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Original Paper

Enhanced expression of *CYP2C9* and tolerance to sulfonylurea herbicides in transgenic rice plants

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Abstract We compared three transgenic rice plants transformed with pIJ2C9, pIES2C9, or pIJU2C9, which express the human *CYP2C9* gene under the control of the CaMV 35S, chimeric CaMV 35S, or maize polyubiquitin 1 promoter, respectively. The plants, especially those transformed with pIJU2C9, showed high tolerance to the sulfonylurea herbicides chlorsulfuron, imazosulfuron, and triasulfuron, owing to metabolic detoxification promoted by the introduced *CYP2C9*. The levels of expression of CYP2C9 were highly related to the tolerance to sulfonylurea herbicides. In this study, we achieved to produce transgenic rice plants with high expression of CYP2C9 by use of a maize polyubiquitin 1 promoter. Imazosulfuron is used in rice, but chlorsulfuron and triasulfuron are not, because of the sensitivity of rice plants to them. Transgenic rice plants expressing CYP2C9 will be useful for introducing tolerance to chlorsulfuron and triasulfuron into rice lines.

Key words: Cytochrome P450, chlorsulfuron, imazosulfuron, triasulfuron, ubiquitin promoter.

Sulfonylurea herbicides are widely used in paddy fields in Japan and Korea because of their very high herbicidal activity at low use-rates, broad-spectrum control of broadleaf and grass weeds, and low mammalian toxicities (Brown 1990; Roberts 1998; Tomlin 2000). Since sulfonylurea herbicides were introduced into Japan in 1988, however, resistance has developed in several weed species (Wang et al. 2000). This is due to repeated use of a very limited range of herbicides, necessitated by the sensitivity of rice to most sulfonylurea herbicides. Sulfonylurea-resistant weeds reported in Japan have appeared in paddy fields treated repeatedly with the same herbicide mixture for several years, either bensulfuron-methyl+mefenacet, bensulfuron-methyl+esprocarb, or pyrazosulfuron+mefenacet (Itoh and Wang 1997; Itoh et al. 1997, 1998).

Sulfonylurea herbicides inhibit acetolactate synthase (ALS; EC 4.1.3.18), the first enzyme that catalyzes the biosynthesis of the branched amino acids valine, leucine, and isoleucine (LaRossa and Schloss 1984). Most sulfonylurea-resistant weeds owe their resistance to an altered ALS that is insensitive to the herbicides (Mallory-Smith et al. 1990; Saari et al. 1990).

Almost all sulfonylurea-resistant weeds are cross-tolerant to a variety of sulfonylurea herbicides. Control of such resistant weeds requires the use of herbicides with different modes of action in rotation (Wang et al. 2000; Kuk et al. 2002, 2003a, b).

Mammalian cytochrome P450 (P450 or CYP) monooxygenases are involved in the metabolism of xenobiotics as well as of endogenous substrates (Funae et al. 1998). Our team reported that 11 human P450s metabolized many kinds of chemical, including 27 herbicides and 4 insecticides, in recombinant yeast (Inui et al. 2001b). Transgenic plants harboring such drug-metabolizing P450 genes from mammals were expected to be tolerant to herbicides and to be able to clean up residual agrochemicals (Ohkawa et al. 1999). We have already produced several kinds of transgenic rice plants expressing genes for human P450s (i.e., *CYP1A1*, *CYP2C9*, and *CYP2C19* under the control of CaMV 35S or chimeric CaMV 35S promoters), and evaluated their tolerance to and metabolization of herbicides (Inui et al. 2001a; Kawahigashi et al. 2003). We considered plants expressing CYP2C9 to be an important goal, because the CYP2C9 enzyme metabolizes the sulfonylurea herbicides imazosulfuron, chlorsulfuron, and triasulfuron (Inui et al. 2001b). The latter two herbicides cannot be

Abbreviations: IS, imazosulfuron; ADPM, 2-amino-4,6-dimethoxypyrimidine; IPSN, 2-chloroimidazo[1,2-*a*]pyridine-3-sulfonamide; HMS, 1-(2-chloroimidazo[1,2-*a*]pyridin-3-ylsulfonyl)-3-(4-hydroxy-6-methoxypyrimidin-2-yl)-urea.

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used in rice because of the sensitivity of rice plants to them. Therefore, enhanced expression of CYP2C9 in transgenic rice plants could increase the range of sulfonylurea herbicides that can be used in rice in Japan.

It is important to stop the progression of weed resistance in paddy fields. Our transgenic rice plants expressing human CYP2C9 could be used in breeding programs to increase the range of sulfonylurea herbicides that can be used in Japanese paddy fields and to stop the use of the same limited combinations of sulfonylurea herbicides. Resistant cultivars could also be used to clean up residual imazosulfuron, which remains active for an extended period in paddy fields (Roberts 1998).

In our previous report, the transgenic rice expressing *CYP2C9* under the control of an enhanced chimeric CaMV 35S promoter showed tolerance to chlorsulfuron and imazosulfuron due to metabolism of these herbicides by introduced CYP2C9 (Inui et al. 2001a). In the present study, to obtain transgenic rice plants with higher activities of introduced P450, we examined three different transgenic rice using different promoters; either the CaMV 35S promoter, an enhanced chimeric CaMV 35S promoter with a 5′-untranslated region, or a maize polyubiquitin 1 promoter with the first exon and first intron of its gene (Christensen et al. 1992). The maize polyubiquitin 1 promoter has been successfully used for transformation in maize and other monocots (Christensen and Quail 1996), and promotes higher expression levels in transgenic rice plants than does the CaMV 35S promoter (Cornejo et al. 1993). Thus, we compared the tolerance, gene expression, and metabolization of imazosulfuron among three transgenic rice lines under the control of these three promoters.

Materials and methods

Chemicals

Imazosulfuron, 1-(2-chloroimidazo[1,2-*a*]pyridin-3-ylsulfonyl)-3-(4,6-dimethoxypyrimidin-2-yl)-urea, with a ^{14}C-labeled imidazopyridine ring (sp. act. 5.30 $MBq\,mg^{-1}$) and its unlabeled metabolites ADPM, IPSN, and HMS came from Takeda Chemical Ind., Ltd. (Osaka, Japan).

Plasmid construction and transformation

The β-glucronidase gene of expression vector pIG121Hm was replaced with human *CYP2C9* cDNA to construct pIJ2C9 (Figure 1). The cauliflower mosaic virus (CaMV) 35S promoter region of pIJ2C9 was replaced with maize polyubiquitin 1 promoter and the first exon and intron (1992 bp, accession number S94464) (Christensen et al. 1992), which was provided by Dr. H. Ichikawa and S. Toki (NIAS, Tsukuba, Japan) to construct pIJU2C9. Expression vector pIES6, which carries a chimeric CaMV 35S promoter containing the seven-tandem enhancer region and 5′-untranslated region from the coat protein mRNA of alfalfa mosaic virus (AMV), was used to construct pIES2C9 (Figure 1) (Inui et al. 2001a). The chimeric CaMV 35S promoter with the seven-tandem enhancer sequence region enabled effective expression in transgenic tobacco and rice plants (Mitsuhara et al. 1996). The AMV 5′-UTR region stabilized mRNA and its translation efficiency (Jobling and Gehrke 1987). The constructed expression plasmids were introduced into *Agrobacterium tumefaciens* strain EHA101 by electroporation in an Electro Cell Manipulator 600 (BTX, Holliston, MA, USA). *Oryza sativa* ssp. *japonica* cv. ‘Nipponbare’ was used for *Agrobacterium*-mediated transformation as previously reported (Toki 1997).

Selection of transgenic rice plants

T_0 plants regenerated on medium containing $50\,mg\,l^{-1}$ hygromycin were analyzed by PCR using human *CYP2C9*-specific primers. DNA preparation for PCR and PCR amplification were performed as reported previously (Kawahigashi et al. 2003). The PCR profile was 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and 5 min at 72°C for the final extension. PCR products were analyzed on 1% agarose gels. The sense (5′-TATTGTGTCCCTTGT-GCTCT-3′) and antisense primers (5′-GTTGTGC-TTGTCGTCTCTCTGT-3′) were designated for amplification of a 906-bp DNA fragment in the *CYP2C9* cDNA.

Transgenic plants containing the human *CYP2C9* gene were grown in a greenhouse, and their seeds (T_1) were harvested. The seeds were selected by germination tests in a 9-cm Petri dish with 40 ml of MS solid medium (Murashige and Skoog 1962) containing 25, 50, or 75 nM chlorsulfuron for the pIJ2C9-, pIES2C9-, or pIJU2C9-transformed line, respectively. The selected lines were grown in a greenhouse, and the T_2 seeds were used for further experiments.

Reverse transcription-PCR (RT-PCR) assay

Total RNA was extracted from two of 6-day-old seedlings of non-transgenic Nipponbare and the pIJ2C9 (9112), pIES2C9 (57E), and pIJU2C9 (U949)-transformed plants grown in MS agar medium containing $50\,mg\,l^{-1}$ hygromycin by using the RNeasy Plant Mini Kit (Qiagen K.K. Tokyo, Japan). The RNA was treated by DNase I at 37°C for 60 min to remove contaminated DNA. RT-PCR was performed using a RNA PCR Kit (AMV) Ver 2.1 (TakaraBio, Tokyo, Japan) according to the manufacturer’s instructions. 1 μg of total RNA was used as the template for a 20-μl RT reaction. The RT profile was as follows: 30°C for pre-incubation for 10 min; 50°C for 60 min for reverse transcription; 99°C for 5 min for denaturation. 4 μl aliquots of cDNA

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solution were subjected to 20 μl of PCR amplification using the CYP2C9-specific PCR primers described earlier. PCR was performed for 25 or 30 cycles. 4 μl aliquots of PCR products were analyzed on 1.5% agarose gels. Rice phenylalanine ammonia-lyase (PAL)-specific primers were used as positive control. The sense (5′-GCTCTCGGCGGTGTTCTGCGA-3′) and antisense primers (5′-GAGGTACTGGAGCTCAGAGCTG-3′) were designated for amplification of a 562-bp DNA fragment in the PAL cDNA.

Western blot analysis

The microsomal fraction was prepared as reported previously (Shiota et al. 1994) from pIJ2C9 (9112), pIES2C9 (57E), and pIJU2C9 (U926, U949)-transformed plants. Rabbit anti-human CYP2C9 serum was purchased from Sumitomo Chemical Co. Ltd., Osaka, Japan. Peroxidase-conjugated anti-rabbit IgG antibodies as secondary antibodies were purchased from Amersham Biosciences K.K. (Tokyo, Japan). Ten micrograms of the microsomal fraction of transgenic plants expressing human *CYP2C9* was used for Western blot analysis. Western blot analysis was performed in an ECL protein detection system (Amersham) according to the manufacturer's instructions.

Germination tests of T_2 seeds

Germination tests were carried out in a test tube 2.5 cm in diameter and 15 cm in height. The T_2 seeds of transgenic and non-transgenic control rice plants were embedded in 10 ml of MS solid medium containing a herbicide and cultured at 27°C for 7 to 14 days under 16 h of light (photon flux density 40 μmol m^{-2} s^{-1}). The herbicide concentrations were 25–75 nM for chlorsulfuron, 5 μM for imazosulfuron, and 150 nM for triasulfuron.

TLC analysis

T_2 seeds were embedded in MS solid medium containing 50 mg l^{-1} hygromycin and incubated at 27°C for 6 days under 16 h of light (photon flux density 40 μmol m^{-2} s^{-1}). Six-day-old plants were transferred individually to test tubes (diameter, 2.5 cm; height, 15 cm) with 3 ml of Hyponex 5-10-5 (Hyponex, Osaka, Japan) solution containing 50000 dpm of [^{14}C]imazosulfuron at a concentration of 1.25 μM. The plants were incubated under 24 h light (photon flux density 40 μmol m^{-2} s^{-1}). The culture medium was sampled after 6 and 22 h of incubation. The plants were sampled after 24 h of incubation. Radioactive chemicals were extracted twice from plants with a mixture of acetonitrile and water (5 : 5, v/v). The extracts were evaporated and dissolved in 90% methanol. Radioactive extract (2000 dpm) was applied to each lane of a silica gel 60F$_{254}$ thin-layer chromatography (TLC) plate (Merck, Darmstadt, Germany). The developing solvent was chloroform and acetic acid (9 : 1, v/v). Radioactivity was measured with an FLA-2000 Bio-Imaging Analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Herbicide tolerance in soil

Rice seeds (T_2) were embedded in MS solid medium containing 50 mg l^{-1} hygromycin and incubated at 27°C for 7 days under 16 h of light. Ten 7-day-old plants were transplanted to a glass beaker (diameter, 9 cm; height, 19 cm) with 500 ml of water and 500 g of Kumiai-Ryujyou-Baido K soil (Kureha Chemical, Tokyo, Japan). Both transgenic plants and non-transgenic Nipponbare plants were grown in a greenhouse for 1 week, then each pot received 159 μg chlorsulfuron (final concentration 0.86 μM), 63.7 μg triasulfuron (0.31 μM), or 605 μg imazosulfuron (2.9 μM). The growth was observed after 4 weeks.

Results

Selection and herbicide tolerance of transgenic rice plants expressing CYP2C9

We transformed rice plants (cv. 'Nipponbare') using three expression plasmids—pIJ2C9, pIES2C9, and pIJU2C9—which were constructed to express *CYP2C9* under the control of three different promoters (Figure 1). The presence of *CYP2C9* in each regenerated plant was confirmed by PCR, and the PCR-positive plants (–50) were grown in a greenhouse. The harvested T_1 seeds were screened by germination test. 7 out of 36 lines of pIJ2C9-transformed plants were tolerant to 30 nM chlorsulfuron. For pIES2C9- and pIJU2C9-transformed plants, 12 out of 47 lines were tolerant to 50 nM chlorsulfuron and 14 out of 27 lines were tolerant to 75 nM chlorsulfuron, respectively. We selected nine herbicide-tolerant lines—911, 9112, and 9122 for pIJ2C9 transformation; 1D, 16T, and 57E for pIES2C9;

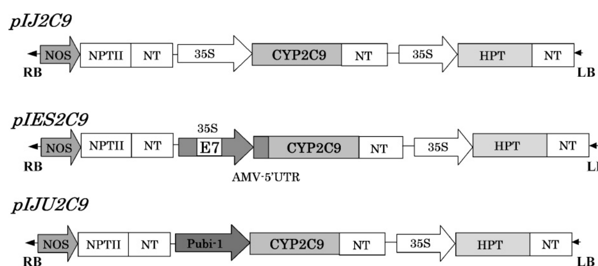

Figure 1. T-DNA regions of the expression plasmids pIJ2C9, pIES2C9, and pIJU2C9 used to introduce human *CYP2C9*. RB, right border; LB, left border; NOS, nopaline synthase promoter; NPTII, neomycin phosphotransferase II; NT, nopaline synthase terminator; 35S, cauliflower mosaic virus (CaMV) 35S promoter; HPT, hygromycin B phosphotransferase; E7, seven-tandem enhancer region (−290 to −90) from CaMV 35S promoter; AMV-5′UTR, alfalfa mosaic virus 5′-untranslated region; Pubi-1, maize polyubiquitin 1 promoter region.

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and U926, U939 and U949 for pIJU2C9. Over 10 chlorsulfuron tolerant plants for each line were grown in the greenhouse, and the T_2 seeds were harvested. Each line of the transgenic rice plants was normal in morphology, growth and fertility compared with non-transgenic Nipponbare. After germination tests in MS solid medium containing $50\,\mathrm{mg\,l^{-1}}$ hygromycin, we selected several plants, which were supposed to be homozygous for further experiments.

Non-transgenic Nipponbare plants did not show tolerance to 50 nM chlorsulfuron, 5 μM imazosulfuron, or 150 nM triasulfuron (Figure 2). One of pIJ2C9-transformed plants showed tolerance to imazosulfuron and triasulfuron, but their growth was severely retarded. pIES2C9-transformed plants showed more tolerance to all herbicides tested; among them, line 57E showed the highest tolerance. All three lines of pIJU2C9-transformed plants showed high tolerance to all herbicides tested. The growth of pIJU2C9-transformed plants in the presence of these herbicides was similar to that of the control Nipponbare plants without herbicides (Figure 2). The observed herbicide tolerance of the *CYP2C9*-expressing rice plants was consistent with *in vitro* catalysis of these herbicides by recombinant yeast microsomes expressing human *CYP2C9* (Inui et al. 2001b).

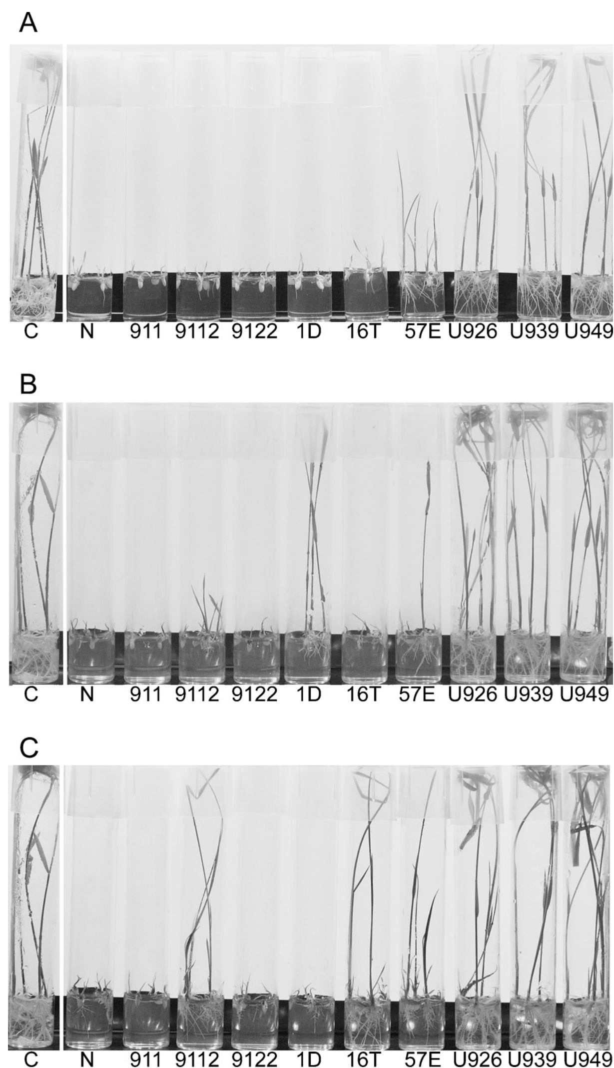

Figure 2. Phytotoxicity of sulfonylurea herbicides to transgenic rice plants expressing *CYP2C9*. Germination tests were performed in 10 ml of MS solid medium containing 50 nM chlorsulfuron (A), 5 μM imazosulfuron (B), or 150 nM triasulfuron (C) at 27°C for 7 to 14 days. Lane C, non-transgenic Nipponbare without herbicide; lane N, non-transgenic Nipponbare with herbicide; lanes 911, 9112, 9122, pIJ2C9-transformed plants with herbicide; lanes 1D, 16T, 57E, pIES2C9-transformed plants with herbicide; lanes U926, U939, U949, pIJU2C9-transformed plants with herbicide.

Expression of the CYP2C9 gene

We assumed that the difference in observed herbicide tolerance between three transgenic rice lines was due to differences in the level of expression of *CYP2C9*. Thus, we confirmed the mRNA expression by RT-PCR (Figure 3A). The patterns from two different cycles of PCR reactions showed that the expression level of *CYP2C9* mRNA was highest in the pIJU2C9-transformed plants and lowest in the pIJ2C9-transformed plants. We also performed Western blot analysis to compare the expression levels of CYP2C9 protein (Figure 3B). Positive bands corresponding to the CYP2C9 protein

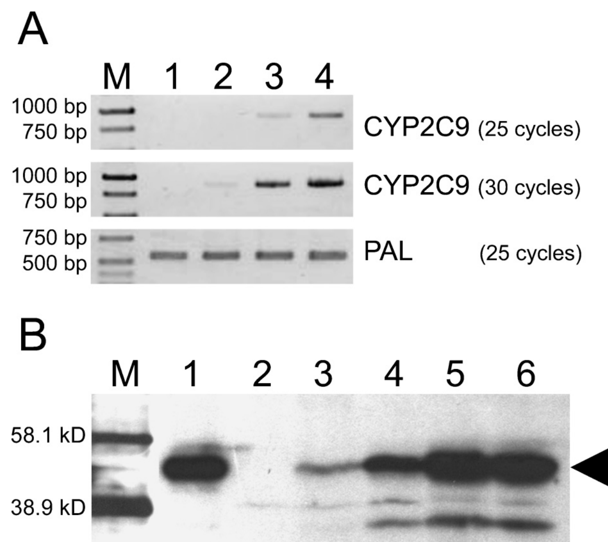

Figure 3. Expression level of CYP2C9 in the transgenic rice plants with pIJ2C9, pIES2C9 and pIJU2C9. (A) RT-PCR analysis of transgenic rice plants expressing *CYP2C9*. RT-PCR was performed by CYP2C9-specific and PAL-specific primers. The number of PCR cycles is shown in parentheses. Lane M, DNA size marker; lane 1, non-transgenic Nipponbare; lane 2, pIJ2C9-transformed plants (9112); lane 3, pIES2C9-transformed plants (57E); lane 4, pIJU2C9-transformed plants (U949). (B) Western blot analysis of transgenic rice plants expressing *CYP2C9*. A 10-μg aliquot of protein from the microsomal fraction of each transgenic rice plant was applied to each lane. Lane M, protein size marker; lane 1, yeast microsome expressing *CYP2C9*; lane 2, non-transgenic Nipponbare; lane 3, pIJ2C9-transformed plants (9112); lane 4, pIES2C9-transformed plants (57E); lanes 5 and 6, pIJU2C9-transformed plants U926 and U949. Black arrowhead indicates bands of CYP2C9 protein of ca. 56 kD.

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were detected in all of the transgenic plants. The expression level of protein was also highest in the pIJU2C9-transformed plants. The result of Western blot analysis using three transgenic rice lines were consistent with that of RT-PCR and suggested their gene expression levels were clearly related to the strength of tolerance to sulfonylurea herbicides of these transgenic lines. These results indicated that the maize polyubiquitin 1 promoter was the strongest among three promoters.

TLC analysis using radioisotope-labeled imazosulfuron

We analyzed the degradation of imazosulfuron in the plants and the culture medium using Nipponbare, pIES2C9-transformed (57E), and pIJU2C9-transformed (U926) plants. The proposed metabolic pathways of imazosulfuron are shown in Figure 4A.

In rice plant extracts, imazosulfuron was detected in Nipponbare, but hardly any was detected in pIJU2C9-transformed plants after 24 h of incubation. The mean amounts of imazosulfuron in pIES2C9- and pIJU2C9-transformed plants were decreased to 18% and 1.2% of that in Nipponbare, respectively (Figure 4B). The metabolite IPSN was detected, but not hydroxy-imazosulfuron. The amount of radioactivity at the origin of the TLC plates that included the conjugated metabolites was greatly increased in both pIES2C9- and pIJU2C9-transformed plants, to 3.2 and 5.1 times, respectively, the value in Nipponbare.

The residual levels of imazosulfuron in the culture medium were also analyzed (Figure 4C). After 22 h of incubation, the residual amounts of imazosulfuron in the culture medium of pIES2C9- and pIJU2C9-transformed plants had decreased to 69% and 13%, respectively, of that in Nipponbare.

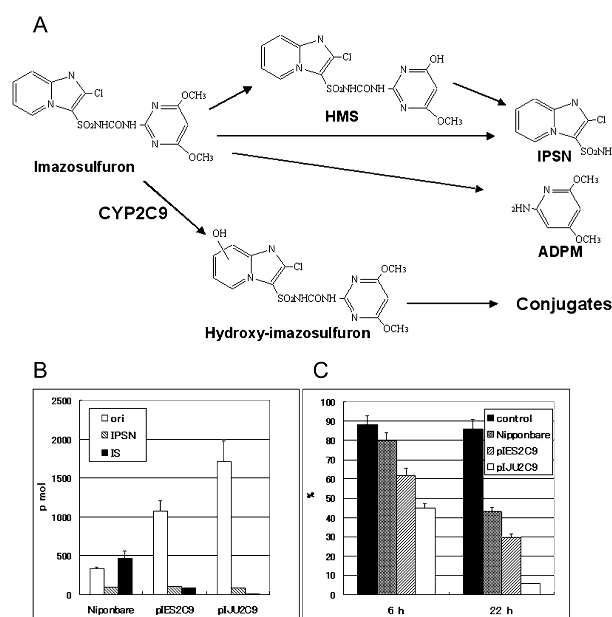

Figure 4. Metabolization of imazosulfuron in transgenic rice plants expressing *CYP2C9*. (A) Schematic diagram of the metabolic pathway of imazosulfuron in transgenic rice plants. (B) Thin-layer chromatography (TLC) analysis of transgenic rice plants treated with 50000 dpm of [^{14}C]imazosulfuron at 1.25 μM for 24 h. Nipponbare, pIES2C9-transformed (57E), and pIJU2C9-transformed (U926) plants were used. The plant extracts (2000 dpm) developed on TLC plates with chloroform and acetic acid (9 : 1, v/v). Values are averages$\pm$standard deviation of three independent experiments ($p<0.05$). ori, the origin of the TLC plate; IPSN, imazosulfuron metabolite (2-chloroimidazo[1,2-*a*]pyridine-3-sulfonamide); IS, imazosulfuron. (C) Residual imazosulfuron in the culture medium was analyzed by TLC. Values are averages of two independent experiments. The radioactivity of the added imazosulfuron was set at 100%. Control, control medium without any plants; Nipponbare, non-transgenic Nipponbare; pIES2C9, pIES2C9-transformed plants (57E); pIJU2C9, pIJU2C9-transformed plants (U926).

Herbicide tolerance in soil

We confirmed the herbicide tolerance of rice plants planted in soil by using Nipponbare plants, and pIJ2C9 (9122), pIES2C9 (57E), and pIJU2C9 (U949)-transformed plants (Figure 5). After 4 weeks of treatment, the growth of Nipponbare plants treated with chlorsulfuron and triasulfuron was severely retarded (Figure 5A, B). pIJ2C9-transformed plants showed weak tolerance and pIES2C9-transformed plants showed medium tolerance. pIJU2C9-transformed plants showed the strongest tolerance among the three transgenic plants. The growth of the transgenic rice plants was consistent with the level of expression of CYP2C9 by Western-blotting analysis. On the other hand, all rice plants showed healthy growth in the presence of imazosulfuron (Figure 5C).

Discussion

We compared three transgenic rice lines transformed with pIJ2C9, pIES2C9, and pIJU2C9, which express *CYP2C9* under the control of the CaMV35S, chimeric CaMV 35S, and maize polyubiquitin 1 promoters, respectively. We previously reported that the pIES2C9 transgenic rice acquired the ability to metabolize chlorsulfuron and imazosulfuron, resulted in their tolerance to these herbicides (Inui et al. 2001a). We focused on the level of gene expression and the strength of tolerance to sulfonylurea herbicides. Non-transgenic Nipponbare rice plants did not germinate or grow in MS medium containing 25 nM chlorsulfuron. pIJ2C9-transformed plants did not show herbicide tolerance in MS medium containing 50 nM chlorsulfuron, and they showed retarded growth in MS medium containing 30 nM chlorsulfuron. However, the pIES2C9-transformed plants (57E) showed some tolerance in culture medium containing 50 nM chlorsulfuron. Thus, we supposed that both the seven-tandem enhancer sequence in the CaMV 35S promoter and the AMV 5′-UTR region contributed to the improvement in herbicide tolerance. The pIJU2C9-transformed plants showed clear

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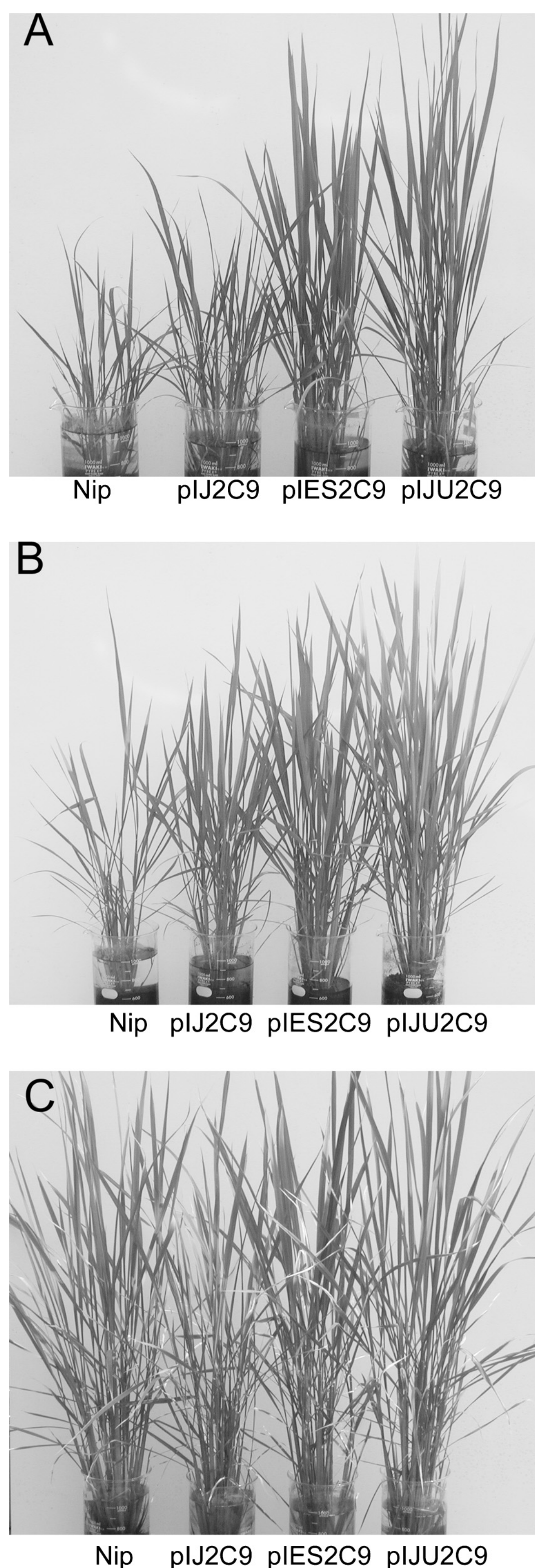

Figure 5. Herbicide tolerance of transgenic rice plants expressing *CYP2C9* in soil. Rice plants were grown in soil containing 0.86 μM chlorsulfuron (A), 0.31 μM triasulfuron (B), or 2.9 μM imazosulfuron (C). The growth was observed after 4 weeks of application. Lane Nip, non-transgenic Nipponbare; lane pIJ2C9, pIJ2C9-transformed plants (9122); lane pIES2C9, pIES2C9-transformed plants (57E); lane pIJU2C9, pIJU2C9-transformed plants (U949).

herbicide tolerance in culture medium containing 50 nM chlorsulfuron, and showed tolerance even in culture medium containing 200 nM chlorsulfuron (data not shown). Therefore, the herbicide tolerance could be increased at least 8-fold by expressing *CYP2C9* under the control of the maize polyubiquitin promoter.

In our TLC analysis, pIJU2C9-transformed plants showed higher metabolic activity than pIES2C9-transformed and non-transgenic plants. The amount of imazosulfuron left by pIJU2C9-transformed plants was only 1.2% of that left by non-transgenic plants. Imazosulfuron is mainly metabolized to HMS and/or IPSN in pea (Shimizu et al. 1996) and rice (Ishida et al. 1996). The proposed metabolic pathway for imazosulfuron in the transgenic rice plants is shown in Figure 4A. CYP2C9 is supposed to produce hydroxy-imazosulfuron (Inui et al. 2001a), but in our experiments only IPSN was detected. The metabolization of imazosulfuron was enhanced in the pIJU2C9-transformed plants relative to the non-transgenic plants. This was probably because CYP2C9 produced hydroxy-imazosulfuron, which was rapidly conjugated with glucose before the hydroxy-imazosulfuron was accumulated in plants. HMS was not detected and is also seemed to be metabolized to conjugated compounds. In fact, the amounts of conjugated compounds ("ori" in Figure 4B) were increased in transgenic rice plants to 3 to 5 times the levels in non-transgenic plants. So conjugation also seems to be an important step in the detoxification of imazosulfuron.

The sulfonylurea herbicide chlorsulfuron is hydroxylated to the phytotoxic 5-OH metabolite, which is immediately converted to glucose conjugates (Sweetser et al. 1982), which do not inhibit ALS (Hutchison et al. 1984). In our study, the introduction of *CYP2C9* added a new route for the metabolization of imazosulfuron rice plants, which resulted in the enhancement of metabolization and herbicide tolerance in transgenic rice plants expressing human CYP2C9.

The decrease of imazosulfuron in the culture medium of pIJU2C9-transformed plants was much greater than that in the culture medium of non-transgenic Nipponbare plants. The level of imazosulfuron remaining decreased to 13% of that remaining with the non-transgenic plants after 22 h of incubation. This also indicates the enhancement of the metabolization of imazosulfuron by CYP2C9.

We also checked the herbicide tolerance of transgenic rice plants grown in soil for 14 days. Chlorsulfuron and triasulfuron are used at 9–25 g ha^{-1} and 5–10 g ha^{-1}, respectively, in cereal fields (Tomlin 2000). Imazosulfuron is used at 75–95 g ha^{-1} in rice paddies (Tomlin 2000). In our study, the pIES2C9- and pIJU2C9-transformed plants showed tolerance to these herbicides in pot cultivation (Figure 5). The strength of the

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herbicide tolerance was consistent with the expression level of CYP2C9. The equivalent dosage used in this experiment was 100 g ha^{-1} for chlorsulfuron, 50 g ha^{-1} for triasulfuron, and 1000 g ha-1 for imazosulfuron, approximately 5–10 times greater than practical use. These results suggest that pIJU2C9-transformed plants could be tolerant enough to these herbicides for cultivation with chlorsulfuron and triasulfuron at an early growing stage in paddy fields.

On the other hand, all rice plants grew well in the presence of imazosulfuron. Imazosulfuron is used on rice, which can detoxify it. Deng and Hatzios (2003) supposed that rice plants have an endogenous P450 to metabolize imazosulfuron, which appears to be enough to detoxify it even at over 10 times the practical dosage.

Although the widely used CaMV 35S promoter is active in monocot cells, its relative strength is substantially less than in dicot cells (Bruce et al. 1989; Christensen et al. 1992). Some improvement had been added to CaMV 35S promoter to obtain higher gene expression (Mitsuhara et al. 1996; Jobling and Gehrke 1987). Meanwhile, the maize polyubiquitin 1 promoter with the first exon and first intron of its gene is significantly more active than the CaMV 35S promoter in monocot cells (Cornejo et al. 1993; Christensen and Quail 1996). In our transgenic rice plants, the expression levels of human CYP2C9 were highest in the plants transformed by pIJU2C9 (ubiquitin), second in the pIES2C9 (enhanced CaMV 35S) and lowest in the plants transformed by pIJ2C9 (CaMV 35S). These results were supported by all of the experiments (germination test, RT-PCR, Western blot analysis, TLC analysis and herbicide tolerance in soil) performed in this study.

Sulfonylurea herbicides were used on 70% to 80% of the paddy fields in Japan in the 1990s, although chlorsulfuron and triasulfuron are not used in Japan, since rice plants are sensitive to them. Some pIES2C9-transformed plants showed some tolerance to imazosulfuron and triasulfuron, but all pIJU2C9-transformed plants showed clear tolerance to these herbicides and grew well. Therefore, the use of transgenic rice plants transformed with pIJU2C9 would be useful for the introduction of tolerance to the herbicides, because the metabolic detoxification was enhanced by the introduced *CYP2C9*.

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 (Ohkawa et al. 1999). In the future, transgenic plants expressing P450 species may be useful for engineering herbicide tolerance and green chemistry.

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