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Transgenic Rice Plants Expressing Human *CYP1A1* Remediate the Triazine Herbicides Atrazine and Simazine

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The human cytochrome P450 *CYP1A1* gene was introduced into rice plants (*Oryza sativa* cv. Nipponbare). One-month-old CYP1A1 plants grown in soil clearly showed a healthy growth and tolerance to 8.8 μM atrazine and 50 μM simazine, but nontransgenic plants were completely killed by the herbicides. Although transgenic and nontransgenic plants metabolized the two herbicides into the same sets of compounds, CYP1A1 plants metabolized atrazine and simazine more rapidly than did control plants. In small-scale experiments, residual amounts of atrazine and simazine in the culture medium of CYP1A1 plants were 43.4 and 12.3% of those in control medium; those of nontransgenic Nipponbare were 68.3 and 57.2%, respectively. When cultivated in soil with 2.95 μM atrazine and 3.15 μM simazine for 25 days, CYP1A1 plants eliminated 1.3 times more atrazine and 1.4 times more simazine from the soil than did control plants. Thus, CYP1A1 rice plants make it possible to remove atrazine and simazine more rapidly from the culture medium and soil than can nontransgenic Nipponbare.

KEYWORDS: Cytochrome P450 (CYP); tolerance; phytoremediation; environmental pollution; endocrine disruptor; paddy field; reduction

INTRODUCTION

Atrazine and simazine are systemic *s*-triazine herbicides that were discovered in the 1950s and inhibit electron transport during photosynthesis. They mainly are absorbed through roots but also through leaves and are translocated acropetally in the xylem. In sensitive plants, unaltered atrazine and simazine accumulate in apical meristems and leaves and cause chlorosis and death through inhibition of electron transport during photosynthesis. Both herbicides are used for pre- and post-emergence control of annual grasses and broad-leafed weeds in many crops, including maize, sorghum, vines, citrus, sugar cane, and grassland. The main uses of atrazine are for maize and sorghum, and simazine is used as an aquatic herbicide and algaecide for the control of algae and submerged weeds (*1*, *2*). Atrazine is used in more than 80 countries, and it is one of the world's most important herbicides. For example, the U.S. Environmental Protection Agency estimates that the annual usage of atrazine in the United States is ∼34000 metric tons (*3*).

Triazine herbicides are slightly hydrophilic (water solubility: atrazine, 33 mg L^{-1}; simazine, 6.2 mg L^{-1}) and are persistent herbicides (*1*). Atrazine and simazine can be transported into surface and ground waters, and they have become common contaminating herbicides of surface and ground waters in the United States and some European countries (*4*). Atrazine has been banned in several European countries, because of human health concerns and the presence of residues in drinking water.

In higher plants, atrazine is metabolized through hydroxylation, dealkylation, and glutathione conjugation. N-Dealkylation occurs in many plant species, with formation of deethylated atrazine in preference to that of deisopropylated atrazine (**Figure 1A**) (*5*). Simazine is metabolized by N-dealkylation in many plant species to form mono- and didealkylated products (**Figure 1B**). N-Dealkylation of a single side chain results in partial loss of phytotoxicity, and that of both side chains causes complete loss of phytotoxicity (*6*). Susceptible and tolerant plant species show important differences in the extent to which they can dealkylate these herbicides (*5*). The responsible enzymes have not been well studied, but this N-dealkylation seems to be catalyzed by cytochrome P450 monooxygenases (CYP). In particular, tulip (*Tulipa generiana* L.) cytochrome P450 causes in vitro metabolism of atrazine, resulting in the production of double-dealkylated metabolites (*7*). The cytochrome P450

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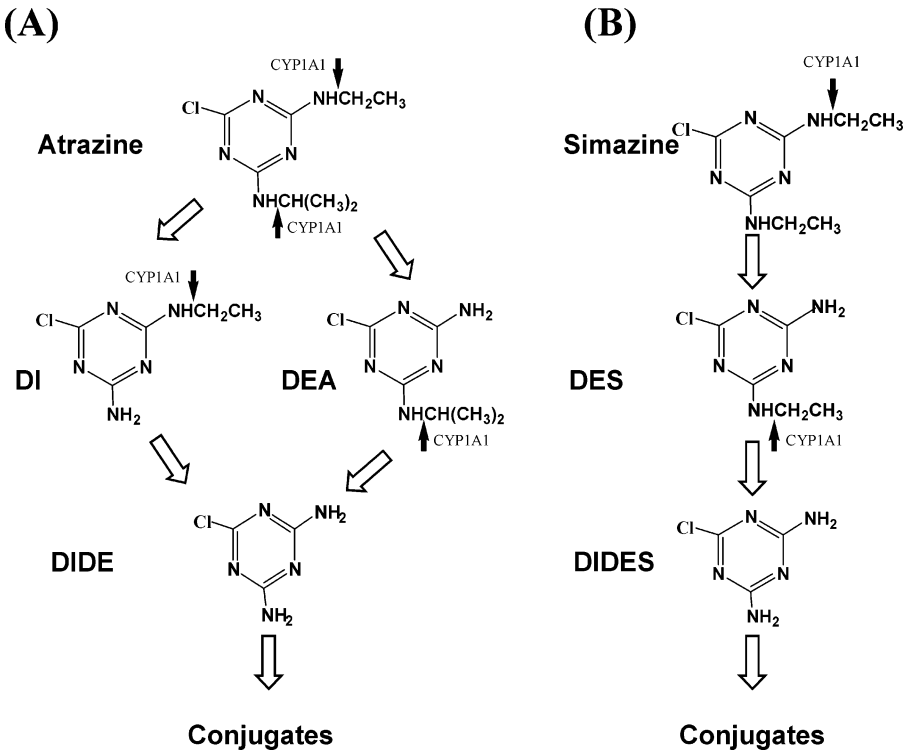

Figure 1. Schematic metabolic pathways of atrazine (**A**) and simazine (**B**) in plants. The black bold arrow indicates the target point of CYP1A1. DI, deisopropylated atrazine; DEA, deethylated atrazine; DIDE, deisopropylated and deethylated atrazine; DES, deethylated simazine; DIDES, di-deethylated simazine.

inhibitor 1-aminobenzotriazole inhibited N-dealkylation of simazine and reduced the plant weights of biotypes of *Lolium rigidum* resistant to triazine herbicides with the combination of simazine (*8*).

P450 monooxygenases insert one atom of oxygen into hydrophobic molecules to make them more reactive and water-soluble (*9*). Genome sequence analysis revealed that plant genomes can include hundreds of P450 genes (e.g., 273 in *Arabidopsis thaliana* and 356 in *Oryza sativa*) (*10*). The varied substrate specificities of these P450s complicate prediction of their functions from sequence similarities only. However, a few P450 genes related to herbicide metabolism have been cloned and characterized from plants, including Jerusalem artichoke (*Helianthus tuberosus*) (*11*, *12*), tobacco (*Nicotiana tabacum*) (*13*), and soybean (*Glycine max*) (*14*).

It is well-known that the microsomes of mammalian livers contain several P450s involved in xenobiotic metabolism, and these enzymes have been reported to show broad, overlapping substrate specificity toward lipophilic xenobiotics, including herbicides (*15*). Atrazine is metabolized through stepwise dealkylation to produce deethylated and deisopropylated atrazine in rats (*16*). Similarly, simazine was metabolized through stepwise dealkylation to form mono-deethylated simazine and eventually the di-deethylated simazine that was the major metabolite in rats (*16*). In vitro metabolism studies using hepatic microsomes from several animals, including rat, goat, pig, and rabbit, showed that atrazine and simazine were converted into dealkylated metabolites (*17*). Inui et al. used an in vitro yeast microsome system to demonstrate that atrazine and simazine were metabolized by human CYP1A1 and CYP1A2 through dealkylation (*18*).

Phytoremediation is the use of plants and plant growth as a technique for detoxifying sites contaminated with organic and inorganic pollutants in relatively low concentrations (*19*). The current physical cleanup method of removing contaminated soil from a site and burying it elsewhere is too costly to be practical and is environmentally destructive. Phytoremediation is also a possible method to remove pesticides from contaminated water. According to Schnoor (*20*), this technology is best suited for sites with shallow contamination (<5 m depth) and moderately hydrophobic pollutants (log K_{ow} = 0.5−3; atrazine, 2.5; simazine, 2.1) (*1*). Rice is a good candidate for phytoremediation because it grows in paddy fields and can remove contaminants from stream water. In many cases, the overexpression of endogenous plant genes or transgenic expression of bacterial or animal genes is required to enhance the phytoremediation properties of plants (*21*).

The use of mammalian P450 genes is one possibility for phytoremediation. The introduction of mammalian P450 species into plants is considered to be a useful technique for producing crops with cross-tolerance to various herbicides (*22*). We have already produced transgenic rice plants that express the human *CYP1A1* gene (*23*). These transgenic rice plants showed herbicide tolerance toward various herbicides, including chlorotoluron, mefenacet, and norflurazon. In addition, transgenic potato plants (*Solanum tuberosum*) expressing rat or human *CYP1A1* show tolerance toward the herbicides chlorotoluron, atrazine, and diuron (*24*, *25*). Our aim was to produce transgenic rice plants by introducing P450s that can be used for phytoremediation, not by accumulating chemicals, but by metabolizing them. In the present study, we produced transgenic rice plants expressing *CYP1A1* that showed herbicide tolerance toward atrazine and simazine by detoxifying them. We also compared the phytoremediation abilities of the plants under three different growing conditions of CYP1A1 rice plants—young seedlings in culture medium, mature plants in culture medium, and mature plants in soil—to remediate these herbicides in practical-use concentrations.

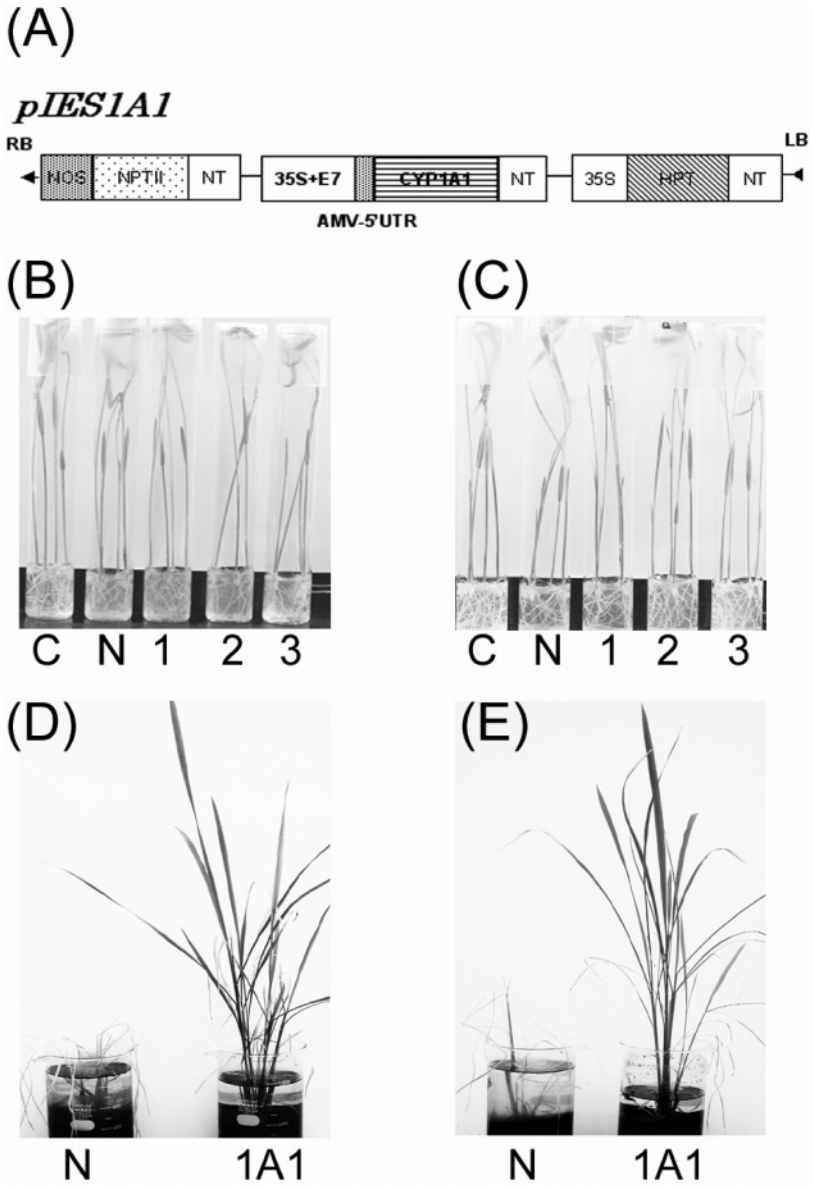

Figure 2. Phytotoxicity of atrazine and simazine toward CYP1A1 rice plants. (**A**) T-DNA regions of the expression plasmid pIES1A1. RB, right border; LB, left border; NOS, nopaline synthase promoter; NT, nopaline synthase terminator; NPTII, neomycin phosphotransferase II; 35S, cauliflower mosaic virus (CaMV) 35S promoter; 35S+E7, 35S promoter with seven-enhancer region (−290 to −90) from CaMV 35S promoter; AMV-5′UTR, alfalfa mosaic virus 5′-untranslated region; HPT, hygromycin B phosphotransferase. (**B**, **C**) Germination tests performed in the MS medium containing 100 μM atrazine (**B**) or 100 μM simazine (**C**). Lane C, nontransgenic plants without herbicide (control); lane N, nontransgenic plants with herbicide; lanes 1–3, CYP1A1 plants (E231, E281, and E441). (**D**, **E**) Herbicide tolerance of CYP1A1 plants in glass pots. Either 953 μg of atrazine (**D**) or 5 mg of simazine (**E**) was added to pots containing 3-week-old rice plants. Growth was recorded 2 weeks after application of herbicide. Lane N, nontransgenics; lane 1A1, CYP1A1 plants (E281).

MATERIALS AND METHODS

Chemicals. ^{14}C-Ring-labeled atrazine [6-chloro-*N*-ethyl-*N*-(1-methylethyl)-1,3,5-triazine-2,4-diamine] (specific activity, 1.99 MBq mg^{-1}; radiochemical purity, 99%) and ^{14}C-ring-labeled simazine [6-chloro-*N*,*N*-diethyl-1,3,5-triazine-2,4-diamine] (specific activity, 1.93 MBq mg^{-1}; radiochemical purity, 97.9%) were provided by Novartis Crop Protection, Inc. (now known as Syngenta, Basel, Switzerland).

Plant Materials. Transgenic rice plants expressing human *CYP1A1* were produced as reported previously (*23*). Briefly, cDNA for human *CYP1A1* was inserted into the expression vector pIES6 to construct the expression plasmid pIES1A1 (**Figure 2A**), which was used for *Agrobacterium*-mediated transformation of *Oryza sativa* cv. Nipponbare (*26*). Regenerated plants were screened for hygromycin resistance and screened by means of Polymerase Chain Reaction (PCR) amplification with human-*CYP1A1*-specific primers. Homozygous R_3 progeny of the selected line, E281, were used in this study. For the germination test, R_3 progeny of the selected lines, E231, E281 and E441, were used.

Germination Tests of R_3 Seeds. Germination tests were done in test tubes (diameter, 2.5 cm; height, 15 cm), each containing 10 mL of MS solid medium (*27*) with atrazine (100 μM) or simazine (100 μM). Five R_3 seeds of transgenic and nontransgenic (control) rice plants were surface-sterilized and embedded in the medium and cultured at 27 °C for 7−14 days under 16 h of light (40 μmol m^{-2} s^{-1}) daily. All experiments were triplicated.

Herbicide Tolerance in Soil. For the tests in a greenhouse, rice seeds were surface-sterilized and embedded in MS solid medium containing 50 mg L^{-1} hygromycin and incubated at 27 °C for 7 days under 16 h of light daily. Ten 7-day-old plants were transplanted to a glass pot (diameter, 9 cm; height, 19 cm) containing 500 mL of water and 500 g of Kumiai-Ryujyou-Baido K soil (Kureha Chemical, Tokyo,

Japan). Transgenic plants and nontransgenic Nipponbare plants were grown in a greenhouse at 28 °C during the day and at 25 °C at night under 13 h of light daily for 2 weeks; we then added 953 μg of atrazine (final concentration, 8.8 μM) or 5 mg of simazine (final concentration, 50 μM) to the water. All experiments were triplicated, and we recorded the growth 2 weeks after application of the herbicide.

Thin-Layer Chromatography (TLC) Analysis. TLC analysis of transgenic rice plants expressing human *CYP1A1* was performed as reported previously (*23*). Briefly, 6-day-old plants were transferred to individual test tubes (diameter, 2.5 cm; height, 15 cm) with 3 mL of sterilized Hyponex 5−10−5 (Hyponex, Osaka, Japan) solution containing 40000 dpm of [^{14}C]atrazine or [^{14}C]simazine at 10 μM. The plants were incubated under 24 h of light (40 μmol m^{-2} s^{-1}) and sampled on day 7 of incubation. Three independent rice plants were analyzed. Radioactive atrazine, simazine, and the metabolites were extracted from plants using a mixture of methanol and water (9:1, v/v). Levels of [^{14}C]-atrazine, [^{14}C]simazine, and the metabolites in the plant extract and culture medium were quantified by a liquid scintillation counter (LS6000TA, Beckman Instruments, Inc., Fullerton, CA). Plant extracts and the culture medium were dried and dissolved in 90% methanol. Aliquotes of 2000 dpm of plant extract and the culture medium were applied to each lane of a silanized silica gel 60F$_{254}$ TLC plate (Merck, Darmstadt, Germany) and developed with chloroform. Radioactivity was measured in an FLA-2000 Bio-Imaging Analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Small-Scale Analysis of Residual Herbicide in Plants and Culture Medium. Rice seeds were surface-sterilized and embedded in MS solid medium containing 50 mg L^{-1} hygromycin and incubated at 27 °C for 7 days under 16 h of light daily. Ten 12-day-old plants were transferred to a 350-mL conical glass beaker with 20 mL of Hyponex solution containing 20 μM atrazine or simazine and incubated at 27 °C for 8 days under 16 h of light daily. All experiments were triplicated. Two hundred microliter aliquots of the culture medium were sampled. The samples underwent high-performance liquid chromatography (HPLC; model LC 10AS, Shimadzu, Kyoto, Japan; column, Cosmosil 5C18-AR-II, 4.6 × 150 mm, Nacalai Tesque, Kyoto, Japan). The solvent systems were acetonitrile/water (50:50, v/v) for atrazine and acetonitrile/water (25:75, v/v) for simazine. The samples were analyzed at 235 nm.

Large-Scale Analysis of Residual Herbicide in the Culture Medium. Residual herbicide analysis of the culture medium was performed using mature rice plants. Hygromycin-resistant 10-day-old plants were transplanted into Kumiai-Ryujyou-Baido K soil in a Wagner pot and grown for 6 weeks in a greenhouse. The roots of the plants were washed carefully, and four rice plants (~100 g of fresh weight) were set into the hole of a polystyrene foam board that covered an enamel pot (diameter, 24 cm; height, 24 cm) holding 9 L of culture medium (10 mM NH_4NO_3, 9.4 mM KNO_3, 1.5 mM $CaCl_2$, 0.75 mM $MgSO_4$, 0.63 mM KH_2PO_4) containing 4.5 μM atrazine or simazine and incubated in a growth chamber at 28 °C during the day and at 25 °C at night under 13 h of light daily. These experiments were duplicated and repeated three times. A 14-mL sample of the culture medium was obtained after 14 days of incubation and loaded onto a Bond Elute LRC-C18 column (Varian Associates, Inc., Harbor City, CA). The bound herbicide was eluted with 2 mL of methanol and analyzed by HPLC as described earlier.

Residual Herbicide Analysis in Plants and Soil. For residual herbicide analysis in soil, 10 7-day-old plants were transplanted into a glass pot (diameter, 9 cm; height, 19 cm) containing 500 mL of water and 500 g of Kumiai-Ryujyou-Baido K soil. Both transgenic plants and nontransgenic Nipponbare plants were grown in a greenhouse at 28 °C during the day and at 25 °C at night under 13 h of light daily for 4 weeks. We then added both 317.9 μg of atrazine (final concentration, 2.95 μM) and 317.9 μg of simazine (final concentration, 3.15 μM) simultaneously into the water. The aerial parts of the plants were harvested, and the soil and water were collected 25 days after herbicide application.

Similarly, both 476.8 μg of atrazine (final concentration, 4.42 μM) and 476.8 μg of simazine (final concentration, 4.72 μM) were simultaneously added to the water of glass pots of both transgenic plants and nontransgenic Nipponbare plants. The soil and water were collected 80 days after herbicide application and subjected to residual herbicide analysis. All experiments were triplicated and repeated twice.

The residual herbicides in the aerial parts of the rice plants and in the soil were analyzed by means of supercritical fluid extraction and gas chromatography−mass (GC-MS) spectrometry (*28*) by Sumitomo Metal Technology Inc. (Hyogo, Japan). The soil and water were mixed well into slurry and filtered through a 2-mm mesh to remove plant roots. Plants were chopped and homogenized; 2-g samples were mixed with 3 g of WetSupport (ISCO Inc., Los Angeles, CA) and extracted using a supercritical fluid extraction system (SFX220, ISCO Inc.) at 13790 kPa and 40 °C for 15 min in stable state and for 15 min in dynamic state. The extracts were trapped in 20 mL of acetone, evaporated to dryness, and dissolved in 1 mL of acetone for analysis by GC-MS (GCMS-2010, Shimadzu) using a DB-5MS column (0.25 mm × 30 m, J&W Scientific, Folsom, CA). The temperature program was raised from 80 °C (2 min) to 200 °C at 20 °C/min and to 260 °C at 5 °C/min, and maintained at 260 °C for 15 min. The injection temperature was 250 °C. A 2-μL aliquot was applied to the column by using the splitless method. The carrier He gas flow rate was 1.46 mL/min. The mass spectrometer was operated in EI ionization mode at 70 eV. The temperature of the source was kept at 250 °C, and the interface and detection temperatures were 250 and 200 °C, respectively. The retention times of atrazine and simazine were 11.4 and 11.2 min, respectively. They were determined at *m*/*z* values of 215 and 201, respectively, by means of selected-ion monitoring (SIM).

RESULTS

Herbicide Tolerance of CYP1A1 Rice Plants. Germination tests were performed using the R_3 seeds of CYP1A1 (E231, E281, E441) and control nontransgenic Nipponbare plants (**Figure 2B**,**C**). CYP1A1 plants in the presence of either 100 μM atrazine or simazine showed healthy growth, which was similar to that of the control plants with and without herbicides. These herbicides did not affect the germination of rice plants under the test conditions. When cultured in soil, CYP1A1 plants (E281) grew well, but the nontransgenic plants did not grow in the presence of atrazine (8.8 μM) or simazine (50 μM) because of inhibition of photosynthesis (**Figure 2D**,**E**). This dosage of atrazine was 1.5 times the value typically used in maize fields; the dosage of simazine was 16 times the value in operational use (*29*). Control plants exhibited growth retardation at 25 μM simazine (8 times the value in operational use in practical use in cornfields) 1 month after application of the herbicide, and they showed more tolerance to simazine than to atrazine. The observed herbicide tolerance of the CYP1A1 rice plants was consistent with in vitro catalysis of these herbicides by recombinant yeast microsomes expressing human *CYP1A1* (*18*).

TLC Analysis Using Radiolabeled Herbicides. We analyzed the metabolism of atrazine and simazine using CYP1A1 plants (E281) and nontransgenic plants (**Figure 3A**,**B**). TLC analysis revealed that the CYP1A1 plants had a high rate of metabolism of these herbicides. On the seventh day after incubation, the mean amount of atrazine in the CYP1A1 plants was decreased to 36% of that in Nipponbare, and that of simazine was decreased to 35% (**Figure 3C**,**D**). The intermediate metabolites produced in the CYP1A1 plants were the same as those in the nontransgenic controls. In the case of atrazine, the total amount of intermediate metabolites, including DEA, DI, and DIDE, was 24.4% of the radioactivity extracted from CYP1A1 plants but only 2% of that for nontransgenics. In the case of simazine, the total amount of intermediate metabolites including DES and DIDES was 28.7% of the radioactivity extracted from the CYP1A1 plants but 11.8% of that extracted from the controls. The metabolism of these herbicides was enhanced by the introduced CYP1A1, which generated the dealkylated metabolites.

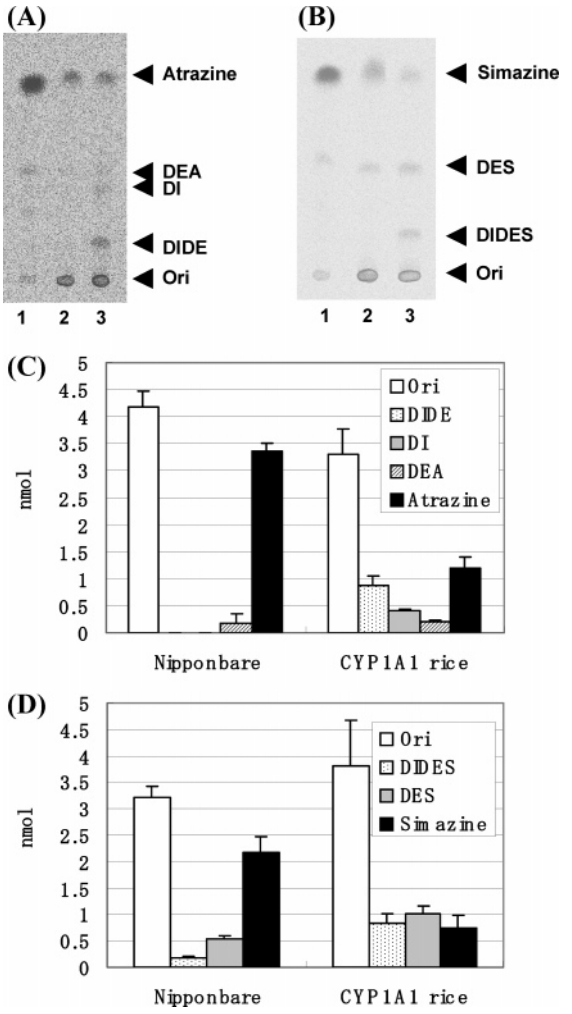

Figure 3. Metabolism of atrazine and simazine by CYP1A1 rice plants: TLC analysis of the extract from plants treated with ^{14}C-labeled atrazine (**A**) or ^{14}C-labeled simazine (**B**). DI, deisopropylated atrazine; DEA, deethylated atrazine; DIDE, deisopropylated and deethylated atrazine; DES, deethylated simazine; DIDES, di-deethylated simazine; Ori, origin of the TLC plate. Lane 1, control medium without plants; lane 2, nontransgenic plants; lane 3, CYP 1A1 plants (E281). (**C**, **D**) Quantities of atrazine (**C**) and simazine (**D**) and their metabolites in rice plants. Values are reported as mean ± 1 standard deviation ($n = 3$, $P < 0.05$ by t test).

Table 1. Residual Herbicides in Culture Medium As Determined by TLC[a]

	TLC (nmol/tube)	
	atrazine	simazine
no plants	26.5 ± 0.013	24.2 ± 1.2
nontransgenic plants	17 ± 0.041	15.6 ± 0.97
CYP1A1 rice plants	11.1 ± 1.7	5.62.4 ± 2.4

[a] Thirty nanomoles of atrazine or simazine was added; samples were collected 7 days later. Values are presented as mean ± 1 standard deviation ($n = 3$, $P < 0.05$ by t test).

Concentrations of both atrazine and simazine decreased markedly in the culture medium of the CYP1A1 plants after 7 days of incubation (**Table 1**). For the CYP1A1 plants, the mean amount of atrazine in the medium decreased to 41.9% of that in control medium, and that of simazine decreased to 23% (**Table 2**). For nontransgenic plants, the mean concentration of atrazine in the medium was 64.1% of the control amount, and that of simazine was 64.4%. Thus, CYP1A1 rice plants absorbed the herbicides and metabolized them more than did nontransgenic rice plants.

Table 2. Residual Herbicide in Culture Medium—Small-Scale Experiment[a]

	conical beaker (nmol/beaker)	
	atrazine	simazine
no plants	451 ± 52	388 ± 6.4
nontransgenic plants	308 ± 36	222 ± 22
CYP1A1 rice plants	196 ± 17	48 ± 0.86

[a] Twenty milliliters of 20 μM atrazine or simazine was added to each beaker. Samples of culture medium were collected 8 days later and analyzed by HPLC. Values are presented as mean ± 1 standard deviation ($n = 3$, $P < 0.05$ by t test).

Small-Scale Analysis of Residual Herbicide in the Culture Medium. When 10 12-day-old CYP1A1 or nontransgenic rice plants were transferred to a conical glass beaker containing 20 μM atrazine or simazine and incubated for 8 days, these herbicides had no effect on growth. For the CYP1A1 plants, the mean amount of atrazine decreased to 43.4% of that in the control medium, and that for simazine decreased to 12.3% (**Table 2**). For nontransgenic plants, the mean concentration of atrazine was 68.3% that in the control medium, and that for simazine was 57.2%. In this small-scale analysis, CYP1A1 plants removed both atrazine and simazine more effectively from the culture medium than did control plants. Both nontransgenic and CYP1A1 plants removed simazine more rapidly than atrazine from the culture medium (**Figure 3**; **Table 2**).

Large-Scale Analysis of Residual Herbicide in Culture Medium. Residual herbicide analysis of the culture medium was performed with 7-week-old mature rice plants in a greenhouse. Rice plants were set on an enamel pot with 9 L of culture medium containing 4.5 μM atrazine or simazine for 2 weeks ($n \geq 3$, $P < 0.05$). The reduction in atrazine and simazine levels (mean ± 1 standard deviation) was 604 ± 208 nmol g^{-1} (dry weight of plants) and 420 ± 152 nmol g^{-1}, respectively, for the CYP1A1 plants. In comparison, the mean reduction of atrazine and simazine by nontransgenic plants was 163 ± 50.6 and 238 ± 143 nmol g^{-1}, respectively. The rate of reduction of atrazine levels was 3.7 times the control value in the CYP1A1 plants, and that of simazine was 1.8 times the control value.

Residual Herbicide Analysis in Plants and Soil. The aerial parts of the rice and the soil treated with both 317.9 μg of atrazine and 317.9 μg of simazine (final concentrations of 2.95 and 3.15 μM, respectively) simultaneously were collected and analyzed 25 days after application of the herbicides (**Figure 4**). Both nontransgenic and CYP1A1 plants showed healthy growth at these herbicide concentrations. The fresh-plant weights (mean ± 1 standard deviation) of control and CYP1A1 plants were 48.4 ± 6.03 and 48.1 ± 5.84 g, respectively. Both atrazine and simazine accumulated in the plants. In the controls, the amounts of atrazine and simazine were 24.4 ± 2.13 and 5.43 ± 1.09 μg plant^{-1}, respectively. In comparison, in CYP1A1 plants, the amounts of atrazine and simazine were 10.5 ± 3.96 and 1.30 ± 0.42 μg plant^{-1}; these concentrations were 43 and 23.9% of the values in nontransgenic plants, respectively (**Figure 4A**).

The residual herbicide concentrations in the soil of the transgenic plants also decreased. The amounts of atrazine and simazine in the soil of CYP1A1 plants were 50.8 ± 11.1 and 94.4 ± 22.2 μg pot^{-1}, respectively, which were 38.1 and 47.9%, respectively, of the values for soil without plants. In comparison, concentrations of atrazine and simazine in the soil of nontransgenic plants were 70.4 ± 11.1 and 125.6 ± 37.4 μg pot^{-1},

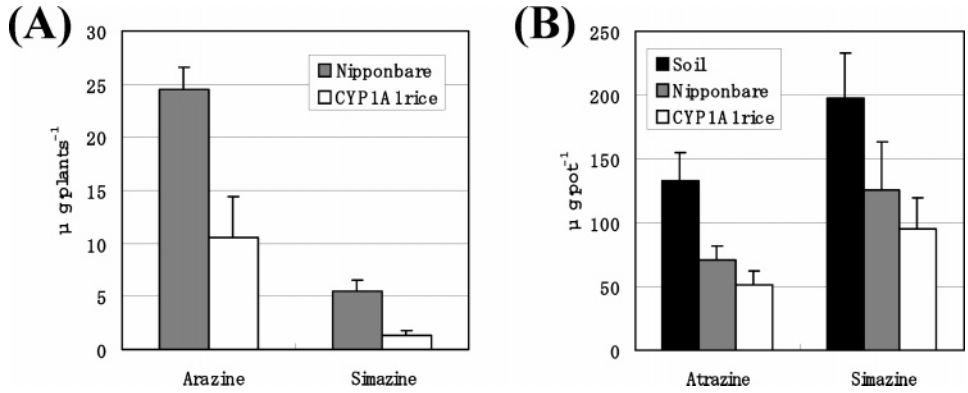

Figure 4. Degradation of atrazine and simazine in CYP1A1 rice plants. (**A**) Total amounts of atrazine and simazine remaining in aerial parts of rice plants ($n = 6$, $P < 0.05$) were analyzed 25 days after application of both 317.5 μg of atrazine and simazine simultaneously. (**B**) Residual atrazine and simazine in soil were analyzed 25 days after application of herbicides. Values are reported as mean ± 1 standard deviation ($n = 6$; $P < 0.05$ for atrazine, $P < 0.1$ for simazine by *t* test).

respectively, which were 52.9 and 63.7%, respectively, of those without plants (**Figure 4B**).

When rice plants were treated with 476.8 μg of atrazine (final concentration, 4.42 μM) and 476.8 μg of simazine (final concentration, 4.72 μM) simultaneously, the CYP1A1 plants showed healthy growth, but nontransgenic plants grew poorly or not at all. In this case, the soils were collected 80 days after application and analyzed. The amounts of atrazine and simazine were 86 ± 27 and 169 ± 51 μg pot^{-1} in the soil without plants. In the CYP1A1 plants, the amounts of atrazine and simazine were 4.7 ± 1 and 6.9 ± 6 μg pot^{-1}, which were only 5.5 and 4.1% of those in soils without plants. In comparison, the amounts of atrazine and simazine in the soil of nontransgenic plants were 54.6 ± 11 and 165 ± 21 μg pot^{-1}, which were 63.6 and 97.7% of those in soil without plants. Under the high concentration of herbicides such as that performed in these test conditions, nontransgenic rice plants were killed by herbicides and only CYP1A1 plants grew healthily and could remediate atrazine and simazine.

DISCUSSION

Phytoremediation is a relatively new field of science and technology that uses plants to clean polluted soil, water, or air (*19*). Many plants—including corn, sorghum, cotton, citrus, black walnut, yellow poplar, Canada thistle, parrot feather, and canna—can take up and metabolize *s*-triazine herbicides (*5*, *30*). It has been proposed that genetic engineering can improve the extent to which plants extract, sequester, and detoxify diverse environmental contaminants (*21*, *31*). We introduced CYP1A1 into rice plants (Nipponbare) to enhance their metabolic activities so as to reduce the residual herbicides in the plants and their environment.

In germination tests, CYP1A1 plants showed strong tolerance to several herbicides with different chemical structures and modes of action at dosages similar to those in practical use (*23*). The photosynthesis inhibitors atrazine and simazine did not affect the growth of nontransgenic rice plants during our germination tests. However, when atrazine or simazine was applied to mature rice plants in soil, CYP1A1 plants showed strong tolerance toward these herbicides, whereas nontransgenic plants were killed due to inhibition of photosynthesis. Germinating rice plants may be able to survive these herbicides because most of their nutrition derives from the seed or because photosynthesis under laboratory conditions does not promote sufficient production of active oxygen species to kill the plants.

Our TLC analysis showed that both control and CYP1A1 plants metabolize atrazine and simazine into the same set of dealkylated metabolites. These results suggest that some endogenous P450 species or oxidases of rice plants metabolize these herbicides in the same manner as does human CYP1A1. More importantly, the intrinsic metabolic pathways of atrazine and simazine in rice plants do not appear to have been perturbed by the introduced CYP1A1. The dealkylated intermediate metabolites, which are less phytotoxic than the parent compounds (*6*), were only just detectable in control plants, mostly at the origin of the TLC plate. This result indicates that the dealkylated metabolites of atrazine and simazine were metabolized quickly into more hydrophilic compounds after oxidation by endogenous P450 enzymes in the control plants. In the CYP1A1 plants, those dealkylated metabolites were detected easily due to enhancement of dealkylation by the introduced CYP1A1. Thus, CYP1A1 rice plants metabolized the herbicides more than nontransgenic rice plants and reduced the residual amount of the herbicides in plants.

In addition, the CYP1A1 plants removed 1.6 times more atrazine and 2.2 times more simazine from culture medium than did nontransgenic controls (**Table 1**) during the incubation time. These results are similar to those from the small-scale analysis of residual herbicide (**Table 2**). Therefore, in two different types of experiments, the CYP1A1 plants removed atrazine and simazine from culture medium more quickly than did the control plants.

In the greenhouse, 25 days after the addition of atrazine and simazine (317.9 μg each) into the soil, the soils of the CYP1A1 plants clearly showed decreased total amounts of both herbicides. The CYP1A1 plants removed 1.3 times more atrazine and 1.4 times more simazine from the soil than did control plants (**Figure 4B**). In contrast, the control plants Nipponbare accumulated 2.3 times more atrazine and 4.2 times more simazine than CYP1A1 plants (**Figure 4A**). This finding shows that the CYP1A1 plants can remediate these herbicides not only within the plants but also by removing them from contaminated soil. Combined with TLC analysis, the herbicide tolerance resulted from CYP1A1-associated enhanced detoxification of these herbicides.

Both atrazine and simazine can be removed by chemical degradation, leaching, and volatilization as well. Because the glass pots did not have any holes, the loss by leaching could be neglected. However, a significant amount of chemical degradation and volatilization could occur in the greenhouse, resulting in the herbicides being removed from the soil. Chemical

degradation and volatilization are considered to occur equally in glass pots with/without plants, and our results showed that rice plants could remove the herbicides from soil. Moreover, CYP1A1 rice plants removed more herbicides than nontransgenic rice plants. The experiments were performed in relatively low concentration, and neither nontransgenic nor CYP1A1 rice plants were damaged by the herbicides. If they were done in higher concentration such as in a herbicide tolerance test (8.8 μM atrazine and 50 μM simazine), only CYP1A1 rice plants could survive and reduce the herbicides from soil. All of the experiments (herbicide tolerance in soil, TLC analysis, and residual herbicide analyses) in the present study support the hypothesis that CYP1A1 rice can reduce the residual amounts of atrazine and simazine in plants as well as in the environment.

Atrazine and simazine are agricultural pesticides commonly used all over the world. In the United States, they are often detected in surface and ground waters (*4*). Because of their widespread use, there is a possibility of nontarget effects of pesticide-associated agricultural runoff. Herbicides and their metabolites may reduce primary productivity by being toxic to phytoplankton, an effect that may change the habitat in aquatic macrophyte communities (*32*, *33*). For example, Hayes and colleagues reported that low levels of atrazine might disrupt the sexual development of wild leopard frogs (*Rana pipiens*) to cause gonadal abnormalities (*34*). The finding suggests that triazine herbicides act as endocrine-disrupting chemicals to wild animals in the environment.

For successful phytoremediation, plants need to have (a) tolerance toward the chemicals or pollutants to be removed, (b) the ability to metabolize or immobilize them, and (c) a sufficiently large biomass to remedy widespread chemicals in the field. Because they meet these criteria in regard to triazine herbicides, CYP1A1 rice plants are good candidates for phytoremediation agents. Similar to the situation reported by Alvord and Kadlec (*35*), who described the effectiveness of wetlands for atrazine removal from river water using constructed ponds and wetlands, transgenic rice plants might remediate soils and water passing through paddy fields. CYP1A1 rice plants appear to be valuable not only as herbicide-tolerant lines but also for the reduction of herbicide residues in the water and soil surrounding the plants themselves. However, further investigations, including safety assessments such as gene flow to wild species from transgenic plants, are needed for the use of transgenic plants.

ABBREVIATIONS USED

DI, deisopropylated atrazine, 2-amino-4-(ethylamino)-6-chloro-1,3,5-triazine; DEA, deethylated atrazine, 2-amino-4-isopropylamino-6-chloro-1,3,5-triazine; DIDE, deisopropylated and deethylated atrazine, 6-chloro-2,4-diamino-1,3,5-triazine; DES, deethylated simazine, 2-amino-4-(ethylamino)-6-chloro-1,3,5-triazine; DIDES, di-deethylated simazine, 6-chloro-2,4-diamino-1,3,5-triazine.

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