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Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides

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ABSTRACT **A strategy based on the random isolation and screening of soybean cDNAs encoding cytochrome P450 monooxygenases (P450s) was used in an attempt to identify P450 isozymes involved in herbicide metabolism. Nine full-length (or near-full-length) P450 cDNAs representing eight distinct P450 families were isolated by using PCR-based technologies. Five of the soybean P450 cDNAs were expressed successfully in yeast, and microsomal fractions generated from these strains were tested for their potential to catalyze the metabolism of 10 herbicides and 1 insecticide. *In vitro* enzyme assays showed that the gene product of one heterologously expressed P450 cDNA (CYP71A10) specifically catalyzed the metabolism of phenylurea herbicides, converting four herbicides of this class (fluometuron, linuron, chlortoluron, and diuron) into more polar compounds. Analyses of the metabolites suggest that the CYP71A10 encoded enzyme functions primarily as an *N*-demethylase with regard to fluometuron, linuron, and diuron, and as a ring-methyl hydroxylase when chlortoluron is the substrate. *In vivo* assays using excised leaves demonstrated that all four herbicides were more readily metabolized in CYP71A10-transformed tobacco compared with control plants. For linuron and chlortoluron, CYP71A10-mediated herbicide metabolism resulted in significantly enhanced tolerance to these compounds in the transgenic plants.**

Cytochrome P450 monooxygenases (P450s) are ubiquitous hemoproteins present in microorganisms, plants, and animals. Composed of a large and diverse group of isozymes, P450s mediate a great array of oxidative reactions. Most of these activities support biosynthetic processes such as phenylpropanoid, fatty acid, and terpenoid biosynthesis. Others metabolize natural products (e.g., the monoterpene camphor) or play a role in xenobiotic detoxification (1). In a typical P450-catalyzed reaction, one atom of molecular oxygen (O_2) is incorporated into the substrate, and the other atom is reduced to water by using NADPH. For most eukaryotic P450s, NADPH/cytochrome P450 reductase, a membrane-bound flavoprotein, transfers the necessary two electrons from NADPH to the P450 (2).

After the initial report of Frear *et al.* (3), who demonstrated the metabolism of monuron by a mixed-function oxidase located in a microsomal fraction of cotton seedlings, an extensive body of evidence has accumulated supporting the involvement of P450s in the metabolism and detoxification of numerous herbicides (1, 2). Differential herbicide-metabolizing P450 activities are believed to represent one of the mechanisms that enables certain crop species to be more tolerant of a particular herbicide than other crop or weedy species. Although the association of P450s with herbicide metabolism has been investigated for several decades, reports on the successful cloning and expression of P450 cDNAs encoding herbicide-metabolizing isozymes are relatively recent. In one example, constructs using the rat CYP1A1 cDNA conferred chlortoluron resistance in tobacco by enhancing herbicide metabolism (4). In another study, a chloroplast-targeted, bacterial CYP105A1 gene expressed in tobacco catalyzed the toxification of R7402, a sulfonylurea proherbicide (5).

To date, only one report has documented the cloning of an endogenous plant P450 gene that encodes an enzyme potentially involved in herbicide metabolism. A trans-cinnamic acid hydroxylase cDNA (CYP73A1) isolated from Jerusalem artichoke and expressed in yeast catalyzed the ring-methyl hydroxylation of chlortoluron (6). *In vivo* experiments with artichoke tubers, however, demonstrated that the ring-methyl hydroxy metabolite represented only a minor portion of the metabolites produced and that the major metabolite was demethylated chlortoluron (6). This, together with the observation that the turnover number of the heterologously expressed enzyme was extremely low (0.014/min), suggested that CYP73A1 plays a minimal role in chlortoluron metabolism *in vivo*.

To increase our understanding of the molecular mechanisms by which plants use P450s to metabolize herbicides, a PCR-based strategy was used to randomly isolate several P450 cDNAs from soybean. Functional expression of the plant cDNAs in yeast permitted the screening of individual P450 isozymes for herbicide-metabolizing activities. In this study we describe the cloning and expression of a soybean cDNA, designated CYP71A10, that encodes a P450 enzyme capable of metabolizing four phenylurea herbicides. Expression of CYP71A10 in transgenic tobacco results in increased linuron and chlortoluron tolerance by enhancing the herbicide-metabolic activities of these plants.

MATERIALS AND METHODS

Substrates. Phenyl-U-[^{14}C]fluometuron, phenyl-U-[^{14}C]chlortoluron, phenyl-U-[^{14}C]metolachlor, phenyl-U-[^{14}C]prosulfuron, pyrimidinyl-2-[^{14}C]diazinon, and phenyl-U-[^{14}C]alachlor were kindly provided by Novartis (Greensboro, NC); phenyl-U-[^{14}C]bentazon was donated by BASF Bioresearch (Cambridge, MA); phenyl-U-[^{14}C]linuron, phenyl-U-[^{14}C]diuron, and carbonyl-[^{14}C]metribuzin were a gift from DuPont; and carboxyl-[^{14}C]imazaquin was provided by American Cyanamid.

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Abbreviations: LC/MS, liquid chromatography/MS; P450, cytochrome P450 monooxygenase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF022157 and AF022457–AF022464).

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Isolation of P450 cDNAs. Random amplification of partial cDNAs encoding P450 enzymes was conducted as described by Meijer *et al.* (7), using a soybean (*Glycine max*) leaf cDNA library as the template (8). To recover full-length versions of the partial cDNAs, a primer (5′-TGTCTAACTCCTTC-CTTTTC-3′) complementary to the pYES2 vector (the vector into which the soybean cDNA library was cloned) and a downstream primer corresponding to a segment of the 3′ untranslated region for each of the unique P450 cDNAs were used in PCRs against the same soybean cDNA library. A final PCR was performed to create full-length versions of the respective P450 ORFs that completely lacked endogenous 5′ untranslated sequences. The forward primers contained a *Bam*HI restriction site immediately followed by the ATG start codon and the next 14–15 bases of the reading frame; the downstream primer was again specific for the 3′ untranslated regions of the respective genes and included sequences specifying either *Eco*RI, *Kpn*I, or *Sac*I restriction sites to facilitate subcloning of the P450 cDNAs into the yeast expression vector pYeDP60 (V-60; ref. 9).

All PCRs, with the exception of the initial amplification of the partial P450 cDNAs described by Meijer *et al.* (7), contained 0.2 ng of template/2 μM each of primer/200 μM each of dNTP/1.5 mM $MgCl_2$ in a final reaction volume of 50 μl. Amplification was initiated by the addition of 1.5 units of Expand High Fidelity enzyme mix using conditions described by the manufacturer (Boehringer Mannheim). DNA sequence was determined by the chain termination method (10) by using fluorescent dyes (Applied Biosystems).

P450 cDNA Expression in Yeast. Yeast transformation was performed as described previously (11). Media composition, culturing conditions, galactose induction, and microsomal preparations were conducted as outlined by Pompon *et al.* (12), with the exception that the galactose-induced yeast cultures were stored for 24 hr at 4°C before microsome isolation. Microsomal protein was quantified spectrophotometrically by using the method of Waddell (13) with BSA as a standard. Dithionite-reduced, carbon monoxide difference spectra were obtained as outlined previously (14) by using a Shimadzu Recording Spectrophotometer UV-240. P450 protein concentrations of yeast microsomes were calculated by using a millimolar extinction coefficient of 91 (15).

***In Vitro* Herbicide Metabolism Assays.** Yeast microsomes enriched for a discrete soybean P450 isozyme were assayed for their capacity to metabolize the 10 herbicides and 1 insecticide listed above. The reaction mixtures contained 10,000 dpm (100–200 ng) of radiolabeled substrate, 0.75 mM NAPDH, and 2.5 mg/ml microsomal protein. Total reaction volumes were adjusted to 150 μl with 50 mM phosphate buffer (pH 7.1). The mixtures were incubated under light for 45 min at 27°C, arrested with 50 μl acetone, and centrifuged at 14,000 $\times$ *g* for 2 min. Supernatant fractions subsequently were analyzed by using TLC. For the four phenylurea compounds (chlortoluron, diuron, fluometuron, and linuron), samples were spotted onto 250-μm Whatman K6F silica plates and developed in a benzene/acetone 2:1 (vol/vol) solvent system. The developed plates were scanned with a Bioscan System 400 imaging scanner (Washington, DC).

Enzyme Kinetics. Substrate conversion was quantified by a combination of TLC analysis and scintillation spectrometry. The location of the metabolic products on the TLC plates was identified by using an imaging scanner, and the bands were scraped and analyzed by scintillation spectrometry. The amount of metabolite produced was calculated based on specific activity and scintillation counts. K_m and V_{max} values were estimated by using nonlinear regression analysis.

Mass Spectral Analysis. Metabolites of fluometuron and linuron produced using yeast microsomes and the metabolites of chlortoluron produced in the transgenic tobacco were analyzed by liquid chromatography (LC)/MS. Mass spectral measurements were made with a Finnigan TSQ 7000 triple quadruple mass spectrometer (QQQ) equipped with an Atmospheric Pressure Ionization (API) interface fitted with a pneumatically assisted electrospray head (Finnigan-MAT, San Jose, CA). Samples were chromatographed on a reverse-phase HPLC column (Inertsil ODS-2, 5μm, 150 $\times$ 2 mm i.d.). The column was eluted at 0.4 ml/min with 95:5 of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in methanol, respectively. Collision-induced dissociation experiments (MS/MS) were conducted by using argon gas with collision energy in the range of 17.5–30 eV at cell pressures of approximately 0.28 Pa. Signals were captured by using a Finnigan 7000 data system.

Tobacco Transformation. To create a plant expression vector capable of mediating the constitutive expression of CYP71A10, the β-glucuronidase ORF of the binary expression vector pBI121 (CLONTECH) was excised and replaced with the full-length CYP71A10 reading frame. This placed the soybean gene under the transcriptional control of the cauliflower mosaic virus 35S promoter. The resulting construct was used to transform *Agrobacterium tumefaciens* strain LBA 4404 (16). Excised leaf discs of *Nicotiana tabacum* cv SR1 were transformed by using the *Agrobacterium,* and kanamycin-resistant plants were selected as described (17).

Herbicide Tolerance. T_1 generation seeds from CYP71A10-transformed tobacco and pBI121-transformed control plants were placed onto Petri dishes containing MS salts and linuron using its commercial formulation (LOROX 50 DF) at active ingredient concentrations ranging from 0.25 to 3.0 μM. Chlortoluron was added at 0, 1.0, 5.0, and 10.0 μM concentrations by using a 99.5% pure analytical standard. The Petri dishes were incubated at 27°C with a 16-hr light/8-hr dark cycle. Phytotoxic effects were determined visually by comparison with control plants and plants grown in the absence of the herbicide.

***In Vivo* Herbicide Metabolism Assays.** Single leaves (approximately 10 cm^2 in size) of control plants and transgenic tobacco plants transformed with CYP71A10 were excised and their petioles were inserted into 100 μl H_2O containing radiolabeled herbicide. The leaves were placed in a growth chamber maintaining a temperature of 27°C and incubated until the entire volume of the herbicide solution was drawn up by the transpirational stream of the leaves (approximately 3 hr). The leaves subsequently were transferred into an Eppendorf tube containing distilled water and incubated an additional 11 hr.

^{14}C-labeled herbicide was extracted from the leaves by grinding for 5 min in 250 μl methanol with a plastic pellet pestle driven by an electric drill. Approximately 70% of the absorbed herbicide was extracted in the methanol-soluble fraction, and an additional 26% was recovered by combusting the methanol-insoluble fraction in a biological materials oxidizer (Model OX-500, R.J. Harvey Instrument Corp., Hillsdale, NJ). After centrifugation for 3 min at 14,000 $\times$ *g*, 75 μl of the supernatant was analyzed by TLC as described above. Substrate conversion was quantified based on the amount of herbicide absorbed, and the ratios of the parent compound and the produced metabolites were determined from the TLC profiles.

RESULTS

Isolation of Soybean P450 cDNAs. To isolate cDNAs encoding P450s from soybean, we employed the PCR strategy described by Meijer *et al.* (7) using a soybean leaf cDNA library as the template. Of 86 randomly selected cloned PCR products that were sequenced, 15 clones representing 10 unique cDNAs were identified that appeared to represent P450 genes based on significant sequence homologies to known P450s. Because this strategy enables only the recovery of sequence corre-

sponding to the C-terminal portion of the proteins, additional PCR-based techniques were used to obtain cDNAs containing the entire reading frames for each clone (see *Materials and Methods*). We were successful in isolating full-length versions for 8 of the 10 individuals and what is likely to be a near full-length cDNA for an additional clone. The individual clones were assigned the following names based on the standardized system of P450 nomenclature (18): CYP71A10, CYP71D10, CYP77A3, CYP78A3, CYP82C1, CYP83D1, CYP93C1, CYP97B2, and CYP98A2. None of these soybean cDNAs fall into P450 families for which an *in vivo* function has been established.

Expression of Soybean P450 cDNAs in Yeast. Because superfluous 5′ untranslated sequences from foreign genes in some cases can impede gene expression in yeast (19), an additional PCR was performed on each cDNA that enabled the cloning of full-length P450 ORFs into the yeast expression vector V-60 without including any of the endogenous 5′ nontranslated flanking sequence (see *Materials and Methods*). For the near-full-length clone, CYP83D1, the 5′ primer also was designed to generate an "artificial" Met start codon and a Val second codon at the 5′ end of the ORF. To maximize the heterologous expression of the soybean P450 cDNAs in yeast, each construct cloned into the V-60 vector was transformed into yeast strain W(R), a cell line engineered to overexpress the endogenous yeast NADPH-dependent cytochrome P450 reductase (12).

In the absence of specific antibodies, reduced-CO difference spectroscopy provides a viable alternative by which the effectiveness of expression of heterologous P450s in yeast may be measured. Microsomal preparations corresponding to five of the soybean constructs (CYP71A10, CYP71D10, CYP77A3, CYP83D1, and CYP98A2) showed characteristic P450 CO difference spectra with Soret peaks at 450 nm (data not shown); no such peaks were observed for the remaining four clones or for the vector-only control. The specific P450 content of the five positive microsomal preparations varied significantly, ranging from 11 pmol P450/mg protein for construct CYP83D1 to 252 pmol P450/mg protein for clone CYP77A3 (data not shown).

***In Vitro* Herbicide Assays.** To determine whether any of the soybean P450 enzymes displayed significant herbicide metabolic activity, microsomal preparations containing each of the five soybean P450s that were expressed effectively in yeast were incubated individually with NADPH and the radiolabeled compounds listed in *Materials and Methods*. These substrates represent six different classes of herbicides and one organophosphate insecticide (diazinon). Upon termination of the reaction, each sample was analyzed by TLC to reveal potential metabolic-breakdown products. The P450 proteins expressed from clones CYP71D10, CYP77A3, CYP83D1, and CYP98A2 displayed no apparent *in vitro* metabolic activity against any of the 11 compounds tested. The P450 enzyme produced from CYP71A10, however, demonstrated substantial metabolic activity against the phenylurea class of herbicides, but no activity against the remaining compounds. As shown in Fig. 1, fluometuron and diuron were converted to a single metabolite; linuron and chlortoluron were transformed into two, a major and minor, metabolites. The chemical structures of the four phenylurea herbicides tested in this study and the derivatives that previously have been characterized as the first metabolites produced during their detoxification in plants known to metabolize these compounds (20–22) are shown in Fig. 2 .

Although each of the four phenylurea-type herbicides were metabolized by the soybean CYP71A10 enzyme, their kinetic properties differed significantly. As shown in Table 1, turnover rates for fluometuron and linuron were considerably greater than those observed for chlortoluron and diuron. The reduced activity for the latter two substrates apparently is not the result

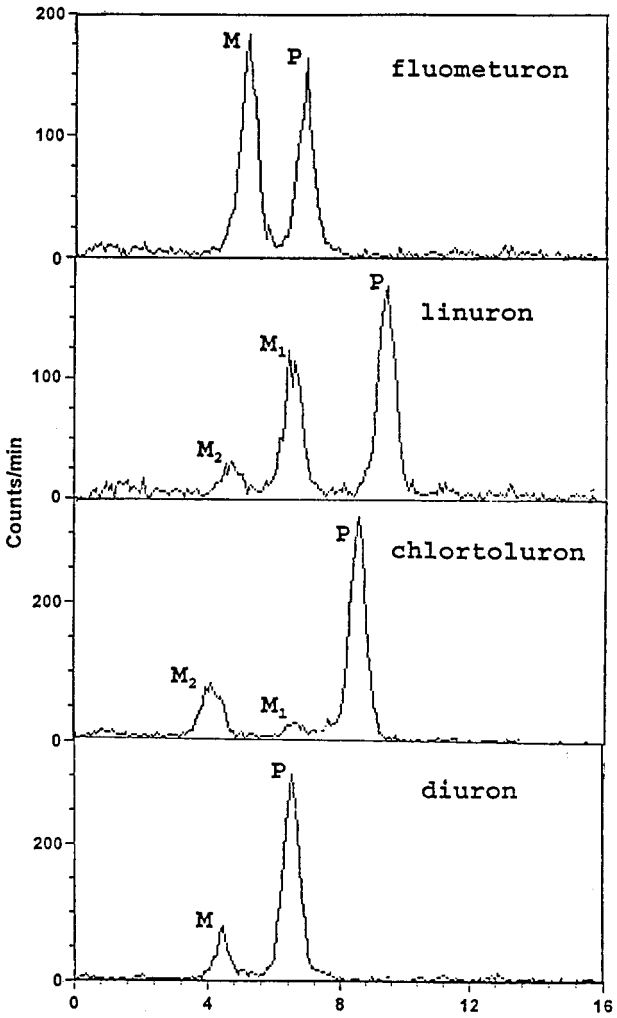

FIG. 1. CYP71A10-mediated metabolism of phenylurea herbicides. Shown are thin-layer chromatograms of ^{14}C-radiolabeled fluometuron, linuron, chlortoluron, and diuron and their respective metabolites after incubation of the radiolabeled herbicides with yeast microsomes containing the CYP71A10 protein. Initial substrate concentrations for fluometuron, linuron, chlortoluron, and diuron were 5.2, 6.5, 4.0, and 3.7 μM, respectively. P, parent compound; M, metabolite.

of decreased binding affinities because the apparent K_m values for chlortoluron and diuron are lower than those measured for fluometuron and linuron.

Analysis of Metabolites. As shown in Fig. 1, CYP71A10-mediated degradation of phenylurea herbicides resulted in the accumulation of either one or two metabolites, depending on the particular substrate used. To determine the structure of the metabolites and thus define the nature of the enzymatic reactions, the single metabolite observed in the fluometuron assay and both the major and minor metabolites generated in the linuron and chlortoluron assays were analyzed by LC/MS. Analysis of the fluometuron metabolite by LC/MS in positive-ion mode resulted in pseudomolecular ions at m/z 219 [$(M+H)^+$, $C_9H_9F_3N_2O$] and m/z 241 $(M+Na)^+$, which corresponds to a sodium adduct (data not shown). Daughter ion spectra of m/z 219 produced a prominent m/z 162 fragment ion because of formation of the protonated trifluoromethylaniline $(C_7H_6F_3N+H)^+$ (Fig. 3*A*). The identity of the fluometuron metabolite was verified further by proton NMR. The fluometuron metabolite displayed an NMR profile that was identical to an authentic standard of *N*-demethyl fluometuron (data not shown).

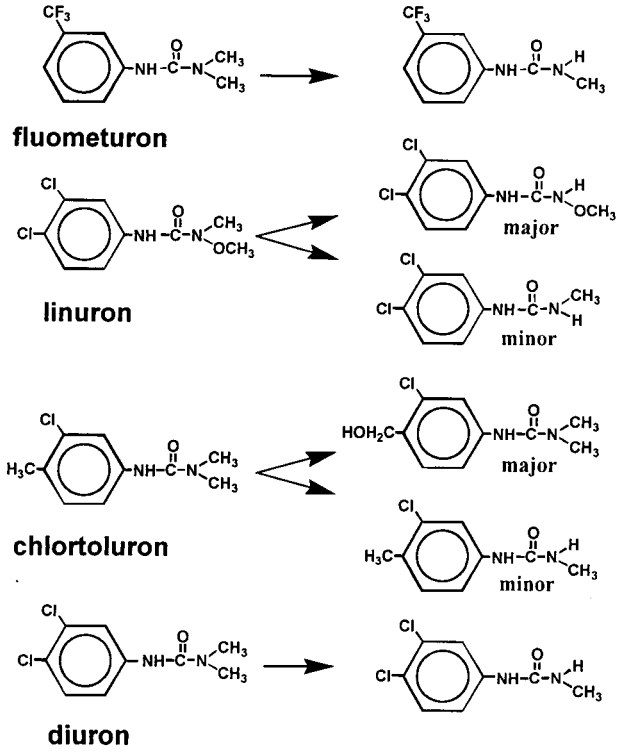

FIG. 2. Chemical structures of fluometuron, linuron, chlortoluron, and diuron, and their metabolites. The linuron and chlortoluron metabolites are designated major or minor depending on their relative abundance in assays using yeast microsomes containing the soybean CYP71A10 protein.

The major linuron metabolite analyzed by LC/MS in the negative-ion mode showed pseudomolecular ions at m/z 233 $(M-H)^-$ and m/z 235 $[(M+2)-H]^-$, which are consistent for a molecule containing two chlorine atoms (data not shown). Daughter ion spectrum at m/z 233 showed a prominent fragment ion at m/z 160 $(C_6H_4Cl_2N-H)^-$ (Fig. 3*B*). The major linuron metabolite was 15 mass units less than the parental compound that is consistent with loss of a methyl group.

LC/MS analysis also was conducted on the minor linuron metabolite and major and minor chlortoluron metabolites (data not shown). In each case, the mass spectral data were consistent with these metabolites representing the compounds depicted in Fig. 2. Although the diuron metabolite has not been analyzed directly, the R_f value of this product observed during TLC separation is consistent with this species representing *N*-demethyl diuron. The CYP71A10 enzyme therefore appears to function primarily as an *N*-demethylase with respect to fluometuron, linuron, and diuron, with some *N*-demethoxylase activity also observed with linuron. Using chlortoluron as a substrate, the enzyme functions primarily as a ring-methyl hydroxylase and, to a lesser extent, as an *N*-demethylase.

Table 1. *In vitro* kinetic parameters of the CYP71A10 enzyme for four phenylurea substrates

Substrate	$K_{m,app}$, μM	V_{max}, pmol/min per mg of protein	Turnover, min^{-1}
Fluometuron	14.9 (1.0)*	303.6 (10.8)	6.8 (0.24)
Linuron	9.8 (2.1)	125.6 (12.0)	2.8 (0.27)
Chlortoluron	1.0 (0.2)	29.4 (2.2)	0.7 (0.05)
Diuron	1.5 (0.3)	16.8 (1.6)	0.4 (0.04)

Assays were repeated three times for linuron and twice for all other substrates. Concentration ranges used for fluometuron, linuron, chlortoluron, and diuron were 3.2–27.7, 3.8–28.3, 0.7–4.0, and 0.7–3.7 μM, respectively.
*Values in parentheses represent standard error.

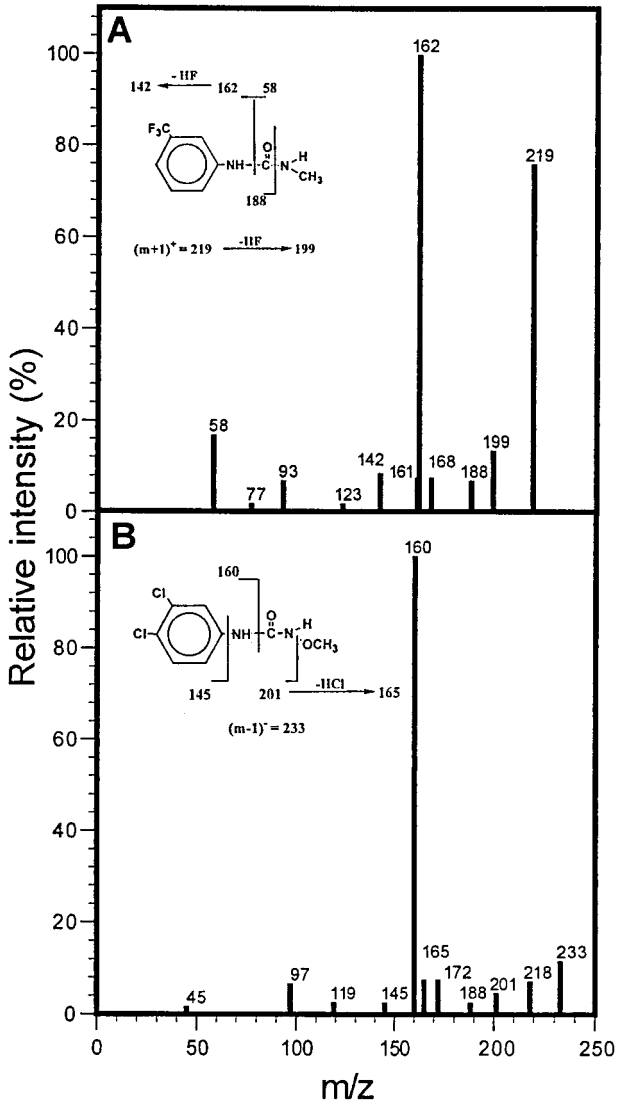

FIG. 3. Mass spectra of the oxidation products of fluometuron and linuron. (*A*) Fluometuron metabolite (predicted to be *N*-demethyl fluometuron). (*B*) Major linuron metabolite (predicted to be *N*-demethyl linuron).

Herbicide Tolerance and Metabolism in Transgenic Plants. To determine whether the overexpression of the soybean CYP71A10 cDNA in a higher plant system could result in enhanced tolerance toward phenylurea herbicides, several independent transgenic tobacco plants were generated by using CYP71A10 constructs under the transcriptional control of the constitutive 35S promoter of cauliflower mosaic virus. Growth of wild-type SR1 plants and transgenic control plants expressing the β-glucuronidase reporter gene was inhibited severely when exposed to 0.25 μM linuron and completely arrested at concentrations of 0.5 μM and higher (Fig. 4*B*). T_1-generation seeds from 21 independent CYP71A10-expressing transgenic plants were germinated and grown on medium containing 0.5 μM linuron. Of these, 20 lines gave rise to progeny that were linuron tolerant (data not shown).

To further characterize the CYP71A10-mediated herbicide tolerance phenotype, one transgenic tobacco line, designated 25/2, was selected for additional analysis. As shown in Fig. 4 *A* and *C*, progeny of plant 25/2 grown in the presence of 0.5 μM linuron appeared indistinguishable from the same seed grown on medium containing no herbicide. Of 23 germinated

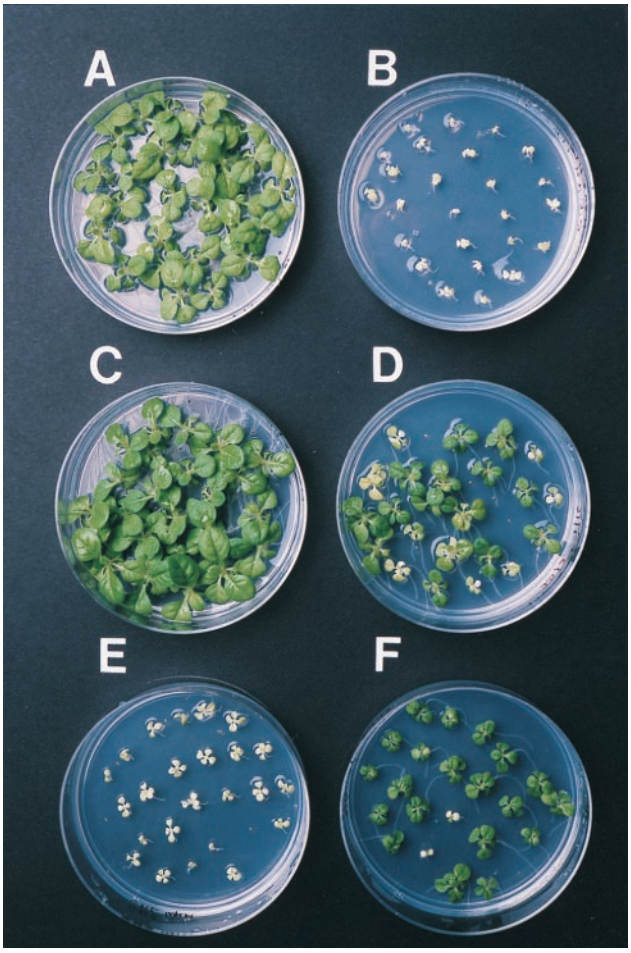

FIG. 4. Tobacco plants transformed with soybean CYP71A10 gene show tolerance to linuron and chlortoluron. Line 25/2 individuals were grown on media containing no herbicide (*A*), 0.5 μM linuron (*C*), 2.5 μM linuron (*D*), or 1.0 μM chlortoluron (*F*). Control tobacco plants transformed with vector pBI121 were grown on medium containing 0.5 μM linuron (*B*) or 1.0 μM chlortoluron (*E*).

seedlings, only 1 plant appeared to be inhibited by the herbicide. This ratio appears to be consistent with that observed when seeds from the same parent were grown on selective medium containing kanamycin; only 1 of 17 seedlings failed to grow in the presence of kanamycin (data not shown). These results suggest that line 25/2 possesses more than one independently segregating transgenic locus. Line 25/2 plants tolerant to levels as high as 2.5 μM linuron were observed, although an increasing percentage of the plants showed growth inhibition as the herbicide concentration was increased (Fig. 4*D*). A likely explanation for this variation in response at high concentrations of herbicide exposure is that transgene segregation may be leading to variability in expression levels among the progeny of 25/2.

Chlortoluron tolerance of line 25/2 was also evident. At 1.0 μM herbicide concentration, chlortoluron completely arrested the growth of the control plants (Fig. 4*E*). Although growth of the 25/2 plants was somewhat inhibited at this herbicide concentration, with the exception of two presumably nontransgenic segregants, the CYP71A10-transformed plants appeared healthy (Fig. 6*F*). In contrast to linuron and chlortoluron, significant tolerance of line 25/2 to fluometuron or diuron was not observed (data not shown).

To access directly the fates of the four phenylurea herbicides when introduced to the CYP71A10-expressing transgenic plants, individual leaves from the 25/2 progeny were excised and incubated with the respective radiolabeled compounds.

Table 2. Phenylurea herbicide metabolism after 14 hr by excised leaves of transgenic tobacco plant 25/2

	Herbicide metabolized, %	
Herbicide*	CYP71A10-transformed	Control†
Fluometuron	91 (4.5)‡	15 (0.6)
Linuron	87 (2.0)	12 (2.6)
Chlortoluron	85 (8.1)§	39 (7.5)§
Diuron	49 (7.0)	20 (2.0)

*Equal amounts of herbicide (1.2 nmol) were added for each experiment.
†Plants transformed with the pBI121 construct were used as controls.
‡Values in parentheses represent standard error. For each herbicide, a single leaf was assayed from four independent 25/2 plants and three independent control plants.
§The major chlortoluron metabolite in the control plants represented *N*-demethyl chlortoluron (63%). The metabolites recovered from the CYP71A10-transformed leaves were ring-methyl hydroxy chlortoluron (47%), *N*-demethyl chlortoluron (8%), and other derivatives (45%).

Because in some circumstances preexposure to herbicides can enhance endogenous P450 activities, these assays were performed by using 25/2 plants that had been germinated only in the presence of kanamycin. As shown in Table 2, leaves of the kanamycin-resistant individuals of line 25/2 metabolized all of the four herbicides tested to a much greater extent than control plants.

The relative migrations of the metabolic products revealed by TLC suggest that for fluometuron, linuron, and diuron, the products observed in the *in vivo* excised leaf assay are the same as were generated from the *in vitro* assays using yeast microsomes (data not shown). For chlortoluron, however, additional metabolites were observed. LC/MS analysis of these metabolites revealed that they represent combinations of hydroxylated and mono- and didemethylated species, as had been observed by Shiota *et al.* (23) in their analysis of chlortoluron-resistant transgenic tobacco that overexpressed the rat CYP1A1 gene (data not shown). Differences in the ratios of the observed chlortoluron metabolites also were observed between the CYP71A10-transformed and the control plants. *N*-demethyl chlortoluron represented 63% of the metabolite produced in the control leaves; in contrast, ring-methyl hydroxy chlortoluron was the most abundant metabolite generated in the CYP71A10-transformed leaves and only 8% of the metabolites represented *N*-demethyl chlortoluron (Table 2).

DISCUSSION

Although the importance of P450 enzymes in catalyzing the metabolism of a variety of herbicides has been well documented, little progress has been made in the identification of the endogenous plant P450s that are responsible for degrading these compounds. Protein purification of specific isozymes involved in the metabolism of a specific herbicide has been hindered by the instability of the enzymes, their low concentrations in most plant tissues, and difficulties in the reconstitution of active complexes from solubilized components (1). Furthermore, it is increasingly apparent that any given plant tissue may possess dozens, if not hundreds, of unique P450 isozymes; therefore, the purification to homogeneity of a particular isozyme found in relatively low abundance presents a major technical challenge.

To circumvent these problems, we employed a molecular strategy that allowed us to examine the herbicide-metabolizing properties of individual P450 isozymes by the heterologous expression in yeast of randomly isolated soybean P450 cDNAs. Although this approach is labor-intensive and time-consuming, it proved to be effective in facilitating the identification of a specific P450 cDNA (CYP71A10) whose product

was able to metabolize herbicides of the phenylurea class. This study shows that this P450 cDNA encodes an enzyme possessing high metabolic activity against a specific class of herbicidal compounds. The specificity of this enzyme is evidenced by its inability to recognize herbicides representative of five other chemical families or the insecticide diazinon as substrates.

Although the overexpression of CYP71A10 in tobacco greatly enhanced the plant's capacity to metabolize all four phenylurea herbicides tested, appreciable levels of tolerance were conferred only to linuron and chlortoluron. Despite the fact that fluometuron was the most actively metabolized compound in both the yeast and transgenic plant systems, only marginal enhancement in tolerance was observed at the whole-plant level. This result is not surprising, however, given our current understanding of the differential toxicities of the various phenylurea derivatives produced in the CYP71A10-transformed yeast and tobacco. Previous studies have shown that the *N*-demethyl derivatives of fluometuron and diuron are only moderately less toxic than their parent compounds (24, 25). In contrast, Suzuki and Casida (21) demonstrated that linuron is a 10-fold-greater inhibitor of the Hill reaction than *N*-demethyl linuron, and Nashed and Ilnicki (26) concluded that *N*-demethyl linuron possessed little or no herbicidal activity. Ultimately, the toxicity of a phenylurea metabolite is a factor not only of dealkylation and hydroxylation, but also of its propensity to become inactivated through conjugation to glucose. In the 14-hr duration of the *in vivo* herbicide metabolism assays shown in Table 2, however, glucosylated derivatives of any linuron or fluometuron metabolite were not observed.

Tolerance toward chlortoluron in the transgenic plants similarly can be explained by the properties of the resultant metabolites. Although considerable chlortoluron metabolism was observed in the susceptible control tobacco lines (Table 2), the majority of this product was the still-toxic *N*-demethyl derivative as opposed to the nontoxic, hydroxylated and didemethylated metabolites (27) produced in the CYP71A10-expressing transgenic plants.

The metabolites of the various phenylurea herbicides produced by yeast microsomes and transgenic tobacco plants expressing CYP71A10 appear to be the same degradation products that previously have been observed when these same compounds have been applied to plants known to metabolize these herbicides (20–22). It is therefore tempting to speculate that CYP71A10 or related genes in plants may play a significant role in crop species that metabolize phenylurea herbicides. From our transgenic tobacco results, one might predict that plants that naturally express significant levels of CYP71A10 during the appropriate stages of plant development would show some level of tolerance toward phenylurea herbicides such as linuron that only require a single demethylation to be substantially detoxified. Crop species such as cotton that show tolerance to fluometuron and diuron by metabolizing these compounds to their nontoxic didemethylated derivatives (25, 27) would be predicted to require additional (or altogether different) enzymatic activities. In this regard, it is interesting to note that soybean, the species from which CYP71A10 was derived, displays a significant level of tolerance to linuron. As a result, herbicides based on this compound have been used extensively by soybean growers both in pre-emergence- and post-emergence-directed applications (28). Northern blot analysis using CYP71A10 as a hybridization probe suggests that the gene is expressed in low to moderate levels throughout the soybean plant (B.S. and R.E.D., unpublished results). The degree with which CYP71A10 is responsible for the linuron tolerance phenotype of soybean, however, cannot be ascertained readily and must await the generation of transgenic soybean plants in which the expression of endogenous gene has been inhibited by antisense CYP71A10 constructs. Such plants also could aid in defining the *in vivo* function and natural substrates of the CYP71A10 gene product, both of which are currently unknown.

Note Added in Proof. Robineau *et al*. (29) recently identified an apparently unrelated plant P450 gene from Jerusalem artichoke (CYP76B1) whose product metabolized certain phenylurea compounds when expressed in yeast.

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