Plant Biotechnology 24, 393–396 (2007)

Short Communication

Expression of *Arabidopsis thaliana* cytochrome P450 monooxygenase, CYP71A12, in yeast catalyzes the metabolism of herbicide pyrazoxyfen

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Received November 10, 2006; accepted May 24, 2007 (Edited by K. Yazaki)

Abstract In higher plants, cytochrome P450 (P450, CYP) monooxygenases play important roles in the biosynthesis and metabolism of endogenous compounds and/or in the oxidative reactions of xenobiotics, such as herbicides, insecticides, and fungicides. In this study, the novel P450 cDNAs encoding CYP71A12 was isolated from wild-type *Arabidopsis thaliana* ecotype Ws using an RT-PCR strategy. Northern blot analysis revealed that *cyp71A12* is expressed specifically in roots but not in flowers, leaves, or stems. To determine the function of CYP71A12, the coding sequences of the corresponding cDNAs were expressed in *Saccharomyces cerevisiae* AH22 cells. Microsomal fractions expressing CYP71A12 metabolized the herbicide pyrazoxyfen. Liquid chromatography mass spectrometry (LC/MS) analysis indicated that the metabolite of pyrazoxyfen might be *N*-demethylated at the pyrazole ring and 5-hydroxylated at the dichlorobenzene ring. This is the first report that a pyrazole herbicide is metabolized by a plant P450 species.

Key words: *Arabidopsis thaliana*, Cyp71A12, cytochrome P450 monooxygenase, herbicide metabolism.

Cytochrome P450 monooxygenases catalyze oxidative reactions of a wide variety of endogenous and exogenous lipophilic compounds. In higher plants, P450 enzymes are mainly involved in the biosynthesis of secondary metabolites, such as phenylpropanoids, fatty acids, and terpenoids. In addition, it is elucidated in many reports that plant P450 species possess xenobiotic detoxification activity and can sometimes activate chemicals (Schuler 1996; Durst 1991). For example, CYP71A10 from soybean was reported to metabolize phenylurea herbicides, fluometuron, linuron, chlortoluron, and diuron in an *in vitro* assay (Siminszky et al. 1999; Sheldon et al. 2000). The CYP71 family comprises 84 known P450 species from several types of plants. Estimates from current genome projects imply the number of 273 P450 genes in *Arabidopsis thaliana* (*A. thaliana*; http://www.p450.kvl.dk/). *Arabidopsis* contains 54 P450 species of CYP71 family, which make up about 20% of all *Arabidopsis* genes, although many of the functions of these genes have not been elucidated. In this study, we isolated CYP71A12 from *A. thaliana* and functionally expressed them in yeast to investigate the novel function of CYP71 family. The molecular cloning and characterization of CYP71A12 are reported.

A. thaliana (L.) Heynh, ecotype Wassilevskija (Ws) was grown with a 16-hr-light and 8-hr-dark cycle at 23°C after vernalization at 4°C for 3 days. An RT-PCR strategy was used for the cloning of P450 cDNAs. Poly(A)$^+$ mRNAs were isolated from whole plants of 30-day-old wild-type *Arabidopsis* Ws. One microgram of poly(A)$^+$mRNA was reverse transcribed to single-stranded cDNAs with the Thermo Script^{TM} RT-PCR System (Life Technologies, Grand Island, NY). Two sets of gene-specific primers were designed based on the genome sequences available in GenBank, (CYP71A12, AC002340): sense primer 5′-AAGGTACCATGGAGA-TGATATTGATGGTCTCTT-3′ and reverse primer 5′-AAGAGCTCTTAAATAACGGAAGATGGAAATGCA-3′. *Kpn* I/*Sac* I and *Sal* I/*Xba* I sites were introduced by PCR upstream and downstream of the *CYP71A12* coding sequence, respectively. The PCR temperature program was 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, for 30 cycles. The PCR products were cloned into pBluescript II SK+ (Stratagene, La Jolla, CA) and used to transform *Escherichia coli* JM109 (Toyobo Biochemical, Tokyo, Japan). The purified full-length cDNAs of CYP71A12 was digested with *Kpn* I/*Sac* I and *Sal* I/*Xba* I, and the full-length coding sequence was

Abbreviations: EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; KPB, potassium phosphate buffer; LC/MS, liquid chromatography mass spectrometry; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcription-polymerase chain reaction
This article can be found at http://www.jspcmb.jp/

inserted between the yeast alcohol dehydrogenase I (ADH) promoter and the terminator of the pAUR123 plasmid vector (Takara Shuzo Co., Ltd., Shiga, Japan) to construct the pA71A12 plasmid. The resulting plasmids were introduced into cells of *Saccharomyces cerevisiae* strain AH22 and were cultured in YPD medium. (Oeda et al. 1985). Reduced carbon monoxide difference spectra of microsomal fractions prepared from the transformed yeast cells were measured according to the method of Oeda et al. (Oeda et al. 1985), in which the P450 content of the microsomal fractions was measured from the reduced carbon monoxide difference using a differential absorption coefficient (450 versus 490) of 91 $mM^{-1}\,cm^{-1}$ (Omura et al. 1964). Six milligrams of microsomal proteins were prepared in suspension buffer containing 1 mM PMSF, 6.4 nM mercaptoethanol, and 20% glycerol in KPB (pH 5.7). After bubbling the microsomal fraction with CO for 1 min in the sample cuvette, the proteins were reduced by adding solid $Na_2S_3O_4$ and the spectra were measured.

The metabolism of various herbicides, insecticides, and an endocrine disruptor summarized in Table 1 were examined in microsomal fractions of the recombinant yeast expressing *CYP71A12*. The assay was carried out according to the methods of Imaishi et al. (Imaishi et al. 2000). The metabolites were analyzed by high performance liquid chromatography (D-7000 HPLC system, HITACHI Tokyo, Japan). The HPLC analytical methods for each substrate are shown in Table 1.

The metabolite of the herbicide pyrazoxyfen was identified by atmospheric pressure chemical ionization (APCI) LC/MS (M-8000 LC/3DQMS system; HITACHI, Tokyo, Japan). The conditions for LC/MS were positive ion mode with drift voltages of 80–150 V and 10–50 V for pyrazoxyfen and its metabolites, respectively.

Northern blot analysis was carried out according to the method of Imaishi et al. (Imaishi et al. 1999). Two micrograms of poly(A)$^+$ RNA were isolated from flowers, leaves, stems, and roots of 7-week-old *A. thaliana*. For Northern hybridization, the 1.5-kb full-length CYP71A12 cDNA was prepared as a probe and labeled with [^{32}P]dCTP using a random primer DNA labeling kit ver.2 (Supelco, Inc.). Hybridization was carried out at 65°C in 6×SSC, 0.1% SDS, 5×Denhardt's solution, and 100 $\mu g\,ml^{-1}$ of denatured salmon sperm DNA at 65°C for 16 hr with the ^{32}P-labeled probe. The membrane was pre-washed with 6×SSC at room temperature for 10 min and then washed with 6×SSC at 65°C for 15 min three times.

Using RT-PCR, the novel P450 cDNA encoding CYP71A12 was isolated from 30-day-old *A. thaliana*. DNA sequence analyses showed that CYP71A12 contains 1494 base pairs (bp). The deduced polypeptide encoded by CYP71A12 contains 498 amino acids, which included the consensus sequence for a heme-binding region (PFG-G-R-C-G), a proline-rich motif (LPPSPWRLPXIG), and a membrane-targeting hydrophobic region next to the *N*-terminus (Mizutani et al. 1998). To determine the catalytic activity of CYP71A12, the coding sequence of the corresponding cDNA was introduced into the pAUR123 vector to produce pA71A12, and the plasmid was expressed in *S. cerevisiae* AH22 cells. The reduced carbon monoxide difference spectra of the microsomal fractions expressing CYP71A12 showed an absorption peak at 449 nm (Data not shown). The microsomal fraction was estimated to have 71 pmol CYP71A12 equivalent per milligram protein.

Microsomal fractions prepared from the recombinant yeast strain expressing CYP71A12 was subjected to metabolism assays of 14 herbicides, 2 insecticides, and a foreign xenobiotic compound. As a result, the herbicide pyrazoxyfen was metabolized by CYP71A12 among them, (Table 1, Figure 1). The activity was calculated from the area of HPLC chromatogram to be approximately 386 $pmol\,min^{-1}\,mmol^{-1}$ P450 protein. The metabolite was not formed when NADPH was omitted from the incubation mixture nor when heat-treated microsomes were used (data not shown), supporting the role of the catalytic activities of CYP71A12 in the metabolism of pyrazoxyfen. The metabolite of pyrazoxyfen was analyzed by LC/MS. The profile showed a prominent ion peak at m/z 405 and an ion peak at m/z 285 which was presumed to be a fragment (Figure 2), indicating that pyrazoxyfen might have been *N*-demethylated at the pyrazole ring and hydroxylated at the dichlorobenzene ring. Based on both the electron-withdrawing properties of the two chloride atoms in the ring and the ion peak of the fragmentation pattern, we presume that the hydroxylation occurred at the position of C5 of the dichlorobenzene moiety. It has been reported that a similar herbicide, pyrazolate, is hydrolyzed at the ester bond to give *p*-toluenesulfonic acid, which does not possess herbicidal activity. This is the first report that pyrazoxyfen is metabolized by the plant enzyme.

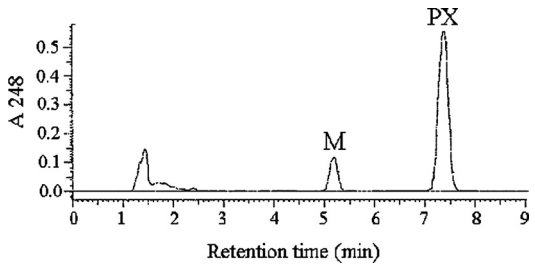

Figure 1. HPLC chromatogram of pyrazoxyfen metabolism. Pyrazoxyfen was assayed with the microsomal fractions prepared from the recombinant yeast strains expressing CYP71A12. PX: pyrazoyfen, M: metabolite.

Table 1. HPLC condition for enzyme assay and catalytic activity of CYP71A12

Substrate	HPLC condition		CYP71A12 activity (pmol/min/mmol protein)
	UV length (nm)	Mobile phase	
Atrazine	254	45% CH3CN (0.1% acetic acid)	ND
Benfuresate	240	40% CH3CN	ND
Bifenox	220	60% CH3CN (0.1 acetic acid)	ND
Bisphenol A	275	0–60% CH3CN (0.1% acetic acid)/0–8 min	ND
Chroltoluron	254	35% CH3CN	ND
Diurn	254	38% CH3CN	ND
Esprocarb	215	67% CH3CN (0.1% acetic acid)	ND
Ethofumesate	230	45% CH3CN (0.1% acetic acid)	ND
Fenitrothine	270	40–60% CH3CN (0.1% acetic acid)/0–10 min	ND
Fluoroglicofen-	210	65% CH3CN (0.1% acetic acid)	ND
Fluvalinate	254	80% CH3CN	ND
Linuron	250	57% CH3CN (0.1% acetic acid)	ND
Mefenacet	240	45% CH3CN (0.1% acetic acid)	ND
Piributicarb	280	50–75% CH3CN (0.1% acetic acid)/0–4 min	ND
Pyrazosulfuron-	254	45% CH3CN (0.1% acetic acid)	ND
Pyrazoxyfen	248	58% CH3CN (0.1% acetic acid)	386
Simazine	240	25% CH3CN (0.1% acetic acid)	ND

ND: not detected.

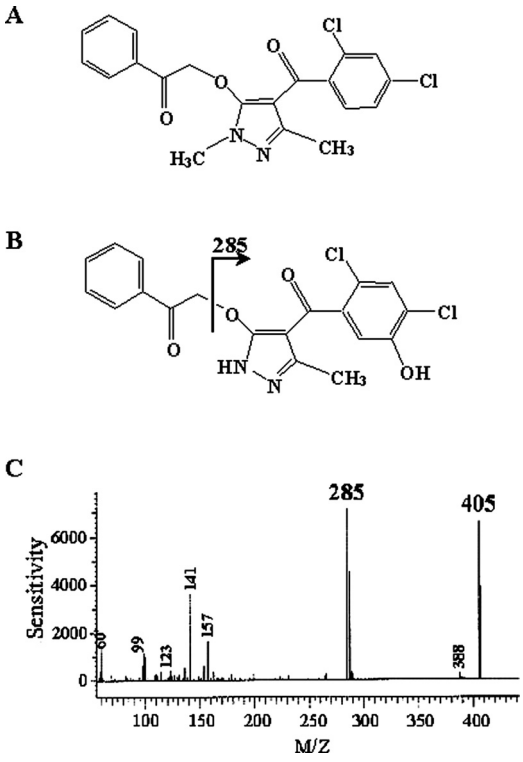

Figure 2. LC/MS analysis of the pyrazoxyfen metabolite. (A) Chemical structure of pyrazoxyfen. (B) Chemical structure of the estimated pyrazoxyfen metabolite (MW 405), which was determined to be *N*-demethylated at the pyrazole ring and hydroxylated at the dichlorobenzene ring. (C) Mass spectrum of the pyrazoxyfen metabolite.

The characteristic of CYP71A12 was further investigated by using Northern blot analysis. To investigate the tissue-specific expression of CYP71A12, a Northern blot analysis was carried out using mRNA from flowers, leaves, stems, and roots of 7-week-old wild-type Ws plants. As a result, CYP71A12 transcripts were detected only in roots, as shown in Figure 3. It suggested that CYP71A12 might function specifically in the roots of *A. thaliana*.

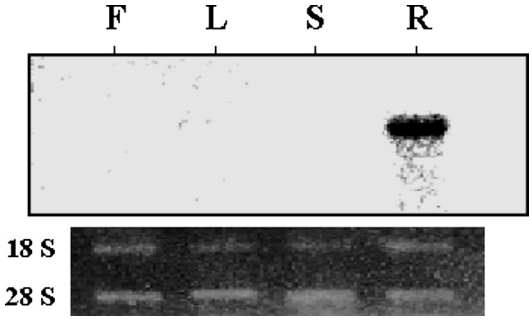

Figure 3. Northern blot analysis of CYP71A12 in 7-week-old wild-type *A. thaliana* ecotype Ws. The 2 μg of poly(A)$^+$RNA obtained from each tissue was electrophoresed, transferred to a nylon membrane, and hybridized with a 1.5 kb ^{32}P-labeled CYP71A12 cDNA probe. F: flower, L: leaf, S: stem, R: root. 18S and 28S represent ribosomal RNA bands used as loading controls.

In conclusion, CYP71A12 metabolized the herbicide pyrazoxyfen to yield a metabolite which might be *N*-demethylated at the pyrazole ring and 5-hydroxylated at the dichlorobenzene ring. This is the first report that a plant P450 is involved in the metabolism of a pyrazole herbicide, which is a new class of herbicides that acts as P450 substrates. This uniquely characterized P450 species could be useful for the studies with differentiation of herbicide selectivity in higher plants.

Acknowledgments

This work was supported by Scientific Research Grant No. 11760066 to H. Imaishi from the Ministry of Education of Japan.

We thank Dr. Hideo Ohkawa of Fukuyama University for his comments on this study.

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