron and its related compounds.\textsuperscript{7} The other P450 species involved in herbicide metabolism have been also cloned from bacteria.\textsuperscript{35-38} These P450 species showed distinct substrate specificity in the metabolism of herbicides. However, this study demonstrated that the human CYP1A1 and CYP1A2 fusion enzymes metabolized not only...
chortoluron but also atrazine. Therefore, expression of the multi-functional human P450 species including CYP1A1 and CYP1A2 on herbicide metabolism in the transgenic plants may confer cross-tolerance towards multiple herbicides as described.39)

Acknowledgments

We express our gratitude to Professor Tukey, R. H. of University of California, San Diego for a generous gift of human CYP1A2 cDNA. We also thank to Y. Motoi for technical assistance. This work was supported by a grant from the BRAIN (Bio-oriented Technology Research Advancement Institution) of Japan.

References

24) Sakaki, T., Shibata, M., Yabusaki, Y., Murakami, H., and Ohkawa, H., Expression of bovine cytochrome P450c21 and its fused enzymes with yeast NADPH-cytochrome P450 reductase in Sac-
Human Cytochrome P450 Expressed in Transgenic Tobacco Plants

2033

25) Burke, M. D. and Mayer, R. T., Inherent specificities of purified cytochromes P-450 and P-448 toward biphenyl hydroxylation and ethoxyresorufin deethyla-

26) Omura, T. and Sato, R., The carbon monoxide-bind-

27) Cabanne, F., Gaillardon, P., and Scalla, R., Phyto-
toxicity and metabolism of chlortoluron in two wheat


29) Gautier, J.-C., Urban, P., Beaune, P., and Pompon, D., Engineered yeast cells as a model to study coupling between human xenobiotic metabolizing en-


38) Shao, Z. Q. and Behki, R., Characterization of the expression of the tBPB gene, coding for a pesticide-