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Phytoremediation of the Herbicides Atrazine and Metolachlor by Transgenic Rice Plants Expressing Human *CYP1A1*, *CYP2B6*, and *CYP2C19*

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This study evaluated the expression of human cytochrome P450 genes *CYP1A1*, *CYP2B6*, and *CYP2C19* in rice plants (*Oryza sativa* cv. Nipponbare) introduced using the plasmid pIKBACH. The transgenic rice plants (pIKBACH rice plants) became more tolerant toward various herbicides than nontransgenic Nipponbare rice plants. Rice plants expressing pIKBACH grown in soil showed tolerance to the herbicides atrazine, metolachlor, and norflurazon and to a mixture of the three herbicides. The degradation of atrazine and metolachlor by pIKBACH rice plants was evaluated to confirm the metabolic activity of the introduced P450s. Although both pIKBACH and nontransgenic Nipponbare rice plants could decrease the amounts of the herbicides in plant tissue and culture medium, pIKBACH rice plants removed greater amounts in greenhouse experiments. The ability of pIKBACH rice plants to remove atrazine and metolachlor from soil was confirmed in large-scale experiments. The metabolism of herbicides by pIKBACH rice plants was enhanced by the introduced P450 species. Assuming that public and commercial acceptance is forthcoming, pIKBACH rice plants may become useful tools for the breeding of herbicide-tolerant crops and for phytoremediation of environmental pollution by organic chemicals.

KEYWORDS: Cytochrome P450 (CYP); tolerance; environmental pollution; norflurazon; simazine; paddy field

INTRODUCTION

Atrazine is a systemic *s*-triazine herbicide that inhibits electron transport during photosynthesis and causes chlorosis. Metolachlor is a chloroacetanilide herbicide that inhibits very-long-chain fatty acid (VLCFA) synthesis in plants (*1*). Both herbicides are used for pre- and postemergence control of annual grasses and broad-leaved weeds in many crops, including maize, sorghum, and turf grasses (*2*).

Pesticides have played very important roles against food shortages by reducing risk of crop loss due to plant diseases. However, nontarget effects of pesticides are an important environmental consideration, especially when runoff contaminates surface water. It is estimated that ∼1−5% of field-applied herbicides are removed by surface runoff (*3*). Atrazine has become a common contaminating herbicide of surface water and groundwater in the United States and in European countries because of its water solubility and its widespread use (*4*). Similarly, metolachlor and its metabolites have been detected in streams, rivers, ponds, and wells (*5*, *6*).

Phytoremediation, the use of living plants to remove and/or detoxify organic and inorganic compounds, is an appealing strategy for cleaning contaminated sites (*7*, *8*). It is a possible method for reducing the risks of exposure of people and the environment to pesticides. The main benefit of phytoremediation is economic. Current physical cleanup methods such as removing contaminated soil from a site and chemical remediation treatments are very costly and sometimes environmentally destructive. Plants can remove organic pollutants, including pesticides, by root and leaf uptake of contaminants, biochemical degradation, and subsequent accumulation of non-phytotoxic metabolites in plant tissue.

According to Schnoor et al. (*7*), this technology is suitable for sites with shallow contamination (<5 m in depth), moderately hydrophobic pollutants [log K_{ow} (log octanol/water partition coefficient) = 0.5−3], short-chain aliphatic chemicals, and nutrients. The log K_{ow} values for atrazine and metolachlor are 2.5 and 2.9 at 25 °C, respectively, and their water solubilities

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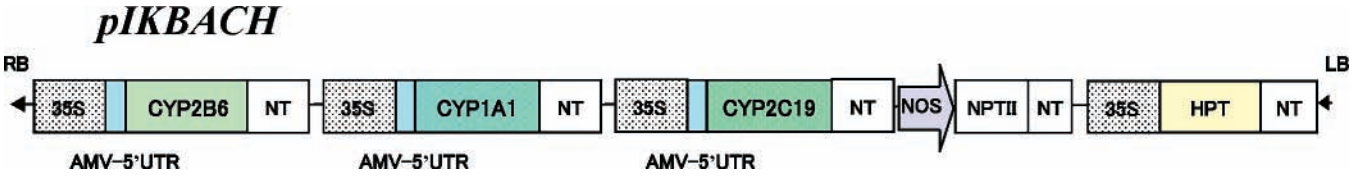

Figure 1. T-DNA region of the expression plasmid pIKBACH. CYP1A1, CYP2B6, and CYP2C19 were expressed constitutively. RB, right border; LB, left border; NOS, nopaline synthase promoter; NT, nopaline synthase terminator; NPTII, neomycin phosphotransferase II; 35S, cauliflower mosaic virus (CaMV) 35S promoter; AMV-5′UTR, alfalfa mosaic virus 5′-untranslated region; HPT, hygromycin B phosphotransferase.

are 33 and 488 mg/L (*2*). Therefore, atrazine and metolachlor are candidates for removal from the environment by phytoremediation.

Phytoremediation has been shown to be useful in the dissipation of atrazine (*9*), chlorpyrifos (*10*), metolachlor (*11*), and other chlorinated compounds (*7*, *12*). Moreover, rice plants are good candidates for phytoremediation because they grow in paddy fields. Therefore, rice plants can remove contaminants from both soil and streamwater. In many cases, overexpression of endogenous plant genes or transgenic expression of bacterial or animal genes is required to enhance the phytoremediation properties of plants (*13*).

The use of plants expressing high activity of P450s is a potential strategy for phytoremediation of certain xenobiotics. P450 monooxygenases insert one atom of oxygen into hydrophobic molecules, making them more reactive and water-soluble, through hydroxylation, oxidative dealkylation, and epoxidation (*14*). One pathway for metabolism of atrazine in plants is N-dealkylation. If both the ethyl and isopropyl groups are removed by N-dealkylation, the metabolite is no longer phytotoxic (*15*). In sorghum microsomes, metolachlor was catalyzed by O-demethylation to a single demethylated metabolite (*16*). The enzymes responsible for N-dealkylation and O-demethylation in plants have not been elucidated but are thought to be P450s. More than 1000 plant P450 sequences have been registered in the P450 database (*17*, *18*), but molecular level information on plant P450s involved in xenobiotic metabolism is limited.

In contrast, several microsomal P450s involved in xenobiotic metabolism have been well studied in mammals, and individual P450s show overlapping, broad substrate specificity. These animal P450s have abilities to metabolize a variety of hydrophobic compounds, including herbicides (*19*). Using an in vitro yeast microsome system, Inui et al. found that human CYP1A1, CYP2B6, and CYP2C19 metabolized 14, 10, and 17 herbicides, respectively (*20*). Those human P450s metabolize atrazine and metolachlor to N-dealkylated atrazine and O-demethylated metolachlor, which are the same compounds as those reported in plants (*20*).

The introduction of these animal P450s into plants is considered to be a useful technique for producing crops with cross-tolerance to various herbicides (*19*), although the major metabolism pathway of both herbicides for detoxification is glutathione conjugation. We have already produced transgenic rice plants that coexpress human *CYP1A1*, *CYP2B6*, and *CYP2C19* genes (pIKABCH rice plants) (*21*). The transgenic rice plants showed cross-tolerance to eight herbicides that were classified into five different groups according to their site of action and chemical structure by detoxifying them in germination tests (*21*). In the present study, we found that pIKBACH rice plants grown in soil had tolerance toward several herbicides, including atrazine and metolachlor. We also evaluated the ability of pIKBACH rice plants to decrease atrazine and metolachlor concentrations under two different conditions: remediation of a hydroponic medium by young seedlings on a small scale and remediation of soil in glass pots and stainless steel pools. We discuss the potential use of pIKBACH rice plants for phytoremediation of herbicides, including atrazine and metolachlor.

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%) was provided by Novartis Crop Protection, Inc. (currently Syngenta, Basel, Switzerland).

Plant Materials. Transgenic rice plants transformed with pIKBACH were produced as reported previously (*21*). Briefly, the expression plasmid pIKBACH, which harbors *CYP1A1*, *CYP2B6*, and *CYP2C19* in tandem (**Figure 1**), was used for *Agrobacterium*-mediated transformation of *Oryza sativa* cv. Nipponbare (*22*). Regenerated plants were screened by hygromycin resistance and by means of Polymerase Chain Reaction (PCR). Homozygous R_3 progeny of the selected line, 3r35, was used in this study.

Herbicide Tolerance in Soil. For herbicide tolerance tests in a greenhouse, rice seeds were surface-sterilized, 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 25 °C at night under 13 h of light daily for 2 weeks. Atrazine, metolachlor, and norflurazon were added to attain concentrations of 5.9, 12.4, and 16.7 μM, respectively. A mixture of herbicides treated at a rate of 4.4, 9.5, and 12.5 μM atrazine, metolachlor, and norflurazon, respectively, was also evaluated. The growth was observed 2 weeks after application of the herbicides.

Analysis of Atrazine Metabolites in Transgenic and Nontransgenic Rice Plants. The metabolism of atrazine was determined in hydroponically grown plants. 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 670 Bq of [^{14}C]atrazine 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. Thin-layer chromatography (TLC) analysis of pIKBACH rice plants was performed as reported previously (*23*). Radioactive chemicals were extracted from whole plants by 90% (v/v) methanol and the culture medium. The samples were dried up and dissolved in 90% methanol and analyzed by a silanized silica gel 60F_{254} TLC plate (Merck, Darmstadt, Germany) with chloroform as developing solvent. RF values of atrazine, DE, DI, and DIDE are 0.70, 0.44, 0.37, and 0.13, respectively. Radioactivity was measured in an FLA-2000 Bio-Imaging Analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Small-Scale Analysis of Residual Herbicides in Hydroponic Medium. Rice seeds were surface-sterilized, embedded in MS solid medium containing 50 mg L^{-1} hygromycin, and incubated at 27 °C 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 or 30 μM metolachlor and incubated at 27 °C for 6 days under 16 h of light daily. All experiments were triplicated. Two hundred microliter aliquots of the hydroponic medium were sampled. The samples were subjected to 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 and metolachlor. Atrazine and simazine were detected at 235 nm, and metolachlor was detected at 230 nm.

Small-Scale Analysis of Residual Herbicides in Plants and Soil. For analysis of residual herbicides 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 25 °C at night under 13 h of light daily for 4 weeks. We then added the mixture of herbicides that consisted of 317.9 μg of atrazine (final concentration, 2.95 μM), 880 μg of metolachlor (final concentration, 6.2 μM), and 633 μg of norflurazon (final concentration, 4.18 μM) into the water. Four replicate pots were used for transgenic and nontransgenic rice plants. The aerial parts of the plants were harvested, and the soil and water were collected 20 days after herbicide application.

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 (*24*) by Sumitomo Metal Technology Inc. (Hyogo, Japan), as described previously (*25*). Briefly, the herbicides from plants and soil (*24*) were extracted by supercritical carbon dioxide using a supercritical fluid extraction system (SFX220, ISCO Inc.) and analyzed 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, to 260 °C at 5 °C/min, and held at 260 °C for 15 min. The carrier He gas flow rate was 1.46 mL/min. The mass spectrometer was operated in EI mode at 70 eV. The retention times of atrazine and metolachlor were 11.5 and 15.1 min, respectively. They were determined at *m*/*z* values of 215 and 238, respectively, by means of selected-ion monitoring (SIM).

Large-Scale Analysis of Residual Herbicides in Plants and Soil. For a large-scale experiment in a greenhouse, 24 7-day-old plants were transplanted to a stainless steel pool (length, 90 cm; width, 55 cm; height, 32 cm) with 50 L of water and 60 kg of Andosol (local soil) supplied with 19.8 g of chemical fertilizer (N−P−K, 14−14−14) and fused magnesium phosphate fertilizer. The rice plants were planted 5 cm from the edge of the pool. A distance of 15 cm was left between rows running parallel to the 55 cm side, and 16 cm was left between rows running parallel to the 90 cm side. Both transgenic plants and nontransgenic Nipponbare plants were grown in each pool in the greenhouse for 5 weeks. Then both 99 μL of Gesapurim (Novartis Crop Protection Inc.), which contained 210 μmol of atrazine (final concentration, 4.2 μM), and 99 μL of Dual (Novartis Crop Protection Inc.), which contained 146 μmol of metolachlor (final concentration, 2.9 μM), were added to the water simultaneously. At 1 month after herbicide application, the aerial parts of the rice plants were harvested. Soil and water were mixed well in the pools, and the slurry samples were collected at four points in each pool.

The residual herbicides in the aerial parts of the rice plants and in the soil were analyzed by supercritical fluid extraction and GC-MS spectrometry (*24*) by Sumitomo Metal Technology Inc. as described under Residual Herbicide Analysis in Plants and Soil.

RESULTS

Herbicide Tolerance of pIKBACH Transgenic Rice Plants. R_3 seeds of the pIKBACH and nontransgenic rice plants Nipponbare were used for tests of herbicide tolerance in soil. pIKBACH rice plants showed healthy growth in soil containing atrazine (5.9 μM), metolachlor (12.4 μM), and norflurazon (16.7 μM) (**Figure 2A**−**C**). These concentrations are ∼2-fold higher than the recommended label rate used in fields (*26*). In comparison, the growth of nontransgenic Nipponbare plants was inhibited by treatment with either herbicide at those concentrations (**Figure 2**). In addition, pIKBACH rice plants showed tolerance toward the mixture of herbicides (4.4 μM atrazine, 9.5 μM metolachlor, and 12.5 μM norflurazon), whereas nontransgenic rice plants were severely damaged (**Figure 2D**).

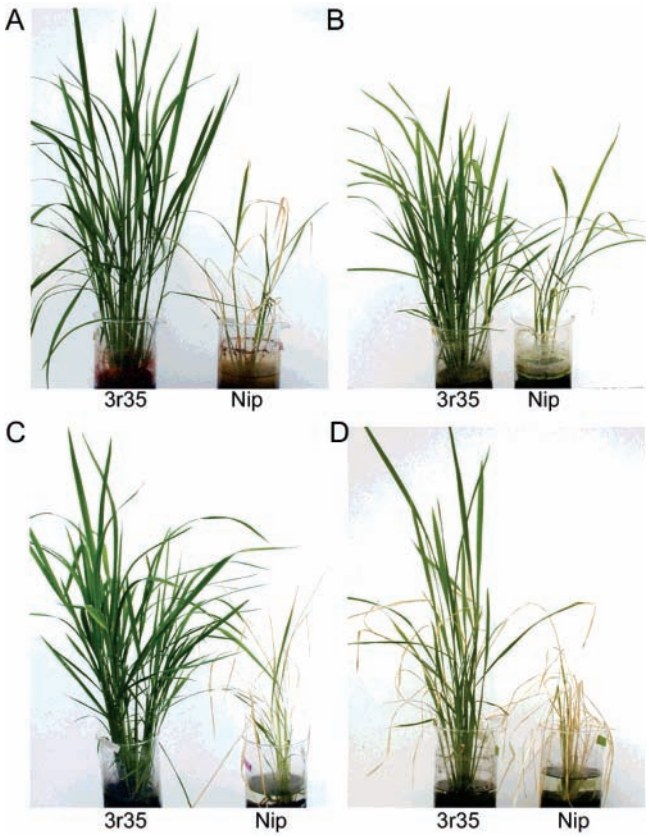

Figure 2. Herbicide tolerance of pIKBACH rice plants: (**A**) tolerance to atrazine (2.18 mg of atrazine was applied); (**B**) tolerance to metolachlor (1.76 mg of metolachlor was applied); (**C**) tolerance to norflurazon (1.76 mg of norflurzaon was applied); (**D**) tolerance to the mixture of herbicides (476.8 μg of atrazine, 1.32 mg of metolachlor, and 1.9 mg of norflurazon). Plant growth was recorded 2 weeks after application of herbicides. 3r35, pIKBACH rice plants; Nip, nontransgenic rice plants of Nipponbare.

Analysis of Atrazine Metabolites in Transgenic and Nontransgenic Rice Plants. The enhanced metabolism of atrazine was confirmed in pIKBACH rice plants (3r35) (**Figure 3A**). Although atrazine was metabolized in both transgenic and nontransgenic rice plants, it was metabolized more rapidly in the pIKBACH rice plants. On the seventh day of incubation, the mean amount of atrazine in the pIKBACH rice plants was 62.4% of that found in nontransgenic plants (**Figure 3B**). The intermediate N-dealkylated metabolites produced in the pIKBACH rice plants were the same compounds as those in the nontransgenic controls. The total amount of those metabolites, including DE, DI, and DIDE, was increased in the pIKBACH rice plants compared to that in the nontransgenic rice plants, which was 19.4% of the radioactivity extracted from pIKBACH rice plants. In both pIKBACH and nontransgenic rice plants, a significant amount of polar metabolites accumulated (at the origin).

The pIKBACH rice plants decreased the mean amount of atrazine in the medium to 75% of that in the case of nontransgenic plants (**Figure 3C**). Thus, pIKBACH rice plants metabolized atrazine in the plants and decreased the residual amount of atrazine in the culture medium effectively by the introduction of P450s. The metabolism of metolachlor by pIKBACH and CYP2B6 rice plants was previously reported (*21*, *27*). pIKBACH rice plants metabolize metolachlor to its O-demethylated metabolites.

Small-Scale Analysis of Residual Herbicides in Hydroponic Medium. When both pIKBACH rice plants and the

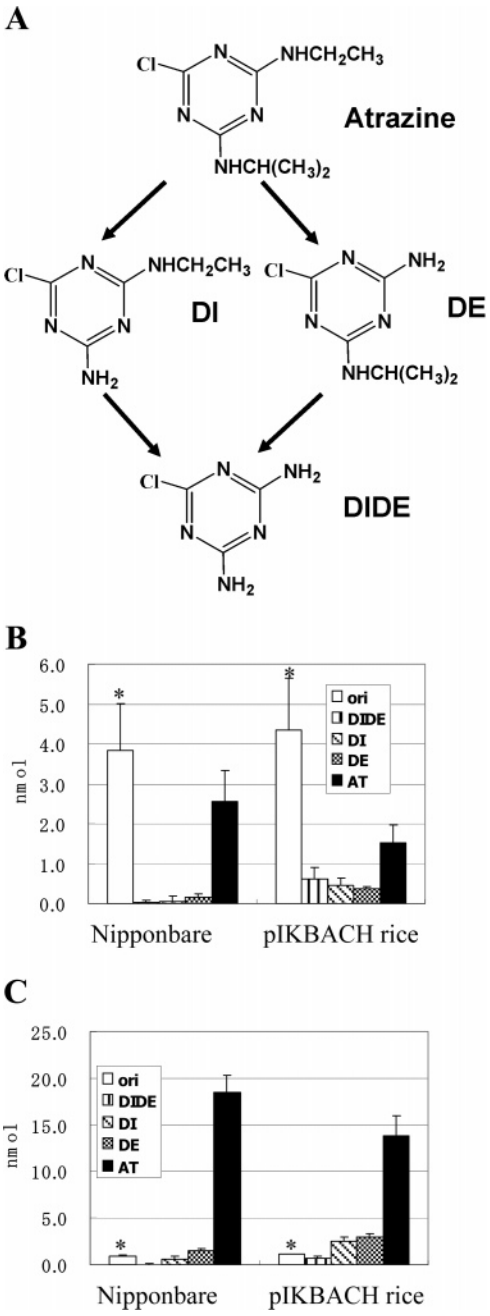

Figure 3. Metabolism of atrazine by pIKBACH rice plants: (**A**) schematic metabolic pathways of atrazine in plants (DI, deisopropylated atrazine; DE, deethylated atrazine; DIDE, deisopropylated and deethylated atrazine); (**B**, **C**) thin-layer chromatography analysis of pIKBACH rice plants treated with ^{14}C-labeled atrazine [(**B**) plant extracts; (**C**) culture medium]. Values are reported as means ± SD ($n = 3$, $p < 0.05$ by *t* test; *, no significant difference).

control plants were transferred to glass beakers and incubated for 6 days, none of the herbicides had an effect on their growth during the incubation period. The pIKBACH rice plants were able to reduce the amounts of atrazine, metolachlor, and simazine in the hydroponic medium by 42.6, 18.3, and 30.7%, respectively, of those of the control, whereas Nipponbare plants reduced the amounts in the medium by 68.1, 38.5, and 60.5%, respectively (**Table 1**). Therefore, the pIKBACH rice plants removed these herbicides 1.3−1.8 times more effectively from the culture medium than did the Nipponbare plants during the incubation period.

Table 1. Small-Scale Analysis of Residual Herbicides in Hydroponic Medium[a]

	atrazine		metolachlor		simazine	
	nmol/beaker	%	nmol/beaker	%	nmol/beaker	%
control	413 ± 13.6	100	566 ± 9.73	100	397 ± 28.1	100
Nipponbare	281 ± 36.2	68.1	218 ± 18.5	38.5	240 ± 35.3	60.5
pIKBACH rice	176 ± 20.2	42.6	106 ± 13.3	18.7	122 ± 10.6	30.7

[a] Atrazine or simazine (20 mL of 20 μM) or metolachlor (20 mL of 30 μM) was added to each beaker. Samples of hydroponic medium were collected 6 days later and analyzed by HPLC. Values are presented as means ± SD ($n = 3$) and significantly different at $p < 0.05$ by Duncan's multiple-range test.

Table 2. Small-Scale Analysis of Residual Herbicides in Plants and Soil[a]

		plants	soil	
herbicide	line	μg/g of fresh wt	μg/pot	%
atrazine	soil	–	118 ± 16a	100
	Nipponbare	0.64 ± 0.1a	108 ± 16ab*	91.5
	pIKBACH rice	0.46 ± 0b	87.7 ± 14b*	74.3
metolachlor	soil	–	794 ± 99a	100
	Nipponbare	0.5 ± 0.2a	559 ± 72b	70.4
	pIKBACH rice	0.05 ± 0b	288 ± 107c	36.3
norflurazon	soil	–	160 ± 17a	100
	Nipponbare	1.36 ± 0.3a	177 ± 15a	111
	pIKBACH rice	0.24 ± 0.1b	114 ± 11b	71.3

[a] The mixture of herbicides (final concentrations: 2.95 μM atrazine, 6.2 μM metolachlor, and 4.18 μM norflurazon) was applied into each glass pot with 5-week-old plants. Aerial parts of rice plants and the soil were analyzed 20 days after application. Each value presented is the mean ± SD of four samples. –, not determined. Values within columns followed by different letters are significantly different at $p < 0.05$ by Duncan's multiple-range test. *, values are significantly different between the samples at $p < 0.1$.

Small-Scale Analysis of Residual Herbicides in Plants and Soil. The aerial parts of the rice plants and the soil treated with the herbicide mixture containing 2.95 μM atrazine, 6.2 μM metolachlor, and 4.18 μM norflurazon were collected and analyzed 20 days after application of the herbicides (**Table 2**). Nontransgenic rice plants were showed damaged growth, but pIKBACH rice plants showed healthy growth at these herbicide concentrations. The fresh plant weights of nontransgenic and pIKBACH rice plants were 20.3 ± 2.8 and 38.6 ± 5.5 g (mean ± SD, $n = 4$, $p < 0.01$ by *t* test), respectively. In pIKBACH rice plants, the amounts of atrazine, metolachlor, and norflurazon were clearly decreased compared to those in nontransgenic rice plants. The concentrations of atrazine, metolachlor, and norflurazon were 71.8, 10, and 17.6% of the values in nontransgenic plants, respectively (**Table 2**).

The residual herbicide concentrations in soil with pIKBACH rice plants also decreased. The amounts of atrazine, metolachlor, and norflurazon in soil with pIKBACH rice plants were decreased to 74.3, 36.3, and 71.3%, respectively, of the amounts in soil without plants. In comparison, concentrations of the herbicides in soil with nontransgenic plants were 91.5, 70.4, and 111%, respectively, of those in soil without plants.

Large-Scale Analysis of Residual Herbicides in Plants and Soil. The fate of atrazine and metolachlor in soil as affected by rice plants was assessed in larger scale studies (**Table 3**). Both 4.2 μM atrazine and 2.9 μM metolachlor (final concentrations) were applied to the soil of plants grown in stainless steel pools, and the amounts of herbicides present 1 month after application were analyzed. The pIKBACH and the nontransgenic rice plants showed healthy growth (fresh weights of plants, 12.2 ± 6.3

Table 3. Large-Scale Analysis of Residual Herbicides in Plants and Soil[a]

	plants		soil		
herbicide	line	μg/g of fresh wt	μg/g of dry wt	%	% remaining[b]
atrazine	soil only	–	0.562 ± 0.05a	100	65.2
	Nipponbare	2.08 ± 0.29a	0.524 ± 0.08a	93.2	60.8
	pIKBACH rice	1.32 ± 0.36b	0.394 ± 0.07b	70.1	45.7
metolachlor	soil only	–	0.355 ± 0.04a	100	45.1
	Nipponbare	0.072 ± 0.02a	0.355 ± 0.06a	100	45.2
	pIKBACH rice	0.023 ± 0.02b	0.261 ± 0.07b	73.5	33.0

[a] Both 4.2 μM atrazine and 2.9 μM metolachlor (final concentrations) were applied simultaneously into a stainless pool with 1.5-month-old plants. Aerial parts of rice plants and the soil were analyzed 1 month after application. Each value presented is the mean ± SD of four samples. Values within columns followed by different letters are significantly different at $p < 0.05$ by Duncan's multiple-range test. [b] Percent of remaining herbicides compared with those added into the soil.

and 10.9 ± 4.3 g, respectively, $n = 24$). The mean concentrations of atrazine and metolachlor in the pIKBACH rice plants were decreased to 63.5 and 31.9%, respectively, of those in nontransgenic rice plants. The mean concentrations of atrazine and metolachlor in soil with pIKBACH rice plants were decreased to 70.1 and 73.5%, respectively, of those in control soil without any plants, whereas the mean concentrations in soil with nontransgenic rice plants were 93.2 and 100%, respectively. The residual amounts atrazine and metolachlor in soil with pIKBACH rice plants were calculated to be 45.7 and 33.0%, respectively, of those initially applied to soil, whereas those in soil with nontransgenic rice plants were 60.8 and 45.2%, respectively. Therefore, pIKBACH rice plants can remediate both atrazine and metolachlor from the soil in large-scale conditions.

DISCUSSION

Phytoremediation is a relatively unexploited technology that uses plants to clean up polluted soil, water, or air economically. Using the tools of genetic engineering, the potential ability of plants for phytoremediation can be enhanced, and it is expected that such plants can be used to extract and detoxify a wide variety of environmental contaminants, including herbicides (*13*, *28*). The expression plasmid pIKBACH was introduced into Nipponbare rice plants to enhance their metabolic activity and decrease residual herbicides in the plants and their environments.

In germination tests, pIKBACH rice plants showed strong tolerance to eight herbicides and to two types of mixture of herbicides with different chemical structures and modes of action at dosages similar to those in practical use (*22*). The photosynthesis-inhibiting herbicide atrazine did not affect the growth of nontransgenic rice plants during germination tests (*25*). However, when atrazine was applied to rice plants grown in soil, pIKBACH rice plants showed strong tolerance, whereas nontransgenic plants were killed because of the inhibition of photosynthesis.

In addition, pIKBACH rice plants grew well in the presence of the VLCFA synthesis-inhibiting herbicide metolachlor and in the presence of the carotenoid synthesis-inhibiting herbicide norflurazon, but nontransgenic Nipponbare plants did not. When the mixture of atrazine, metolachlor, and norflurazon was applied to rice plants in soil, pIKBACH rice plants showed strong tolerance during vegetative growth, whereas the growth of nontransgenic Nipponbare plants was severely impaired. pIKBACH rice plants in soil also showed tolerance toward chlorotoluron, diuron, simazine, and mefenacet (data not shown). The observed herbicide tolerance of pIKBACH rice plants resulted from the combination of metabolic activities of CYP1A1, CYP2B6, and CYP2C19. Therefore, pIKBACH rice plants exhibited multiple herbicide tolerance, and they can be used for effective weed control by herbicides with different modes of action.

Our TLC analysis showed that both nontransgenic and pIKBACH rice plants metabolize atrazine into the same set of N-dealkylated metabolites, which are less phytotoxic (*29*). The amounts of intermediate metabolites produced by pIKBACH rice plants were increased compared to those produced by nontransgenic rice plants. Our previous study showed that pIKBACH rice plants metabolize metolachlor into its nonphytotoxic or less phytotoxic O-demethylated metabolite (*27*). These studies indicated that similar metabolites are formed in both transgenic and nontransgenic rice plants.

In small-scale analysis in glass beakers (**Table 1**), the residual herbicides in the hydroponic medium of pIKBACH rice plants were clearly decreased more than were those in the hydroponic medium of nontransgenic rice plants. Therefore, combining those results, pIKBACH rice plants can metabolize both atrazine and metolachlor by detoxifying them and also have abilities to decrease residual herbicides in a hydroponic medium in laboratory experiments.

In small-scale greenhouse experiments using soil, the residual concentrations of atrazine, metolachlor, and norflurazon were decreased in soils grown with pIKBACH rice plants compared with nontransgenic rice plants. Likewise, lower concentrations of these herbicides were found in pIKBACH rice plants compared with nontransgenic rice plants (**Table 2**). All herbicides can be removed by chemical degradation and volatilization as well. Actually, norflurazon can be degraded by sunlight (*2*). However, chemical degradation and volatilization are considered to occur equally in glass pots with and without plants. Thus, our results showed that rice plants could remove the herbicides from soil.

We also checked the remediation ability of pIKBACH rice plants grown in a stainless steel pool for 1 month. The half-life of atrazine was 16−77 days (median, 41 days) in the field and that of metolachlor was 20 days in the field (*2*). The remaining amounts of atrazine and metolachlor in the soil were 65.2 and 45.1% of the herbicides added, respectively, which is consistent with the half-lives of former studies. The remaining amounts of atrazine and metolachlor in the soil of nontransgenic Nipponbare were 60.8 and 45.2% of the herbicides added, respectively, and those of pIKBACH rice plants were 45.7 and 33.0%, respectively. The results showed that pIKBACH rice plants can remove greater amounts of the herbicides from soil. The differences between total residual amounts of atrazine and between total residual amounts of metolachlor in the soil of Nipponbare and pIKBACH rice plants were 6.86 and 5.04 mg. Therefore, in this experimental condition, the differences between remediation abilities of Nipponbare and pIKBACH rice plants are estimated to be 13.69 g/1000 m^2 for atrazine and 10.2 g/1000 m^2 for metolachlor in 1 month.

Our combined results (small- and large-scale evaluations of remediation of culture medium and soil) show that pIKBACH rice plants can decrease the amounts of atrazine and metolachlor in contaminated water and soil. In addition, Inui et al. reported that human P450s involved in xenobiotic metabolism can metabolize herbicides, insecticides, and industrial chemicals (*20*). Presumably, the chemicals and pollutants that can be

metabolized by CYP1A1, CYP2B6, and CYP2C19 can be remediated by pIKBACH rice plants.

Water quality and agriculture are closely linked, because nonpoint-source pollution of lakes, rivers, and streams is caused by agricultural runoff. The long-term effects from exposure to low levels of herbicides are difficult to detect, but herbicides and their metabolites may reduce primary productivity by being toxic to phytoplankton, an effect that may change the organisms within adjacent aquatic and terrestrial ecosystems (*30*). For example, reduction of species richness in aquatic communities caused by insecticides and herbicides has been reported (*31*). Also, low levels of atrazine might disrupt the sexual development of wild leopard frogs (*Rana pipiens*) to cause gonadal abnormalities (*32*).

For phytoremediation of atrazine and metolachlor, we have already shown the remediation abilities of CYP1A1 and CYP2B6 rice plants (*25*, *27*). pIKBACH rice plants are more suitable for phytoremediation because they have a much broader spectrum of herbicide tolerance and metabolic activities toward these chemicals to be removed, both in the field and in water passing through paddy fields. Moreover, pIKBACH rice plants can be expected to metabolize other chemicals. However, further investigations, including the safety assessment of these transgenic plants, are needed. In particular, the possibility of gene flow from transgenic plants to their wild species must be evaluated. In the future, pIKBACH rice plants may become useful for degrading and decreasing the environmental loads of these herbicides and other chemicals in paddy fields.

ABBREVIATIONS USED

DI, deisopropylated atrazine, 2-amino-4-(ethylamino)-6-chloro-1,3,5-triazine; DE, 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.

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