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Transgenic Note

Broad range of herbicide tolerance of glutinous upland rice variety 'Yumenohatamochi' carrying human cytochrome P450 genes

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Abstract We generated three types of herbicide-tolerant Yumenohatamochi rice plants (*Oryza sativa* L cv. 'Yumenohatamochi') transformed with pIES1A1, pIJ2B6, or pIKBACH that express human cytochrome P450 gene *CYP1A1* or *CYP2B6* or co-express *CYP1A1*, *CYP2B6*, and *CYP2C19*, respectively. The transformed plants were screened by a combination of hygromycin resistance, PCR, and herbicide tolerance. We compared the tolerance towards various herbicides with different modes of action. The herbicides tested include photosynthesis inhibitors (chlorotoluron, diuron), very long-chain fatty acid (VLCFA) synthesis inhibitors (acetochlor, alachlor, metolachlor, and mefenacet), a carotenoid biosynthesis inhibitor (norflurazon), and microtubule assembly inhibitors (pendimethalin, trifluralin, and amiprophos-methyl). The pIES1A1- or pIJ2B6-transformed Yumenohatamochi showed tolerance towards five and four different chemical classes of herbicides, respectively. The spectrum of herbicide tolerance of pIKBACH-transformed Yumenohatamochi was broader compared to transformants expressing the single human P450 gene. Transformed rice plants expressing P450s involved in xenobiotic metabolism may become useful tools for the breeding of herbicide-tolerant crops.

Key words: CYP1A1, CYP2B6, detoxification, transgenic plants, xenobiotic metabolism.

Yumenohatamochi is a glutinous upland rice and a highly drought tolerant variety in Japan because of the deep elongation of its thick roots (Hirasawa et al. 1998). At present, this variety is of the highest eating quality of upland glutinous rice. Because upland rice is grown in dry fields, weed infestation is a major problem. Herbicides are viewed as a laborsaving means of improving crop yield and quality because weed infestation adversely affects crop production by reducing yields and decreasing market prices of the crop (Lockhart et al. 1990). The proper use of chemicals for weed control maintains a biomass residue that helps conserve soil and moisture, thereby promoting sustainable agriculture, whereas mechanical weed control disturbs the soil and results in both loss of soil moisture and erosion.

Herbicides are now widely used for crop cultivation and sprayed herbicides are usually removed from the environment by chemical degradation and degradation by bacteria and plants. In higher plants as well as animals and bacteria, herbicides are metabolized to more hydrophilic metabolites by cytochrome P450 (P450 or CYP) monooxygenases. Mammalian cytochrome P450 monooxygenases in liver are involved in the metabolism of foreign chemicals and endogenous substrates (Funae et al. 1998). Human P450s that metabolize xenobiotic chemicals are almost exclusively in the gene families CYP1, CYP2, and CYP3 and to a lesser degree, CYP4 (Nebert and Russell 2002). Inui et al. (2001b) using a recombinant yeast system found that 11 human P450s in the CYP1, 2, and 3 families metabolized many kinds of agro-chemicals, including 27 herbicides and 4 insecticides. Transformed plants harboring such drug-metabolizing P450 genes from mammals were expected to be tolerant to herbicides and to be able to eliminate residual agrochemicals (Ohkawa et al. 1999).

We have already produced several transformed Nipponbare rice plants (*Oryza sativa* L cv. Nipponbare) expressing human P450s. The transformed Nipponbare expressing human CYP1A1 showed tolerance towards various herbicides, including chlorotoluron, norflurazon, and quizalofop-ethyl (Kawahigashi et al. 2003). Transformed Nipponbare expressing CYP2B6 showed a strong herbicide tolerance towards chloroacetamide

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herbicides such as metolachlor (Hirose et al. 2005). Transformed Nipponbare expressing human CYP2C9 or CYP2C19 showed tolerance towards the herbicides chlorsulfuron or norflurazon, respectively (Inui et al. 2001a). All transformed rice plants expressing a single human P450 gene had a certain range of herbicide tolerance. However, when P450 genes are used in combination, the tolerance is even more pronounced. For example, the Nipponbare transformed by pIKBACH, which co-expresses three human P450s—CYP1A1, CYP2B6, and CYP2C19—showed a much broader spectrum of herbicide tolerance (Kawahigashi et al. 2005).

The wider cross-tolerance to herbicides having different modes of action and different chemical structures seems to be a special feature of transformed plants expressing the mammalian P450 genes involved in xenobiotic metabolism. This cross-tolerance would prove useful to prevent the development by weeds of herbicide resistance, because this cross-tolerance would allow use of several herbicides in rotation without harming the crop. Use of several herbicides in rotation would impede the development of herbicide resistant weeds. If multiple herbicide tolerance can be introduced into Yumenohatamochi, the Yumenohatamochi plants are useful for practical applications, especially in direct seedling systems in upland fields where rice plants must compete with many weeds. Therefore, in this study, we introduced P450 genes into Yumenohatamochi and evaluated the resulting plants' herbicide tolerance to assess their potential in producing herbicide-tolerant crops.

Expression plasmids pIKBACH, harboring human *CYP1A1*, *CYP2B6*, and *CYP2C19* cDNA in tandem, and pIES1A1 and pIJ2B6, harboring single human P450 species *CYP1A1* or *CYP2B6*, respectively, were constructed and reported previously (Figure 1) (Kawahigashi et al. 2003; Hirose et al. 2005; Kawahigashi et al. 2005). The expression plasmids were each introduced into *Agrobacterium tumefasciens* strain EHA101, which was subsequently used for transformation of *Oryza sativa* L cv. 'Yumenohatamochi.' Yumenohatamochi exhibited a low callus formation ability and low regeneration ability compared to a standard rice variety in Japan, 'Nipponbare,' making it difficult to obtain regenerated plants. Thus, we modified the previous method (Toki 1997) as follows: a) The concentration of acetosyringone for the infection of *Agrobacterium* was twice as high ($20\,mg\,l^{-1}$) as that used for Nipponbare. b) The period of the selection by hygromycin in N6D solid medium was shortened as much as possible. c) The concentration of hygromycin in the regeneration medium was reduced from $50\,mg\,l^{-1}$ to $30\,mg\,l^{-1}$. The transformation efficiency was approximately four times greater than that of the previous method.

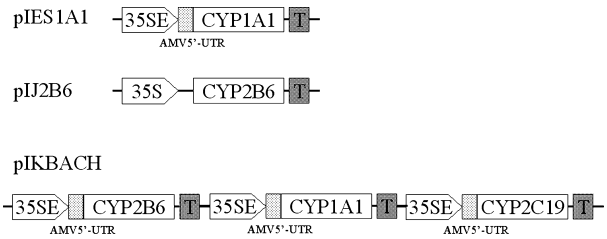

Figure 1. Schematic structure of the expression plasmids pIES1A1, pIJ2B6, and pIKBACH used to transform Yumenohatamochi. 35SE, cauliflower mosaic virus (CaMV) 35S promoter with seven-tandem enhancer region (−290 to −90) from CaMV 35S promoter; T, nopaline synthase terminator; 35S, CaMV 35S promoter; AMV-5′UTR, alfalfa mosaic virus 5′-untranslated region (Jobling and Gehrke 1987). Each expression plasmid contains a hygromycin resistance gene (hygromycin B phosphotransferase) and a kanamycin resistance gene (neomycin phosphotransferase II) for selection purposes.

Regenerated plants (T_0) on culture medium containing $30\,mg\,l^{-1}$ hygromycin were screened by PCR as described previously (Kawahigashi et al. 2003). The transformed plants containing the P450 genes were grown in a greenhouse and their seeds (T_1) were harvested. T_1 seeds were screened by germination tests in a test tube (diameter, 2.5 cm; height, 15 cm) with 10 ml of the MS solid medium (Murashige and Skoog 1962) containing a herbicide. Eight out of 24 lines of pIJ2B6-transformed plants were tolerant to 5 μM metolachlor. For pIES1A1-transformed plants, 9 out of 21 lines were tolerant to 0.4 μM norflurazon. For pIKBACH- transformed plants, 9 out of 25 lines were tolerant to both 0.4 μM norflurazon and 1.5 μM mefenacet. These herbicide tolerant lines harbored corresponding P450 genes confirmed by PCR. Among them, 5 lines (T_1) of each transgenic rice plants (Dr1A-6, 8, 9, 11 and 12 for pIES1A1- transformed Yumenohatamochi, Dr2B-2, 8, 10, 16 and 24 for pIJ2B6-transformed Yumenohatamochi, Dr3r-3, 12, 14, 15 and 20 for pIKBACH- transformed Yumenohatamochi) were used for further germination tests. Transformed Nipponbare rice plants expressing human P450 species were also generated previously (Kawahigashi et al. 2003; Hirose et al. 2005; Kawahigashi et al. 2005). Three homozygous lines (T_4) were used for experiments: E281 for the pIES1A1- transformed Nipponbare, A11 for pIJ2B6-transformed Nipponbare, and 3r35 for the pIKBACH-transformed Nipponbare.

Germination tests for herbicide tolerance were carried out in test tubes 2.5 cm in diameter and 15 cm in height. Four seeds of transformed or non-transformed control Yumenohatamochi were embedded in 10 ml of the MS solid medium containing a herbicide in each test tube and cultured at 27°C for 7 to 14 days under 16 h of light (photon flux density 40 $\mu mol\,m^{-2}\,s^{-1}$). The herbicide concentrations were shown in Table 1.

The results of germination tests of transformed

Table 1. Herbicide tolerance of transformed Nipponbare and Yumenohatamochi rice plants in germination test.

HRAC group[1]	Mode of action (Chemical family)	Herbicide	Concentration[2] (μM)	Transgenic rice plants transformed with[3]					
				pIES1A1		pIJ2B6		pIKBACH	
				NIP	YUME	NIP	YUME	NIP	YUME
C2	Inhibition of photosynthesis								
	(Urea)	Diuron	150	+	ND	−	ND	+	+
	(Urea)	Chlorotoluron	100	+	+	−	ND	+	+
F1	Inhibition of Carotenoid biosynthesis								
	(Pyridazinone)	Norflurazon	0.4	++	++	+	+	++	++
K1	Inhibition of microtubule assembly								
	(Dinitroaniline)	Pendimethalin	10	+	−	++	+	++	+
	(Dinitroaniline)	Trifluralin	7.5	ND	ND	++	+	+	+
	(Phosphoroamidate)	Amiprophos-methyl	2.5	++	+	+	+	++	+
K2	Inhibition of mitosis/microtubule organization								
	(Carbamate)	Chlorpropham	7.5	++	++	+	+	++	++
K3	Inhibition of VLCFAs								
	(Chloroacetamide)	Acetochlor	0.1	−	+	++	++	++	++
	(Chloroacetamide)	Alachlor	1.4	−	−	++	+	+	+
	(Chloroacetamide)	Metolachlor	2.5	−	ND	++	+	++	++
	(Oxyacetamide)	Mefenacet	1.5	++	++	+	+	++	++

VLCFA, very long chain fatty acid; NIP, Nipponbare; YUME,Yumenohatamochi.

[1] As defined in HRAC (http://www.plantprotection.org/HRAC/index.html).

[2] Final concentration of herbicide used for germination test.

[3] ++, Transgenic plants grew well but non-transgenic plants did not grow at all in the herbicide-containing medium; +, Transgenic plants grew but retarded and non-transgenic plants did not grow at all in the herbicide-containing medium; −, Neither transgenic or non-transgenic plants grew in the herbicide-containing medium; ND, not determined. In case of norflurazon, ++ indicates that transgenic plants showed healthy green shoots, + and indicates transgenic plants showed pale green shoots whereas the control plants showed white shoots by inhibition of carotenoid synthesis. More than 5 independent lines from each transgenic plant were used for germination tests.

Yumenohatamochi are summarized in Table 1. The pIES1A1-, pIJ2B6-, and pIKBACH-transformed Yumenohatamochi showed tolerance in the germination tests to 7 out of 8, 9 out of 9, and 11 out of 11 herbicides, respectively, which belong to different chemical families (The Herbicide Resistance Action Committee 2005). These are carbamate (chlorpropham), chloroacetamide (acetochlor, alachlor, and metolachlor), dinitroaniline (pendimethalin and trifluralin), oxyacetamide (mefenacet), phosphoroamidate (amiprophos-methyl), pyridazinone (norflurazon), and urea (chlorotoluron and diuron). The transformed Yumenohatamochi showed a similar spectrum of herbicide tolerance as their corresponding Nipponbare transformants (Table 1). As we expected, pIES1A1- and pIJ2B6- transformed Yumenohatamochi expressing single P450 species showed tolerance only to the corresponding herbicides that could be metabolized by each P450. The pIKBACH-transformed Yumenohatamochi showed tolerance to all these herbicides. The observed herbicide tolerance of the pIKBACH-transformed Yumenohatamochi resulted from the combination of the metabolic activity of CYP1A1, CYP2B6 and CYP2C19. Thus, we concluded that the three introduced P450s worked additively, not synergistically, in the transgenic rice plants.

The results of some germination tests are shown in Figure 2. With the addition of microtubule assembly inhibitor amiprophos-methyl, pIKBACH-transformed Yumenohatamochi showed healthy shoots and roots (Figure 2A), but non-transformed plants showed retarded growth. Similar results were obtained with the addition of the other inhibitors of microtubule assembly/organization, chlorpropham, pendimethalin, and trifluralin. With the addition of photosynthesis inhibitor chlorotoluron (Figure 2B), pIKBACH-transformed Yumenohatamochi showed healthy growth, but non-transformed plants showed retarded growth. Similar results were obtained with the addition of diuron. In the case of inhibitors of very long-chain fatty acid (VLCFA) synthesis, metolachlor and mefenacet (Figure 2C, D), transformed Yumenohatamochi showed healthy growth, but non-transformed plants did not grow at all. Similar results were obtained with the addition of acetochlor and alachlor. When carotenoid biosynthesis inhibitor norflurazon was added, pIKBACH-transformed Yumenohatamochi developed green healthy shoots, but non-transformed plants had white shoots–an indication that carotenoid synthesis had been disrupted. The observed herbicide tolerance of the human P450-expressing rice plants was consistent with *in vitro* catalysis of these herbicides by recombinant yeast microsomes expressing human P450s (Inui et al. 2001b).

To evaluate herbicide tolerance of plants grown in soil, rice seeds (T_1) were embedded in MS solid medium containing 50 mg l^{-1} hygromycin and three 7-day-old plants were transplanted to a Wagner pot (1/5000 a) with Andosol (local soil). Both pIKBACH-transformed and non-transformed Yumenohatamochi were grown in a

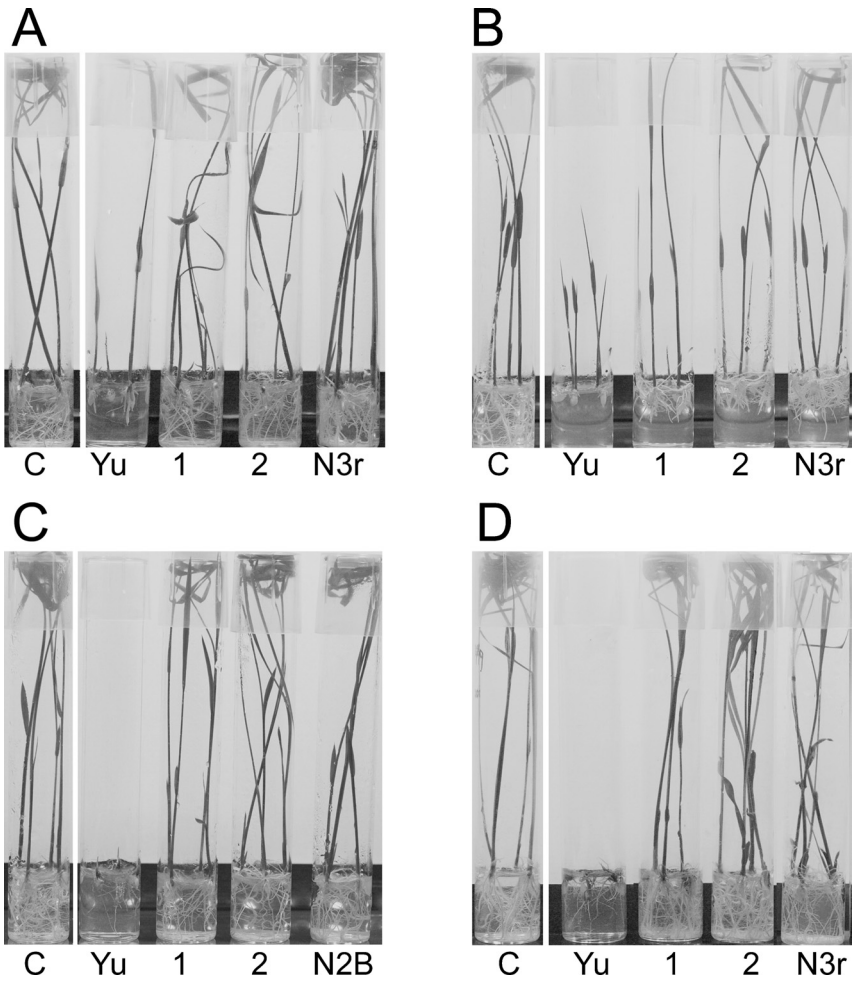

Figure 2. Herbicide tolerance of pIKBACH-transformed Yumenohatamochi in test tubes. Germination tests were performed in 10 ml of the MS solid medium containing 2.5 μM amiprophos-methyl (A), 100 μM chlorotoluron (B), 2.5 μM metolachlor (C), or 1.5 μM mefenacet (D). Lane C, non-transformed Yumenohatamochi without herbicide; lane Yu, non-transformed Yumenohatamochi with herbicide; lanes 1 and 2, pIKBACH-transformed Yumenohatamochi with herbicide; lane N3r, pIKBACH-transformed Nipponbare with herbicide; lane N2B, pIJ2B6-transformed Nipponbare with herbicide.

greenhouse for 2 weeks with 400 mg of Dairon (Sumikatakeda, Tokyo, Japan), which contains 3% diuron (twice what is used in fruit fields), or 16 μl of Dual (Novartis, Basel, Switzerland), which contains 45% metolachlor (twice what is used in cornfields) (Nouyaku Hand Book 2001). The pIKBACH-transformed Yumenohatamochi showed healthy growth despite the herbicide treatment. However, the growth of non-transformed plants was stopped by metolachlor or severely damaged by diuron's inhibition of photosynthesis (Figure 3). These results suggest that pIKBACH-transformed Yumenohatamochi could survive exposure to these herbicides at an early growing stage in dry upland fields.

In the field, repeated use of one herbicide tends to induce the rapid evolution of herbicide-resistant weeds. To avoid serious damage to crops by such herbicide-resistant weeds, several herbicides with different modes of action and chemical structures should be used in rotation (Putwain 1990). A combination of transformed Yumenohatamochi expressing human P450s and rotation of different herbicides would be useful to prevent the emergence of herbicide-resistant weeds in the direct-seeding system for upland rice, where there is no water cover and the germinated rice has to compete with many weeds.

The herbicide tolerance of transformed rice plants expressing human P450s were shown both in paddy and upland rice plants. In a greenhouse, the transformed Yumenohatamochi showed normal growth and morphology including plant height, leaf color, and seed size compared with non-transformed Yumenohatamochi. Although there was no morphological difference observed between the transformed and non-transformed rice plants, further investigations, including safety assessment of transformed rice plants and experiments for practical use, are needed. Because the wider cross-tolerance to herbicides having different modes of action and different chemical structures seems to be a special feature of transformed plants expressing human P450

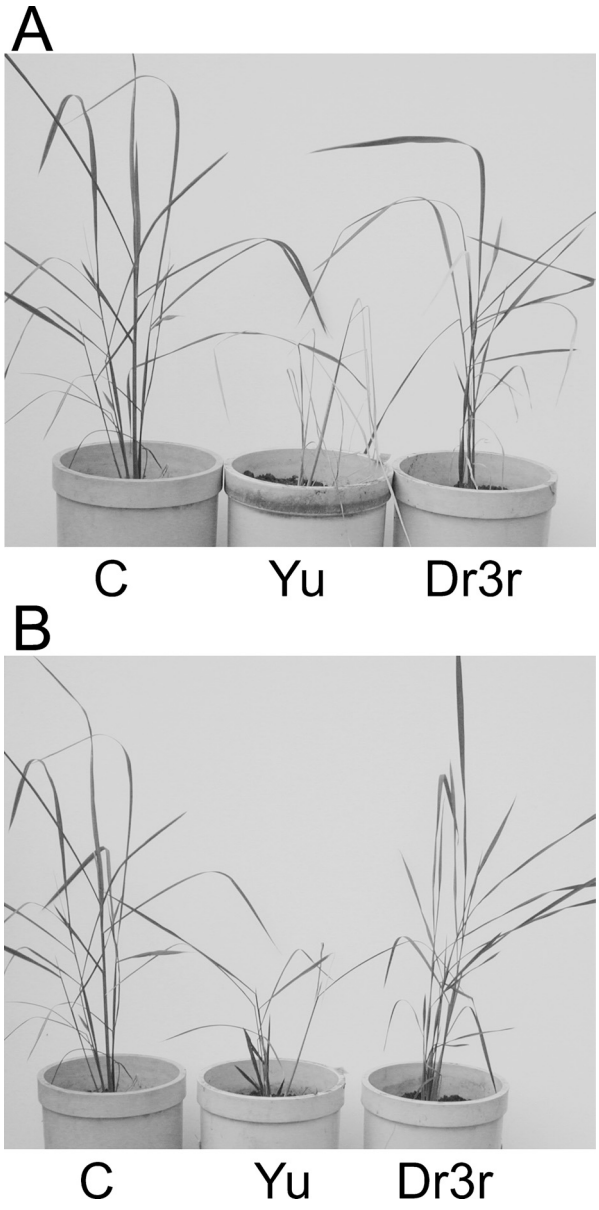

Figure 3. Herbicide tolerance of pIKBACH-transformed Yumenohatamochi rice plants in soil. Yumenohatamochi plants were grown in soil for 2 weeks and then each pot received (A) 400 mg of Dairon, or (B) 16 μl of Dual. Growth was observed 2 weeks after application of herbicide. Lane C, non-transformed Yumenohatamochi without herbicide treatment; lane Yu, non-transformed Yumenohatamochi with herbicide treatment; lane Dr3r, pIKBACH-transformed Yumenohatamochi with herbicide treatment.

genes involved in xenobiotic metabolism, these plants could be utilized for engineering herbicide-tolerant crops in the future.

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