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Transgenic Rice Containing Human CYP2B6 Detoxifies Various Classes of Herbicides

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The human gene for CYP2B6, a cytochrome P450 monooxygenase that inactivates xenobiotic chemicals, was introduced into *Oryza sativa* cv. Nipponbare by *Agrobacterium*-mediated transformation. At germination, R_1 seeds of transgenic rice plants expressing CYP2B6 (CYP2B6 rice) showed a high tolerance to 5 μM metolachlor, a preemergence herbicide that is degraded by CYP2B6. Thin-layer chromatography after culture with ^{14}C-labeled metolachlor revealed that the amounts of residual metolachlor decreased in plant tissues and the medium of CYP2B6 rice faster than those of untransformed Nipponbare. CYP2B6 rice plants were able to grow in the presence of 13 out of 17 herbicides: five chloroacetamides and mefenacet, pyributicarb, amiprofos-methyl, trifluralin, pendimethalin, norflurazon, and chlorotoluron. These herbicides differ in their modes of action and chemical structures. Transgenic rice expressing a xenobiotic-degrading human CYP2B6, which has broad substrate specificity, should be good not only for developing herbicide tolerant rice but also for reducing the environmental impact of agrochemicals.

KEYWORDS: Human P450; herbicide tolerance; phytoremediation; metolachlor; chloroacetamides; dinitroanilines

INTRODUCTION

Weed infestation adversely affects crop production both quantitatively and qualitatively by reducing yields and decreasing market prices of the crop. Simple economics dictates that for cost-effective land use, crop yield and price must be maximized and the cost of weed control must be minimized (*1*). Herbicides are viewed as a labor-saving means of improving crop yield and quality. In addition, using chemicals for weed control leaves a biomass residue that helps conserve soil and moisture, whereas mechanical weed control disturbs the soil and results in a loss of soil moisture and in erosion. Herbicides are now widely used for crop cultivation and for management of lawns, railroad rights-of-way, highway margins, and other purposes, although herbicide residues themselves can damage the environment. To decrease the net herbicide load on the environment, intentional degradation of these chemicals should be as much a goal as reduction of their use.

Another problem with the use of herbicides is that their use can selectively accelerate the evolution of herbicide resistant weeds. Indeed, many herbicide resistant weeds to various types of herbicides have been reported in agricultural fields and gardens worldwide (http://www.weedscience.org/in.asp). Repeated use of the same herbicide in a field tends to promote the emergence of resistant weeds. To avoid the appearance of resistant weeds, using several herbicides in rotation or in a mixture is recommended for a weed control system (*2*). To enable crop plants to grow under such an herbicide regime, it should be helpful to develop transformed plants containing a gene for a single mammalian P450 enzyme, which would detoxify several types of herbicides, thereby giving these plants cross-tolerance to herbicides. Two major mechanisms of resistance, modification of the target site and development of enhanced detoxification, have been identified (*2*). Crop tolerance to certain herbicides is also often due to the ability of the crop to metabolize the herbicide and thereby prevent injury.

Two enzyme systems that play major roles in conferring tolerance to herbicides by degrading them have been reported. One of them, the cytochrome P450 monooxygenase (P450) system, consisting of cytochrome P450 and NADPH-cytochrome P450 oxidoreductase, catalyzes monooxygenation of lipophilic xenobiotic compounds, including herbicides (*3*). The products of monooxygenation are conjugated with water soluble molecules such as glucose by other enzyme systems (*4*). Subsequently, the conjugated metabolites are converted to secondary conjugates or insoluble bound residues. These metabolites are deposited in vacuoles or into cell walls of plants.

Recently, over 1000 plant P450 sequences have been listed in the database (*5*). Some of the P450s are involved in secondary

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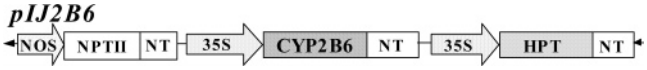

Figure 1. Structure of *CYP2B6* expression plasmid. T-DNA region of pIJ2B6 plasmid used to express human *CYP2B6* gene. NOS, nopaline synthase promoter; NT, nopaline synthase terminator; NPTII, neomycin phosphotransferase II; 35S, cauliflower mosaic virus (CaMV) 35S promoter; and HPT, hygromycin B phosphotransferase.

metabolism, and some are involved in herbicide tolerance; however, molecular information on plant P450s related to xenobiotic metabolism is limited. In contrast, in mammals, several microsomal P450s are involved in xenobiotic metabolism and have been well-studied. The individual P450s appear to show overlapping, broad substrate specificity and thus improve the animal's ability to catabolize a variety of unknown lipophilic compounds, including herbicides (*4*).

Inui et al. (*6*) showed that 11 human P450s metabolize many kinds of chemicals including 27 herbicides, four insecticides, and two industrial chemicals using recombinant yeast expressing human P450s. For instance, human CYP1A1 metabolized 16 herbicides, including triazines, ureas, and carbamates. Human CYP2B6 catabolized more than 10 herbicides, including chloroacetoanilides, oxyacetamides, and 2,6-dinitroanilines, three insecticides, and two industrial chemicals. Some of those chemicals were supposed to be environmental endocrine disruptors.

We have already produced several kinds of transgenic rice plants expressing human P450s, i.e., CYP1A1, CYP2C9, and CYP2C19 under the control of either the cauliflower mosaic virus (CaMV) 35S promoter or an enhanced chimeric CaMV 35S promoter with a 5′-untranslated region from the coat protein mRNA of alfalfa mosaic virus. In some cases of these P450 species, the enhanced chimeric CaMV 35S promoter tends to be more effective to confer the plants strong tolerance to herbicides and activities of metabolizing herbicides.

Here, we show that transgenic rice expressing human P450 2B6 (CYP2B6) under the control of unaltered CaMV 35S promoter can apparently detoxify several types of herbicides, resulting in strong herbicide tolerance. We also discuss the potential use of such plants in phytoremediation, i.e., for degrading chemicals that harm the environment.

MATERIALS AND METHODS

^{14}C Ring-Labeled Chemical. ^{14}C ring-labeled metolachlor (2.33 GBq mmol^{-1}, 98.9% purity) was obtained from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom).

Vector Construction and Transformation. cDNA for human CYP2B6 was kindly provided by Sumitomo Chemical Co. (Osaka, Japan). The vector, pIG121Hm (*7*), containing hygromycin B phosphotransferase (HPT), neomycin phosphotransferase II (NPT II), and β-glucosidase (GUS) units, was kindly provided by Dr. K. Nakamura, Nagoya University. The expression plasmid, pIJ2B6, was constructed by replacing the GUS structural gene with the human *CYP2B6* cDNA in the pIG121Hm vector (**Figure 1**). The pIJ2B6 plasmid was introduced into *Agrobacterium tumefaciens* EHA101 by electroporation with an Electro Cell Manipulator 600 (BTX, Holliston, MA).

Oryza sativa cv. Nipponbare was used for *Agrobacterium*-mediated transformation, which was carried out as reported previously (*8*). Plants (R_0) regenerated on MS medium (*9*) containing 50 mg L^{-1} hygromycin were analyzed by polymerase chain reaction (PCR) using human CYP2B6 specific primers described below. The transgenic plants containing *CYP2B6* were grown in a closed greenhouse, and self-pollinated seeds were harvested. These R_1 seeds were hulled, surface-sterilized, embedded in 40 mL of MS solid medium containing 5 μM metolachlor in a 9 cm Petri dish, and incubated at 27 °C under 16 h/day of light (40 μmol m^{-2} s^{-1}) condition. The homozygous R_2 seeds of the plants tolerant to metolachlor were used for further analyses.

PCR and Southern Blot Analysis. Total DNA for PCR analysis was extracted from about 1 mg of leaf of R_0 plants by the method described by Edwards et al. (*10*). The DNA was mixed with a reaction mixture containing the human CYP2B6 specific primers 5′-GACTCTTGCTACTCCTGGTT-3′ and 5′-CGAATACAGAGCTGATGAGT-3′. The PCR profile was 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min followed by final extension at 72 °C for 10 min. The amplified DNA was detected by electrophoresis.

Genomic DNA for Southern blot analysis was extracted from young rice leaves by the potassium acetate method (*11*). Genomic DNA (5 μg) digested with *Dra* I was fractionated on 0.8% agarose gel by electrophoresis. Then, a 0.87 kb *Sac* I fragment encoding human CYP2B6 was used as a probe in Southern hybridization. Hybridization and detection were done with an ECL nucleic acid detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Western Blot Analysis. The microsomal fraction was prepared from young leaves of rice plants as described previously (*12*). Ten micrograms of microsomal fraction was used for Western blot analysis. The CYP2B6 protein was detected with an ECL Western blotting detection system (Amersham Pharmacia Biotech). Rabbit anti-rat CYP2B1 primary polyclonal antibodies, which cross-react with human CYP2B6, and protein standards for human CYP2B6 were purchased from Daiichi Pure Chemicals (Tokyo, Japan). For secondary antibodies, donkey anti-rabbit IgG antibodies conjugated with horseradish peroxidase were purchased from Amersham Pharmacia Biotech.

Thin-Layer Chromatography (TLC) Analysis of the Metabolism of ^{14}C-Metolachlor. Six day old seedlings of R_2 (line, A11) cultured in MS solid medium at 27 °C under light were transferred into 3 mL of Hyponex (N:P:K = 5:10:5) (Hyponex Japan, Osaka) solution containing 25 000 dpm of ^{14}C-metolachlor at 5 μM (27.7 MBq/mol). Plants and culture medium were sampled 0, 1, 2, and 3 days after incubation at 27 °C under continuous light. Plants were extracted with a mixture of methanol and water (8:2, v/v). Radioactivities in the plant extract and culture medium were quantified by a liquid scintillaion counter (LS6000TA, Beckman Instruments, Inc., United States). The extracts and the culture medium were dried and dissolved in 90% methanol. Samples of extracts equivalent to 2000 dpm of radioactivity were applied to each lane on 60F$_{254}$ TLC plates (Merck, Darmstadt, Germany). The developing solvent was ethyl acetate/hexane (4:6, v/v). After development, the plates were dried and radioactivity was measured with an FLA-2000 BioImaging Analyzer (Fuji Film, Tokyo, Japan).

Germination Tests. Germination tests were carried out in 25 mm × 150 mm (diameter × height) test tubes. R_1 and R_2 seeds of transgenic plants and the seeds of untransformed (control) rice plants were hulled, surface-sterilized, and embedded in 10 mL of MS solid medium containing various herbicides, which were demonstrated to be degradable by human CYP2B6 using microsomes of transformed yeast (*6*). They were incubated at 27 °C under 16 h/day light (40 μmol m^{-2} s^{-1}) conditions, and seedling vigor was observed 7−30 days after sowing.

Herbicide Tolerance in an Enamel Pot. Rice seeds of line A11 (R_2) were embedded in MS solid medium containing 50 mg L^{-1} hygromycin and incubated at 27 °C under 16 h/day light. Ten of the 14 day old plants were transplanted to an enamel pot (diameter, 57 cm; height, 30 cm) with 27 L of water and 30 kg of Kumiai-Ryujyou-Baido K soil (Kureha Chemical, Tokyo, Japan). Both transgenic plants and untransformed Nipponbare plants were grown in a greenhouse for 3 weeks, and 162 μmol of metolachlor was added into the water (final concentration, 6 μM). The growth was observed after 3 weeks.

RESULTS

Production of Transgenic Rice Plants Expressing Human CYP2B6. A total of 133 PCR positive rice plants were transplanted to soil and grew to maturity in a greenhouse. R_1 seeds from 71 plants showed high tolerance to the herbicide by the germination tests in the presence of 5 μM metolachlor. For further analysis, we selected three tolerant plants, A11, A4117,

Table 1. Metabolism of [^{14}C]metolachlor in Untransformed Nipponbare and CYP2B6 Rice[a]

		amounts of metolachlor and the metabolites in plant (nmol/plant)			amounts of metolachlor and the metabolites in medium (nmol/tube)			
		day			day			
line	metolachlor and metabolites	1	2	3	0	1	2	3
Nipponbare	metolachlor	0.48 ± 0.08	0.63 ± 0.35	0.59 ± 0.07***	12.7 ± 0.22	5.96 ± 0.70**	4.58 ± 1.26*	3.94 ± 0.40***
	demethylated metolachlor	0.03 ± 0.008	0.026 ± 0.007	0.02 ± 0.004*	0.69 ± 0.09	0.96 ± 0.33***	0.74 ± 0.02***	0.72 ± 0.01***
	origin	2.86 ± 0.30	2.70 ± 0.86	2.18 ± 0.31*	0.75 ± 0.001	3.01 ± 0.27*	4.41 ± 0.007	6.23 ± 1.05
CYP2B6 rice (A11)	metolachlor	0.25 ± 0.12	0.19 ± 0.16	0.03 ± 0.03***	12.5 ± 0.23	3.29 ± 0.96**	1.77 ± 1.25*	0.29 ± 0.30***
	demethylated metolachlor	0.07 ± 0.04	0.09 ± 0.04	0.09 ± 0.04*	0.72 ± 0.06	5.88 ± 1.04***	5.96 ± 0.71***	4.62 ± 0.79***
	origin	2.58 ± 0.40	2.49 ± 0.66	3.43 ± 0.65*	0.90 ± 0.19	2.48 ± 0.22*	4.10 ± 1.13	4.82 ± 0.49

[a] Fifteen nanomoles of ^{14}C-metolachlor was added per tube. These values are the average of three independent experiments ± SD. *, **, and *** indicate a significant difference at 0.07, 0.05, and 0.01 probability levels, respectively, between Nipponbare and CYP2B6 rice.

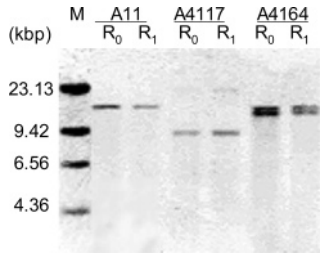

Figure 2. Southern blot analysis of R_0 and R_1 plants of CYP2B6 rice. M, DNA size marker. A11, A4117, and A4164, CYP2B6 rice.

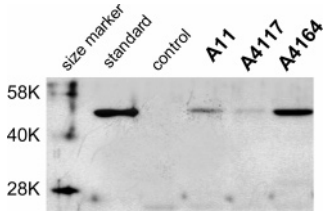

Figure 3. Western blot analysis of CYP2B6 rice. Ten micrograms of microsomal fraction was used for each lane. Size marker, protein standard marker; standard, membrane preparation of a human cell line expressing CYP2B6 cDNA purchased from Daiichi Pure Chemicals; control, microsomal fraction from untransformed Nipponbare. A11, A4117, and A4164, microsomal fraction from CYP2B6 rice.

and A4164, and harvested their self-pollinated seeds (R_2). These three lines showed normal morphological characteristics, just like those of untransformed Nipponbare.

Southern blot analysis of *Dra* I-digested genomic DNA from these three plants revealed that a single band that hybridized with human *CYP2B6* cDNA was present in A11, whereas two bands were present in A4117 and A4164 (**Figure 2**). The band patterns were the same in the R_0 and R_1 generations. The CYP2B6 protein with a molecular mass of 52 kDa was produced in all three lines (**Figure 3**).

Metabolism of Metolachlor in CYP2B6 Rice and Untransformed Nipponbare. During the culture period, 18.5−24% of the radiolabeled metolachlor added (total 15 nmol/tube) was detected in the plant tissue in both transformed and untransformed plants. Meanwhile, 65−79% of the added radioactivity was detected in the culture medium (**Table 1**). On the TLC plate, we found distinct spots of demethylated metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-hydroxyl-1-methylethyl) acetamide; DMMC], one of the metabolites of metolachlor, in the medium of CYP2B6 rice after 1 day of culture (**Figure 4B**). The spots at the origin in **Figure 4A**, **B**, containing water soluble metabolites, were detected in both plant tissue and culture media of both CYP2B6 rice and Nipponbare after

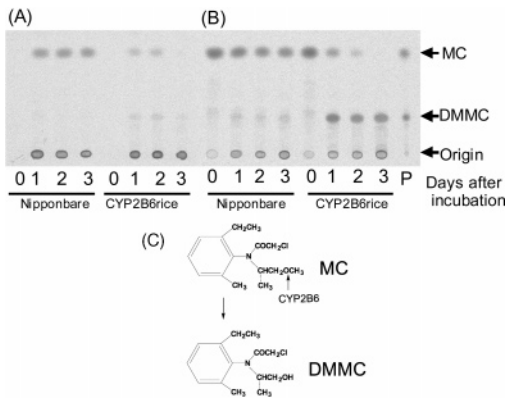

Figure 4. Metabolites of metolachlor in rice plants (**A**) and culture medium (**B**) detected by TLC. MC and DMMC indicate the locations of metolachlor and its demethylated metabolite, respectively. P is a mixture of authentic MC and DMMC. Nipponbare, untransformed Nipponbare; CYP2B6 rice, line A11 plants. (**C**) Schematic diagram of the first step of the metabolic pathway of metolachlor in transgenic rice plants.

1 day of culture. Very little intact metolachlor (0.2% of the added radioactivity) was detected in CYP2B6 plants, whereas considerable metolachlor (4%) remained in Nipponbare plants after 3 days of culture.

Although the proportion of metolachlor in the culture medium rapidly decreased within the first day of culture of both CYP2B6 rice and Nipponbare, less metolachlor was detected in the medium of CYP2B6 rice (22%) than of Nipponbare (40%) (**Table 1** and **Figure 5**). The metolachlor concentration in the culture medium continued to decrease during the incubation period, and only about 2% of the added radiolabeled metolachlor remained in the CYP2B6 medium after 3 days, as compared to 26% in the Nipponbare medium. The amount of DMMC increased to 39.2% in the medium of CYP2B6 rice on the first day of culture and remained constant for the remainder of the incubation period. Little DMMC (4.8−6.4%) was detected in Nipponbare medium during the incubation period. Concentrations of metabolites that remained at the origin increased throughout the culture period in the culture media of both CYP2B6 rice and Nipponbare.

Herbicide Tolerance of Transgenic Rice Expressing Human CYP2B6. Each of the three lines of the transgenic rice plants (A11, A4117, and A4164) expressing human CYP2B6 showed a high tolerance to the chloroacetanilide herbicides alachlor and metolachlor, which inhibit the synthesis of very long chain fatty acid (**Figure 6**). Four R_2 seeds sown on MS medium containing 2.5 μM alachlor or 5 μM metolachlor germinated and grew well, whereas untransformed Nip-

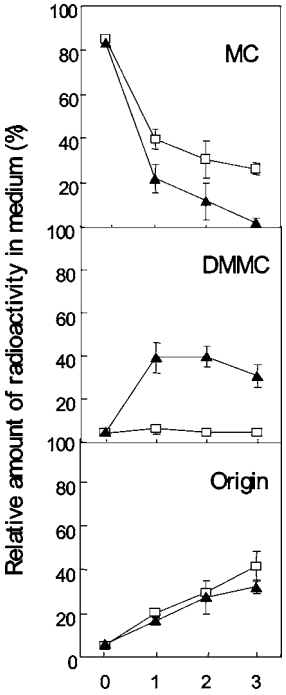

Figure 5. Relative amount of metolachlor and its metabolites in the culture medium of rice plants. Nipponbare (□); CYP2B6 rice (A11) (▲).

ponbare did not grow at all. Other chloroacetanilide herbicides—acetochlor (0.1 μM), thenylchlor (5 μM), and pretilachlor (2.5 μM)—completely prevented the germination of untransformed Nipponbare, whereas the CYP2B6 rice plants germinated and grew well in the medium with these herbicides just like the control plants did in the medium without the herbicide [**Table 2**; the mode of action and chemical family for each herbicide is also mentioned in this table (http://www.plantprotection.org/HRAC)]. Butachlor was an exception: Both untransformed Nipponbare and CYP2B6 rice showed nearly normal growth on medium containing 10 μM butachlor.

Pyributicarb (2.5 μM), pendimethalin (10 μM), and trifluralin (15 μM) inhibited the root growth of Nipponbare, but CYP2B6 rice plants produced roots and grew better than Nipponbare. Amiprofos-methyl inhibited the root growth and terrestrial part of the seedlings of Nipponbare, while CYP2B6 rice plants grew equally well in the medium with or without herbicide. Chlortoluron inhibited the growth of Nipponbare plants, although the CYP2B6 plants grew more vigorously than Nipponbare on a medium containing 120 μM chlortoluron (**Figure 6** and **Table 2**).

Mefenacet inhibited the germination of Nipponbare but had little effect on the growth of CYP2B6 rice plants. CYP2B6 rice was slightly tolerant to norflurazon (0.3 μM), which caused bleaching of shoots of Nipponbare. CYP2B6 rice plants did not show any tolerance to chloropropham (7.5 μM), quizalofop-ethyl (0.4 μM), or isoxaben (3 μM) (**Table 2**).

Untransformed Nipponbare and CYP2B6 rice were grown in the enamel pots with soil and water for 1 month and then treated with 6 μM metolachlor. After 1 month of culture, CYP2B6 rice plants showed clear tolerance and grew well but untransformed Nipponbare was almost killed by the treatment of metolachlor (**Figure 7**).

DISCUSSION

The xenobiotic-degrading P450 systems in mammalian liver show an overlapping, broad substrate specificity toward xenobiotics, including herbicides. As Funae mentioned, only 11 P450s are responsible for more than 90% of P450-dependent drug metabolism in the human liver (*13*). Therefore, the transgenic rice producing human CYP2B6 was reasonably able to metabolize a broad spectrum of herbicides and showed cross-tolerance to several herbicides having different modes of action and different chemical structures.

The results of TLC analysis showed that the amounts of metolachlor decreased in CYP2B6 rice plants and in the medium of CYP2B6 rice faster than those of untransformed Nipponbare (**Figures 4** and **5**). The metabolism of metolachlor seemed to be enhanced by the introduced CYP2B6 enzyme in the transgenic plants although metolachlor was metabolized not only by CYP2B6 rice but also by control plants in the same pathway. In the culture media and plant tissue, the amounts of water soluble metabolites remaining at the origin in TLC were similar for both plants, but the residual metolachlor was lower in CYP2B6 rice than in Nipponbare. The first metabolite of metolachlor, DMMC, was detected in the culture medium of CYP2B6 rice, suggesting that it was probably not converted into more hydrophilic compounds rapidly enough to prevent its rapid accumulation, owing to the introduced human CYP2B6. On the other hand, the metabolism of metolachlor to DMMC in Nipponbare seems to be relatively slow and only little accumulation of DMMC was detected.

DMMC seemed to be released from CYP2B6 rice plants, probably from roots, into the culture medium. However, the amount of DMMC was constant after the first day of culture, whereas residual metolachlor kept decreasing and hydrophilic metabolites kept accumulating. It seemed that the DMMC, once released into the culture medium, was absorbed back into CYP2B6 rice plants and further metabolized into hydrophilic compounds. We have reported that intermediate metabolites of chlortoluron, atrazine, and norflurazon were also detected in the culture medium of CYP1A1 rice (*14*). We suggest again in this paper that rice plants may secrete herbicide metabolites into the culture medium through channels or transporters to remove overproduced chemical compounds from their roots to the surrounding water.

Several studies have been reported on the metabolism of metolachlor in soil microorganisms, plants, and animals. O-demethylation and hydrolytic dechlorination of metolachlor to form eight or nine metabolites have been reported to be the major pathways of metabolism in rat liver (*15*), bluegill sunfish (*16*), soil fungus *Chaetomium* (*17*), and soil actinomycete (*18*). In the case of sorghum microsomes, metolachlor was catalyzed by O-demethylation and formed DMMC, a single metabolite (*19*), which is the same metabolite by our transgenic rice plants and untransformed Nipponbare. This metabolite is also one of the major metabolites formed by the other organisms mentioned above. As far as we know, there is no report on toxicity of this compound to plants or crops. In this study, the transgenic rice with CYP2B6 rapidly metabolized metolachlor to DMMC, which was detected in the culture medium. Meanwhile CYP2B6 rice showed strong tolerance to metolachlor by germination tests. These facts indicate that DMMC is not or less toxic to rice plants than metolachlor itself.

Our transgenic rice plants producing human CYP2B6 showed high tolerance to the herbicides, which belong to different chemical families, norflurazon (pyridazinones), mefenacet (oxyacetamides), trifluralin and pendimethalin (2,6-dinitroanilines), amiprofos-methyl (phosphoamidates), pyributicarb (thiocarbamates), and chlortoluron (ureas). Especially, significantly high tolerance was observed to the five chloroacetoamides (ac-

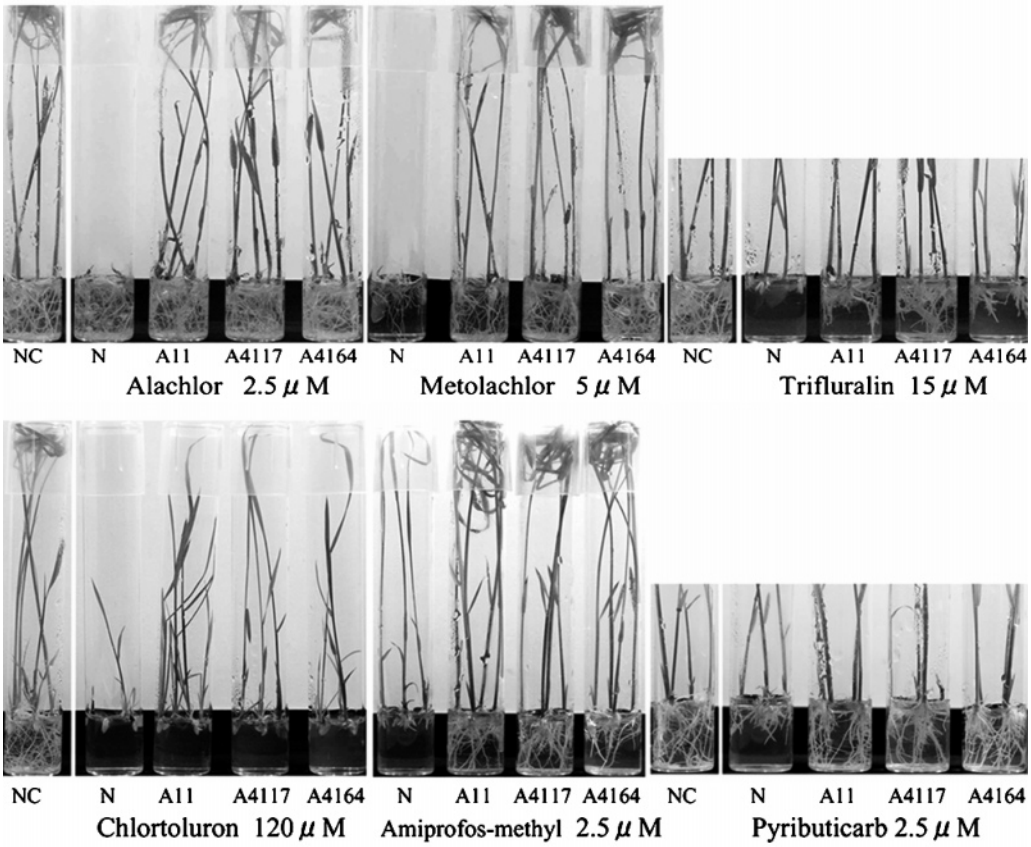

Figure 6. Herbicide tolerance of CYP2B6 rice. NC, Nipponbare control (without herbicide); N, Nipponbare with the indicated herbicide. A11, A4117, and A4164, CYP2B6 rice with the indicated herbicide. Seedlings were photographed after 14 days of incubation for alachlor, metolachlor, trifluralin, amiprofos-methyl, and pyributicarb or after 30 days of incubation for chlorotoluron.

etochlor, alachlor, metolachlor, pretilachlor, and thenylchlor). For instance, CYP2B6 rice seeds were able to germinate and grow in the medium containing 80 μM metolachlor, while untransformed Nipponbare did not germinate in the presence of 2 μM metolachlor.

The CYP2B6 transgenic rice also grew well on medium containing chlorotoluron, norflurazon, amiprofos-methyl, or pyributicarb (**Figure 6**), for which the transgenic yeast did not show any distinct metabolic activity (**Table 2**). Likely, the activity of human CYP2B6 is higher in rice plants than in yeast, and it is probable that the CYP2B6 rice degraded the herbicides gradually during and after germination, keeping the concentration of the herbicide in plant tissues under the lethal threshold. Both cytochrome P450 and NADPH-cytochrome P450 oxidoreductase are associated with the endoplasmic reticulum. Although our transgenic rice received only an exogenous gene for cytochrome P450, without any accompanying gene for the NADPH-cytochrome P450 oxidoreductase, the plants degraded the herbicides and showed tolerance to them. As Porter et al. (*20*) mentioned, a single form of NADPH-cytochrome P450 oxidoreductase is responsible for electron transport to diverse P450 monooxygenase in mammals. Although up to 300 P450 genes are expected in the genome of higher plants (http://drnelson.utmem.edu/CytochromeP450.html), only two isoforms of P450 reductase were identified in *Arabidopsis thaliana* (*21*). From these results, we speculate that endogenous NADPH-cytochrome P450 oxidoreductase transfers electrons to both the introduced CYP2B6 enzymes and the endogenous P450 species.

Over 280 biotypes of herbicide resistant weeds have emerged (http://www.weedscience.org/in.asp). They are resistant to various kinds of herbicides that belong to 18 HRAC (The Herbicide Resistance Action Committee) groups, and 50% of the biotypes are resistant to group B herbicides; inhibitors of acetolactate synthase, such as sulfonylureas, and group C1 herbicides, inhibitors of photosynthesis at photosystem II, such as triazines. Several strategies are used to prevent or reduce herbicide resistance in weed populations. When resistance is polygenic, high initial application rates of a herbicide would delay the appearance of resistance, whereas when resistance is due to a single major gene, low herbicide doses would be effective (*2*). The majority of instances of herbicide resistance appear to be major gene resistances, and herbicide mixtures including lower doses of herbicides are commonly used to cope with such weeds. Using various herbicides in rotation was also proposed as the most practical approach to prevent or delay the appearance of resistance, because the repeated use of a single herbicide for a long time tends to select for weeds that are resistant.

Our transgenic CYP2B6 rice showed cross-tolerance to a number of herbicides that can be classified into different groups according to their site of action and chemical structures (**Table 2**). Transgenic potatoes expressing rat CYP1A1, human CYP1A1, human CYP2B6, and human CYP2C19 (*23*, *24*) and transgenic rice plants expressing human CYP1A1, CYP2C9, or CYP2C19 also show cross-tolerance to several herbicides (*22*, *14*). The wider cross-tolerance to herbicides having different modes of action and different chemical structures seems to be a special feature of transgenic plants expressing mammalian P450 genes. This cross-tolerance would prove useful when weeds are managed according to strategies that aim to prevent the development of herbicide resistance, because the use of several herbicides in rotation would not harm the crop.

Additionally, CYP2B6 rice plants grew vigorously in an enamel pot with soil and water, which contained 6 μM metolachlor, but untransformed Nipponbare plants were almost

Table 2. Germination and Growth of CYP2B6 Rice in the Presence of Various Herbicides

HRAC[a] group: mode of action herbicide	(chemical family)	concn[b] (μM)	rice	yeast microsome
A: inhibition of acetyl CoA carboxylase				
quizalofop-ethyl	(aryloxyphenoxy-propionates)	0.4	–[c]	–[d]
B: inhibition of acetolactate synthases				
pyriminobac-methyl	(pyrimidinylbenzoates)	5	+	–
C2: inhibition of photosynthesis at photosystem II				
chlortoluron	(ureas)	120	+	–
F1: inhibition of carotenoid biosynthesis at the phytone				
norflurazon	(pyridazinones)	0.3	++	–
K1: microtubule assembly inhibition				
trifluralin	(dinitroanilines)	15	++	5
pendimethalin	(dinitroanilines)	10	++	ND
amiprofos-methyl	(phosphoroamidates)	2.5	++	–
K2: inhibition of microtubule organization				
chlorpropham	(carbamates)	7.5	–	7
K3: inhibition of very long chain fatty acid synthesis				
acetochlor	(chloroacetamides)	0.1	+++	42
alachlor	(chloroacetamides)	2.5	+++	18
metolachlor	(chloroacetamides)	5	+++	17
thenylchlor	(chloroacetamides)	5	+++	ND
pretilachlor	(chloroacetamides)	2.5	+++	ND
butachlor	(chloroacetamides)	10	=	ND
mefenacet	(oxyacetamides)	2.5	+	6
L: inhibition of cell wall synthesis				
isoxaben	(benzamide)	3	–	–
Z: unknown (inhibition of sterol and triterpenoid biosynthesis?)				
pyributicarb	(thiocarbamates)	2.5	++	–

[a] Herbicide resistance action committee. [b] Final concentration of herbicide in germination test in the culture medium. [c] +++, Transgenic lines grew as well in the presence of herbicide as did control plants in medium lacking herbicide. ++, Transgenic lines grew better than control plants in the medium with herbicide. +, Some of the transgenic lines grew better than control plants in the medium with herbicide. –, No growth nor did the control plants in the medium with herbicide. =, Transgenic lines and control plants grew equally well in the presence of herbicide. [d] Percentage degradation of herbicide by in vitro yeast microsome system (*6*). –, Not detected (less than 5%). ND, not determined.

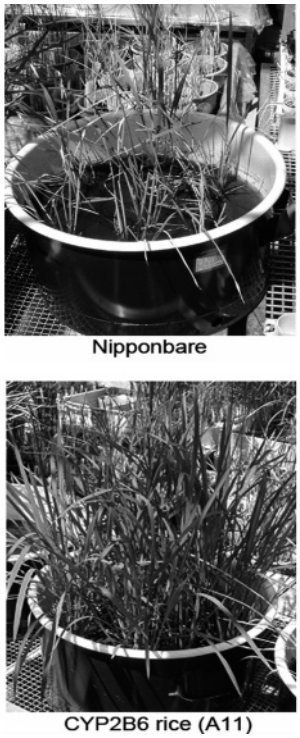

Figure 7. Herbicide tolerance of CYP2B6 rice in the enamel pot in a greenhouse. Both pots were treated with 162 μmol (final concentration, 6 μM) of metolachlor.

killed by the herbicide (**Figure 7**). This result indicated that our transgenic rice is practically useful as an herbicide tolerant crop under the condition of a paddy field. We expect that CYP2B6 rice will also prove useful in degrading and thus decreasing the environmental loads of herbicides, insecticides, industrial chemicals, and endocrine-disrupting pollutants in paddy fields and the connected water streams.

Because human P450 species that can detoxify xenobiotics have broad substrate specificities, modification of the expression of P450 species in plants by transformation may alter the patterns of secondary metabolites produced in the transgenic plants. Therefore, safety assessments of transgenic plants expressing P450 species are needed before practical use (*4*). In the future, transgenic plants expressing P450 species may be useful for engineering herbicide tolerance and green chemistry.

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