

TRANSGENIC RICE AND POTATO PLANTS EXPRESSING HUMAN CYTOCHROME P450S SHOW CROSS-TOLERANCE TO HERBICIDES BY DETOXIFYING THEM

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ABSTRACT

Transgenic plants expressing various species of the detoxifying enzyme, cytochrome P450 monooxygenase from mammals, were bred by Agrobacterium-mediated transformation. The transgenic plants metabolized exogenous chemicals, including herbicides which they were able to tolerate. Because the enzymes had broad substrate specificity, the transgenic plants showed cross-tolerance to several herbicides, even though these had different modes of action and different chemical structures. Plants with cross-tolerance can be used in a herbicide rotation system, to avoid or delay the evolution of herbicide-resistant weeds. The transgenic plants are also expected to reduce the environmental load of agricultural chemicals on farmland.

INTRODUCTION

The commercial cultivation of herbicide-tolerant transgenic crops has increased in recent years. The main transgenic crops cultivated in the world are soybean and corn. These bear a mutant 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase gene, or a bacterial phosphinothricin acetyltransferase (PAT) gene (James 2000, Ohkawa *et al.* 1999). Although the use of appropriate herbicides has made weed management easy, repeated use of one herbicide tends to induce the rapid evolution of herbicide-resistant weeds (Benbrook 1999). One of several strategies to delay the evolution of resistance is the rotation of herbicides. This strategy was proposed many years ago, as the most practical approach (Putwain 1990).

Cytochrome P450 (or CYP) enzymes are involved in the metabolism of herbicides in plants. P450, in cooperation with NADPH-cytochrome P450 oxidoreductase (reductase),

catalyzes oxidation reactions of lipophilic compounds, including certain herbicides (Fig. 1). These P450 species play an important role in herbicide selectivity and resistance (Werck-Reichhart *et al.* 2000).

However, molecular information about these P450 species is quite limited, since it has been rather difficult to identify the function of a P450 species in a large gene family. For example, there are almost 300 species of *Arabidopsis* (Ohkawa *et al.* 1999).

On the other hand, the molecular mechanism of P450 species in mammalian liver microsomes, which are known to be drug-metabolizing enzymes, has been well studied. These P450 species are involved in the oxidative metabolism of xenobiotics, including herbicides. Since one of the mammalian P450s showed high herbicide-metabolizing activity and broad substrate specificity (Ohkawa *et al.* 1999), we produced potato and rice plants expressing mammalian P450.

Keywords: cytochrome P450, detoxification, herbicide resistance, transgenic plants, phytoremediation, weed management

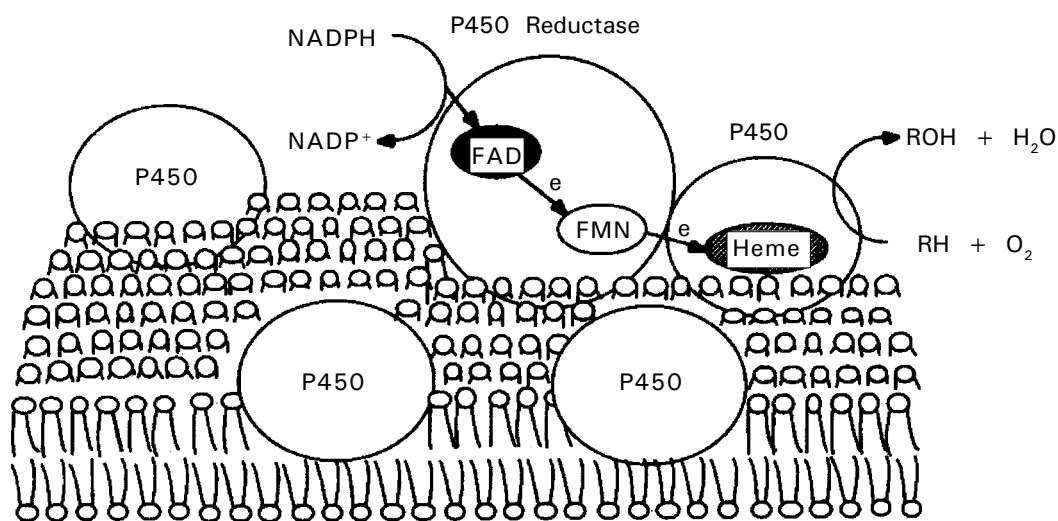


Fig. 1. Cytochrome P450 monooxygenases in plant microsomes.

Source: Ohkawa *et al.* 1998

HERBICIDE TOLERANCE OF TRANSGENIC POTATO EXPRESSING HUMAN P450 ENZYMES

Eleven P450 species in human liver; CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, have been reported to be involved in more than 90% of the P450-dependent metabolism of drugs (Funae *et al.* 1998). We examined the microsomes from the recombinant yeast strains expressing each of the 11 human P450 species. Twenty-seven out of 50 herbicides tested were found to be metabolized mainly by CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2C18, CYP2C19, CYP2D6 and CYP3A4. One herbicide was metabolized by several P450 species in different subfamilies and families, whereas each of the P450s metabolized several herbicides in different classes (Inui *et al.* 2001).

Based on these results, three major P450 species, CYP1A1, CYP2B6 and CYP2C19, belonging to different families and subfamilies, were selected and introduced into potato plants (*Solanum tuberosum* cv. MayQueen) by an *Agrobacterium*-mediated transformation system. The expression plasmids pUHA1, pUHB6 and pUHC19 were constructed by the insertion of human CYP1A1, CYP2B6 and CYP2C19 cDNAs, respectively, into pUTR121H. The

plasmid pIKBAC derived from pBI121 for co-expression of CYP1A1, CYP2B6 and CYP2C19 was also constructed by the insertion of each of the three expression units (Fig. 2).

After selection by a combination of kanamycin-resistance, PCR analysis, 7-ethoxycoumarin o-demethylase assay (standard assay for CYP1A1 activity) and Western blot analysis, the presence of the corresponding genes in the transgenic plant genome and their expression were confirmed by Southern, Northern and Western analyses. Four transgenic potato plants, S1965, S1972, S1974 and T1977 expressing CYP1A1, CYP2B6, CYP2C19, and all three of them, respectively, were finally selected.

The herbicide tolerance of the transgenic potatoes was evaluated 8 to 15 days after spraying the herbicide onto transgenic plants cultivated in pots. The T1977 plant exhibited tolerance toward the photosynthesis-inhibiting herbicides, atrazine (AT), chlortoluron (CT) and methabenzthiazuron (MT). In contrast, the non-transformed potato, MayQueen, was susceptible to these herbicides (Fig. 3). Although the S1965 plant was also tolerant to CT and MT, and the S1974 plant was slightly tolerant to AT, the S1972 plant was susceptible to these herbicides. The transgenic plants T1977, S1972 and S1974 exhibited resistance to the spray of the protein

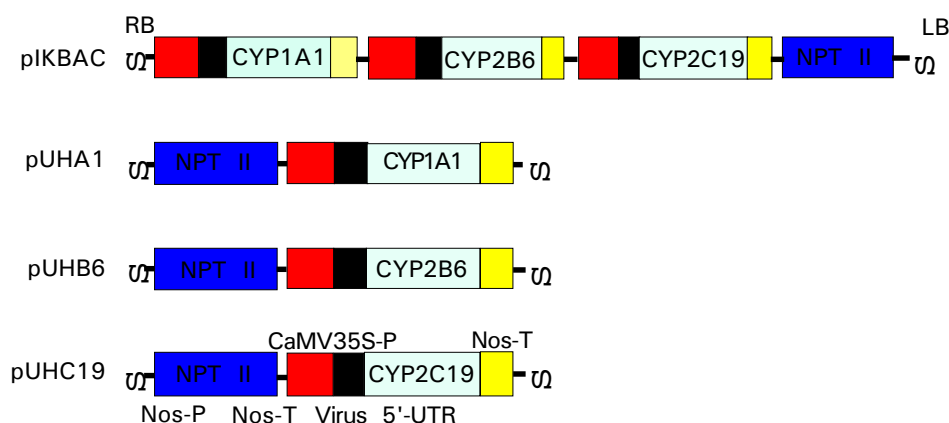


Fig. 2. Structure of the constructed expression plasmids for human CYP species

Source: Inui *et al.* 2000

biosynthesis-inhibiting herbicides, acetochlor (AC) and metolachlor (MC). However, MayQueen and S1965 were susceptible to the herbicides, and showed withered apical buds or did not germinate at all. Furthermore, T1977 and S1965 were highly tolerant toward the carotenoid biosynthesis-inhibiting herbicide, norflurazon (NR), whereas the apical parts of MayQueen and other transgenic plants were completely bleached. The transgenic plant T1977 treated with the lipid biosynthesis-inhibiting herbicide, piributicarb (PC), showed normal root elongation and growth, although MayQueen and other transgenic plants were susceptible and showed retarded root elongation and growth.

Based on these results, T1977 expressing three human P450 species, CYP1A1, CYP2B6 and CYP2C19, exhibited higher cross-tolerance to the herbicides with different modes of action and chemical structures. Even S1965, the transgenic potato expressing only one P450 species, CYP1A1, showed cross-tolerance to a 1,3,5-triazine herbicide, MT, a phenylurea herbicide, CT, and a pyridazinone herbicide, NR. S1972 expressing CYP2B6 and S1974 expressing CYP2C19 also showed cross-tolerance to chloroacetoanilid herbicides, AC and MC and to AT, AC and MC, respectively. In T1977, the three P450 species were considered to cooperatively metabolize and bring tolerance to PC which prohibit root elongation and growth in the

transgenic potatoes expressing single P450, S1965, S1972 and S1974 (Inui *et al.* 2000).

THE METABOLISM OF HERBICIDES IN TRANSGENIC POTATO EXPRESSING HUMAN P450 ENZYMES

The microsomes from the recombinant yeast strain expressing CYP1A1 metabolized AT (Inui *et al.* 2001). The transgenic potato, which expressed CYP1A1, showed tolerance to AT. The metabolism of AT in the CYP1A1 potato was then analyzed by thin-layer chromatography (TLC). ¹⁴C-labeled AT applied to a nutrient solution was rapidly taken up into the plants. ¹⁴C-labeled metabolites extracted from the plants were analyzed by TLC. Four metabolites were found, two of which were identified as deisopropylated (DI) and deisopropylated deethylated (DIDE) metabolites. However, deethylated AT (DE) reported in AT-resistant wheat (Lamoureux *et al.* 1973) was not found in these transgenic plants, nor in the non-transformed plants. The amount of DIDE, which is nonphytotoxic, was five times higher in the CYP1A1 potato than in the non-transformed plant over eight days (Table 1). The amount of the unknown compound (UK2) at the origin of the TLC plate appeared to be higher in the CYP1A1 plant than the non-transformant.

From these results, the transgenic potato

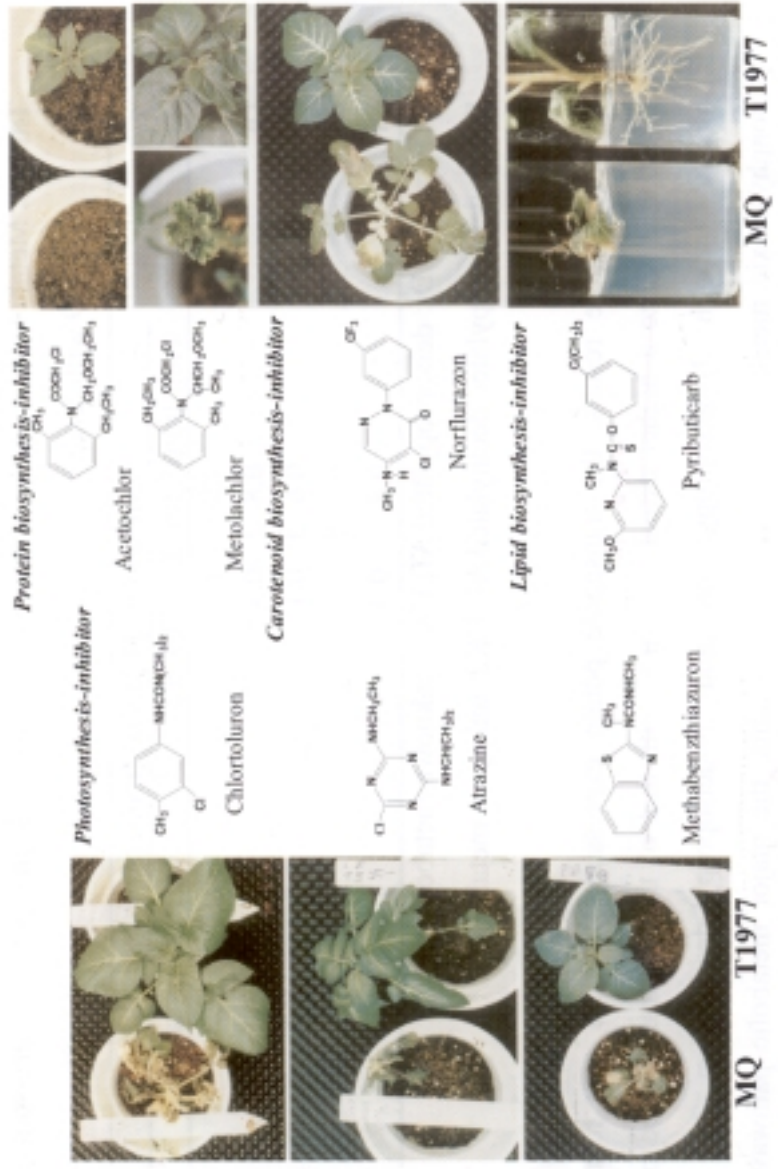


Fig. 3. Tolerance of T1977 to several herbicides with different modes of action and different structures

Table 1. Metabolism of ¹⁴C-labeled atrazine in the transgenic potato plant

AT/metabolite	Metabolite produced (nmol/plant/8 days)	
	Non-transformed MayQueen	CYP1A1 potato
AT	3.0 ± 1.3	2.5 ± 0.9
DI	0.8 ± 0.4	1.1 ± 0.2
UK1	0.6 ± 0.1	0.8 ± 0.2
DIDE	0.2 ± 0.1	1.0 ± 0.3
UK2	0.8 ± 0.3	1.2 ± 0.3

AT: atrazine, DI: deisopropylated AT, UK1: unknown metabolite 1, DIDE: deisopropylated deethylated AT, UK2: unknown metabolite 2

Source: Inui *et al.* 1999

expressing CYP1A1 was found to metabolize AT more than the non-transformed potato, mainly through N-deisopropylation and N-deisopropyl-deethylation to yield nonphytotoxic metabolites. In AT-resistant plants, the major metabolic pathway was glutathione (GSH) conjugation, as found in sorghum (Lamoureux *et al.* 1973). N-dealkylation has also been reported to contribute less to resistance of 1,3,5-triazines compared with GSH conjugation. However, accumulation of the dealkylated metabolite, DIDE, seemed to be important for tolerance toward AT in the transgenic CYP1A1 potato, since it seemed to metabolize AT through deisopropylation and then deethylation to yield DIDE, as shown in Fig. 4, which was five times higher than the non-transformed potato. Therefore, higher tolerance of the CYP1A1 potato seemed to be due to the ability of the CYP1A1 potato plants to form DIDE.

HERBICIDE TOLERANCE OF TRANSGENIC RICE EXPRESSING HUMAN P450 ENZYMES

Five major P450 species, CYP1A1, CYP2B6, CYP2C9, CYP2C18 and CYP2C19, belonging to different families and subfamilies, were selected and introduced into rice (*Oryza sativa* L. var. Nipponbare) plants. The structure of the P450 expression plasmids is shown in Fig. 5. The expression plasmids, pIES1A1, pIJ2B6, pIJ2C9, pIES2C9, pIJ2C18, pIES2C18, pIJ2C19, pIES2C19 were constructed by the insertion of each human P450 cDNA into pIG121Hm. Transgenic rice plants were generated by PEG-mediated transformation or *Agrobacterium*-mediated

transformation (Hiei *et al.* 1994). After selection with hygromycin, genomic DNAs were extracted from the leaves of regenerated rice plants and subjected to PCR analysis and Southern Blot analysis, in order to confirm the presence of the P450 cDNAs. R1 seeds of transformed rice were sown onto medium containing an appropriate herbicide, to screen the transformants expressing P450s. The selected lines of transformed rice were named CYP1A1 rice, CYP2B6 rice etc.

In general, the mature plants are more resistant to herbicides than germinating seeds and seedlings. The herbicide tolerance of the CYP rice plants was then tested by germination tests on MS medium containing appropriate herbicides. R2 seeds from homozygous CYP2B6 rice were seeded in the medium, which contained six kinds of herbicides. These had been metabolized *in vitro* by the microsome from the recombinant yeast strains expressing CYP2B6.

All seeds showed a high tolerance to the protein biosynthesis-inhibiting herbicides, MC, AC and alachlor (AL) and the cell division-inhibiting herbicide, trifluralin (TF). On the other hand, non-transformed rice did not produce any shoots on the medium with MC, AC or AL, nor did it produce any roots on the medium with TF (Fig. 6). CYP2B6 rice was also slightly resistant to the protein biosynthesis-inhibiting herbicide, mefenacet (MF) and the photosynthesis-inhibiting herbicide, chloridazon. R1 seeds from transformants expressing CYP2C19 were seeded in 10 kinds of herbicides and showed tolerance to five of them, CT, PM, NR, MF and PC (Table 2). CYP1A1 rice was tolerant

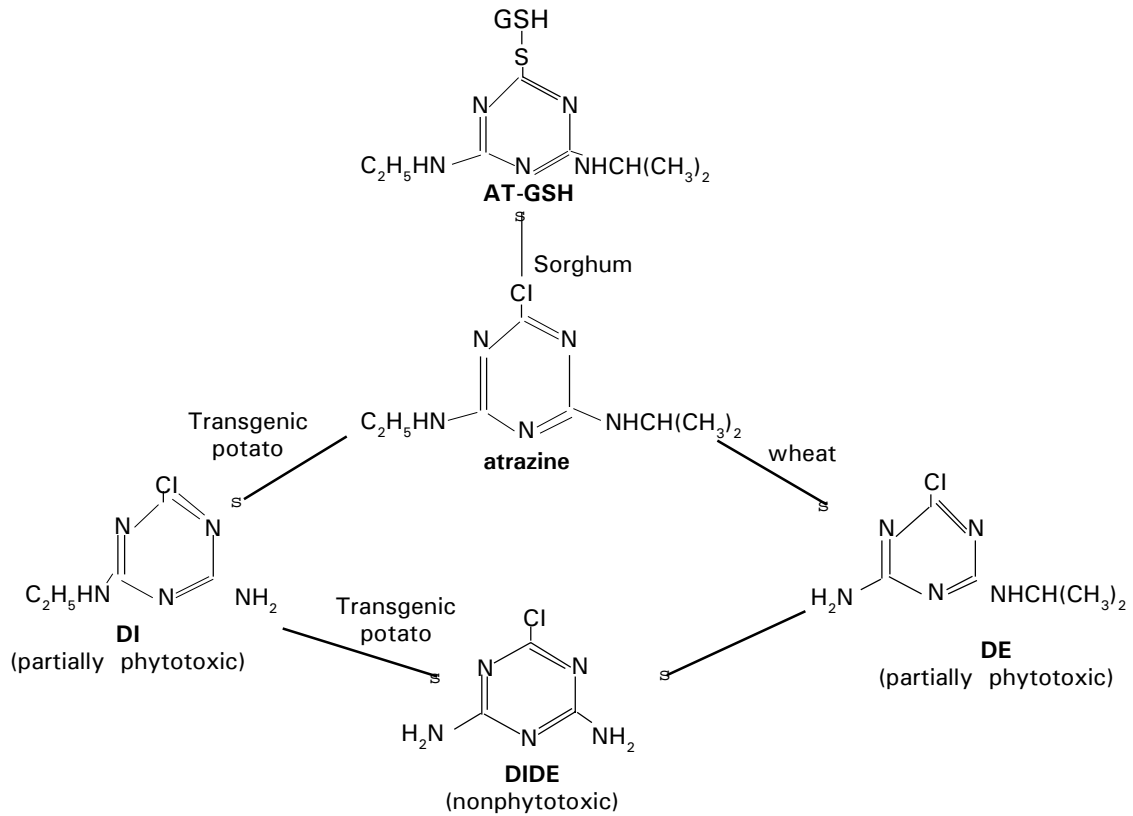


Fig. 4. Major metabolic pathways for the herbicide atrazine in higher plants

Source: Inui *et al.* 1999

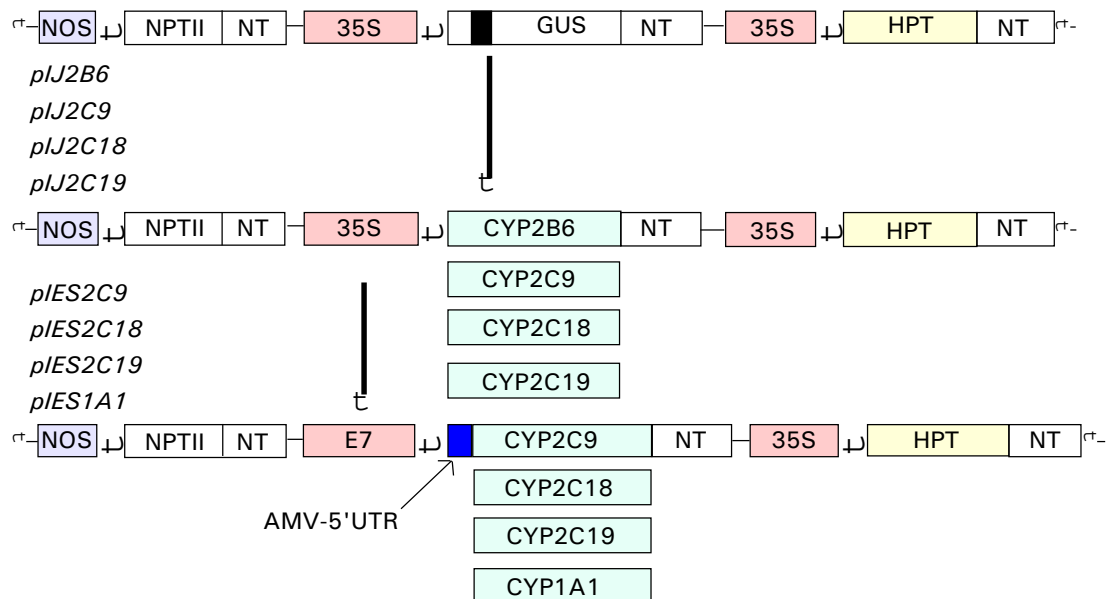


Fig. 5. Structure of the constructed expression plasmids for human CYP species.

Source: Hirose *et al.* 1999

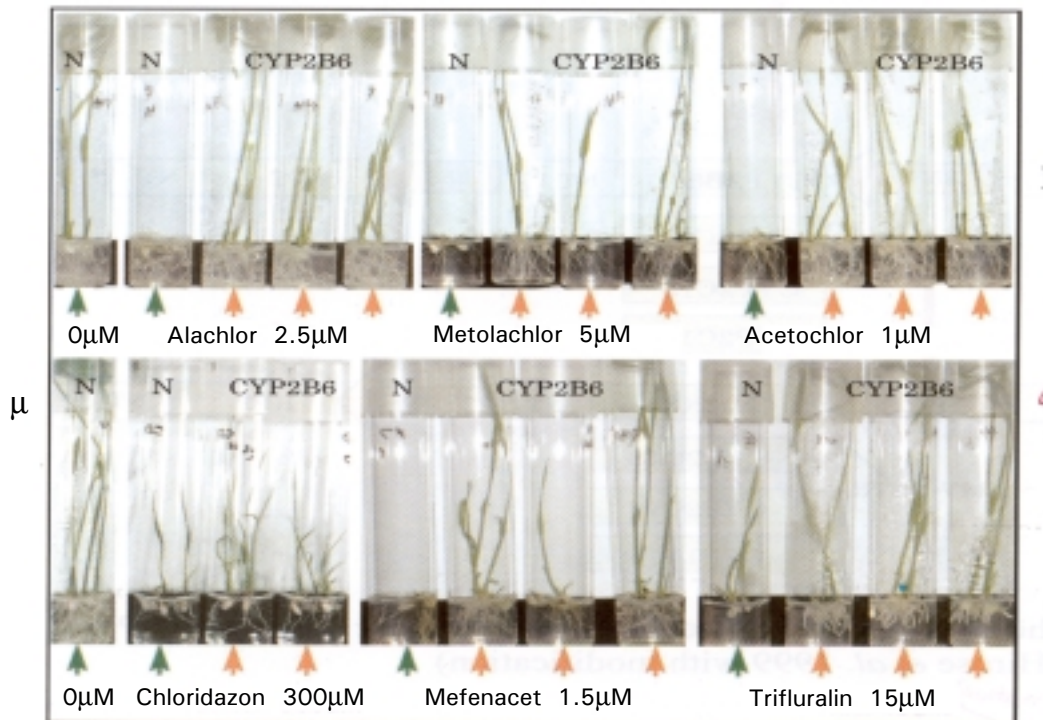


Fig. 6. Tolerance of CYP2B6 rice to herbicides.

N: Nipponbare CYP2B6: CYP2B6 rice

of the photosynthesis-inhibiting herbicides CT and NR, the cell division-inhibiting herbicide MF, and the acetyl CoA carboxylase-inhibiting herbicide quizalofop-ethyl (QE). CYP2C9 rice was tolerant of branched chain amino acid biosynthesis-inhibiting herbicides, chlorsulfuron (CS) and imazosulfuron (IS). However, the transformant for CYP2C18 did not show clear tolerance to any of the herbicides examined.

The transgenic rice plants expressing human P450 species showed tolerance to most of the herbicides corresponding with the P450 species expressed in yeast microsomes metabolized *in vitro*. However, in some cases such as the CYP2C18 rice, the transformed rice plant did not show tolerance to the herbicides. This was possibly because of the low activity of the P450 enzyme *in vivo*. Although there were some exceptions, the catalytic experiment of herbicides in microsomes derived from recombinant yeast strains expressing P450 enzymes suggested possible tolerance to the corresponding herbicides in the transgenic rice plants.

The herbicides tested belong to different classes: MC, AC and AL to chloroacetoanilid, MF to oxyacetamide, TF to 2,6-dinitroaniline, chloridazon and NR to pyridazinone, PC to thiocarbamate, QE to 2-(4-aryloxyphenoxy) propionic acid, CS and IS to sulfonylurea. Like the transgenic potato, the transformed rice expressing human P450 species exhibited cross-tolerance to the herbicides with different modes of action and classes.

METABOLISM OF HERBICIDE IN TRANSGENIC RICE EXPRESSING HUMAN P450 ENZYMES

Homozygous R3 progenies of the CYP2B6 rice were used to analyze the metabolic pathway of metolachlor by TLC analysis using ^{14}C -labeled metolachlor. Two ^{14}C -labeled metabolites, demethylated metolachlor and probable conjugated compounds at the origin of the TLC plate were found. The demethylated metabolite was mainly found in the culture medium, instead

Table 2. Tolerance of CYP2B6 rice and CYP2C19 rice to herbicides

Herbicide	Class	CyP2B6		CYP2C19	
		Yeast	Rice	Yeast	Rice
Photosynthesis inhibitor					
Chloridazon	Pyridazinone	19	+	8	+
Chlortoluron	Phenylurea	-	+	36	++
Branched chain amino acid biosynthesis inhibitor					
Pyriminoabac-methyl	Pyrimidinylxybenzoic	-	±	86	++
Carotenoid biosynthesis inhibitor					
Norflurazon	Pyridazinone	-	++	83	+++
Protein biosynthesis inhibitor					
Acetochlor	Chloroacetoanilide	42	+++	15	ND
Alachlor	Chloroacetoanilide	18	+++	-	ND
Metolachlor	Chloroacetoanilide	17	+++	43	±
Thenylchlor	Chloroacetoanilide	ND	+++	ND	±
Pretilachlor	Chloroacetoanilide	ND	+++	ND	±
Butachlor	Chloroacetoanilide	ND	-	ND	-
Cell division inhibitor					
Mefenacet	Oxyacetamide	6	+	25	++
Trifluralin	2,6-dinitroaniline	5	++	-	ND
Lipid biosynthesis inhibitor					
Pyrbuticarb	Thiocarbamate	-	-	17	++

Numbers in the Yeast column indicate the percentage of metabolism by microsomes from the transgenic yeasts. Symbols in the Rice column indicate herbicide tolerance

- +++ : Grows as well as in medium without herbicide
- ++ : Grows better than control (non-transgenic) plants
- + : Some of the lines grow better than the control plants
- ± : Some of lines grow slightly better than control plants
- : No growth
- ND: Not determined)

Source: Ohkawa *et al.* 2000, Inui *et al.* 2001

of in plants as conjugated compounds. Metolachlor was quickly metabolized to its demethylated form by the CYP2B6 rice within a few days, whereas more than 20% of metolachlor remained in the medium of non-transformed Nipponbare three days after incubation. The demethylated metolachlor seemed to be secreted rapidly into the medium before further degradation (Fig. 7).

The residual metolachlor in plants and in the culture medium were analyzed with a gas-chromatograph-massspectrometer (GC/MS). Only a small portion of the metolachlor was detected in the plants, whereas most of the metolachlor remained in the culture medium. The uptake of metolachlor was considered to

be rather low. However, CYP2B6 rice degraded the metolachlor which it had taken up, and secreted the metabolites rapidly. The amount of metolachlor in the medium seemed to fall quickly. The rate of metolachlor degradation in the CYP2B6 rice plants was about 1.4 times higher than in the non-transformed Nipponbare. These results show that the exogenous CYP2B6 in rice has high activity and potential *in vivo* to catalyze chemical compounds in the xenobiotic pathway.

CONCLUSION

Transgenic potatoes and rice plants

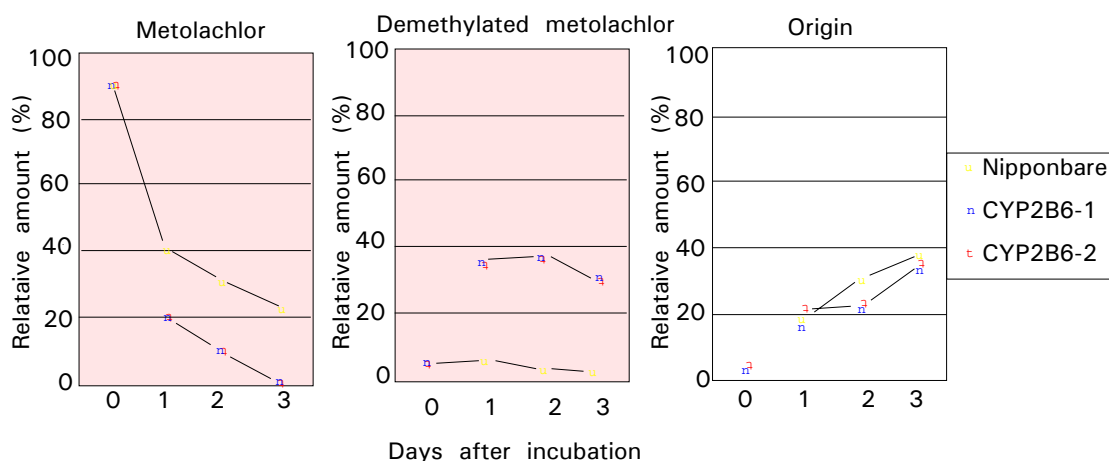


Fig. 7. Changes in relative amounts of metolachlor and its metabolites in the culture medium

which expressed human P450 enzymes were able to degrade several herbicides which had different modes of action and different chemical structures. As a result of the detoxification, the transgenic plants gained cross-tolerance towards several herbicides.

Although most crops which are herbicide-tolerant show tolerance only to one particular herbicide, the transgenic plants with P450s showed cross-tolerance, and were expected to tolerate different herbicides in rotation. A combination of the transgenic plants and the rotation of different herbicides will help prevent the emergence of mutant weeds tolerant to herbicides.

The transgenic potato and rice plants absorbed herbicides present in the medium, and then degraded them. Some of the herbicides, such as simazine, remain in the soil and are effective for a long time. Simazine is a 1,3,5-triazine herbicide containing atrazine, and is degraded with the human (CYP1A1) enzyme. Thus, the transgenic CYP1A1 potato and rice should be able to remove residual simazine from the soil and promote its degradation. The transgenic plants expressing P450 enzymes are also expected to be useful for phytoremediation.

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