Review

The use of cytochrome P450 genes to introduce herbicide tolerance in crops: a review

Hideo Ohkawa,[1]* Hisae Tsujii[1] and Yasunobu Ohkawa[2]

[1]*Department of Biological and Environmental Science, Faculty of Agriculture, Kobe University, Rokkodaicho 1-1, Nada, Kobe 657-8501, Japan*

[2]*Department of Biotechnology, National Institute of Agrobiological Resources, Kannondai 2-1-2, Tsukuba 305–8602, Japan*

Abstract: Mechanisms of herbicide resistance include (1) modified target site, (2) enhanced detoxification or delayed activation, and (3) alterations in the uptake, translocation, or compartmentalization of a herbicide. The first two mechanisms have mainly been identified in plants. Herbicide resistance genes were isolated for several herbicides of different modes of action. Genes that coded for herbicide target or detoxification enzymes were transferred into crop plants. The transgenic plants expressing these genes were tolerant of the active ingredients of herbicides.

Before commercialization, the transgenic plants were tested in the field for risk assessment. In the case of crops with herbicide detoxification enzymes, including cytochrome-P450-species-metabolizing xenobiotics, the substrate specificity of the enzymes as well as the toxicological properties of the herbicide metabolites and the pattern of secondary metabolites in plants must be evaluated.

Keywords: herbicide resistance; transgenic plants; cytochrome P450; risk assessment; metabolic detoxification; gene engineering

1 INTRODUCTION

Herbicides can be grouped according to their site of action and subdivided into chemical classes. All known herbicides were separated into at least 13 groups based on the site of action. Resistance to various herbicides has been found in over 84 different plant species (59 dicotyledons and 25 monocotyledons) world-wide, covering the majority of known herbicide sites of action and their chemical classes. Mechanisms of herbicide resistance include structural changes in the herbicide's target site, metabolic detoxification and alterations in the uptake, translocation, or compartmentalization of a herbicide.[1,2]

Plant gene engineering has been employed to transfer herbicide tolerance into crops. The resulting herbicides-tolerant crops represent one of the first and most widely publicized applications of recombinant DNA technology. Most of the herbicide-resistant cultivars were created through the stable integration of foreign genes related to herbicide target or detoxification enzymes.[3] This paper reviews recent progress on genetic engineering of herbicide-tolerant crops, with special reference to cytochrome P450.

2 MOLECULAR MECHANISMS OF HERBICIDE RESISTANCE IN PLANTS

Mechanisms of herbicide resistance include (1) modified target site, (2) enhanced detoxification or delayed activation, and (3) alterations in the uptake, translocation, or compartmentalization of a herbicide. The first two mechanisms have mainly been identified in plants.

2.1 Modified target site

Since the discovery of *Senecio vulgaris* L resistant to the triazine herbicide, simazine, there have been numerous reports of weed biotypes exhibiting resistance to herbicides. Resistance to triazines is one of the most prevalent types of herbicide resistance found in weeds. In these cases, herbicide resistance in weeds is mostly due to a modification at the target site, the D1 protein of the photosystem II complex in chloroplasts, whereas tolerant crops can detoxify these herbicides. The D1 protein is referred to as the 32-kDa protein or the Q_B protein, and is encoded by the chloroplast *psbA* gene. Triazine and phenylurea herbicides displace plastoquinone at the Q_B binding site on the D1 protein and,

* Correspondence to: Hideo Ohkawa, Department of Biological and Environmental Science, Faculty of Agriculture, Kobe University, Rokkodaicho 1-1, Nada, Kobe 657-8501, Japan

Contract/grant sponsor: Bio-oriented Technology Research Advancement Institution, Japan
(*Received 12 February 1999; accepted 10 May 1999*)

 Pestic Sci 0031–613X/99/$17.50

thereby, block electron flow from Q_A to Q_B. The triazine herbicide atrazine binding in the Q_B niche is thought to be due to hydrogen bonding with Ser264 and Phe265 as well as hydrophobic interactions with Phe255. With triazine-resistant weeds, resistance is mostly due to a modification of amino acid residues in the Q_B binding niche on the D1 protein. In all cases, resistance is due to a point mutation of the *psb*A gene, resulting in a substitution of Gly for Ser at residue 264. This modification greatly reduces the affinity of atrazine at the Q_B binding site, since binding affinity is strongly dependent on hydrogen bonding with the hydroxyl group of Ser 264. Other amino acid substitutions that confer resistance have been identified in or near the Q_B binding niche between residues 211–275 in algae, cyanobacterium, and higher plants.[1,2]

Shortly after the discovery that atrazine resistance in weeds was due to a modified target site, development of atrazine-tolerant crops was examined. The incorporation of this trait into normally susceptible crops would prevent carry-over injury. Atrazine tolerance (Ser264 to Gly) was transferred from *Brassica campestris* L to the oil crop canola (*Brassica napus* L). However, the triazine-tolerant canola variety 'OAC Triton' in Canada exhibited a 10 to 20% decrease in yield compared to triazine-susceptible varieties.[1,2]

2.2 Enhanced detoxification

In most cases, crop tolerance to herbicides is due to the ability of the crop to metabolize the herbicide and thereby prevent injury. Two enzyme systems that play major roles in conferring resistance to herbicides are glutathione *S*-transferase (GST) and cytochrome P450 mono-oxygenase. GSTs are soluble dimeric enzymes that catalyze nucleophilic attack by the thiolate anion of glutathione at electrophilic sites on certain herbicides. The formation of the glutathione conjugate detoxifies the herbicides. Cytochrome P450 mono-oxygenases consisting of cytochrome P450 (P450) and NADPH-cytochrome P450 oxidoreductase (P450 reductase), both of which are associated with endoplasmic reticulum, catalyze mono-oxygenation of lipophilic compounds including certain herbicides. The monooxygenation metabolites are conjugated with water-soluble molecules such as glucose.[4]

Maize is highly tolerant of triazine herbicides. Rapid detoxification of atrazine via glutathione conjugation is the primary mechanism of atrazine resistance. Maize has three GST isozymes catalyzing atrazine conjugation with glutathione. Atrazine can also be metabolized via *N*-dealkylation. Mono-*N*-dealkylation reduces the binding affinity of atrazine for the D1 protein and thereby partially reduces its phytotoxicity. However, removal of both *N*-alkyl groups is necessary for complete detoxification (Fig 1). As a mechanism for herbicide resistance, *N*-dealkylation is considerably less efficient than glutathione conjugation. Although P450 is thought to catalyze *N*-dealkylation, this has not been demonstrated *in vitro*. The phenylurea herbicide chlorotoluron has been used for selective weed control in wheat, which can detoxify chlorotoluron via ring-methyl hydroxylation and *N*-demethylation. Both reactions appear to be mediated by P450. Metabolism via ring-methyl hydroxylation is primarily responsible for chlorotoluron resistance in wheat, whereas *N*-demethylation makes a relatively minor contribution toward chlorotoluron resistance (Fig 2).[1]

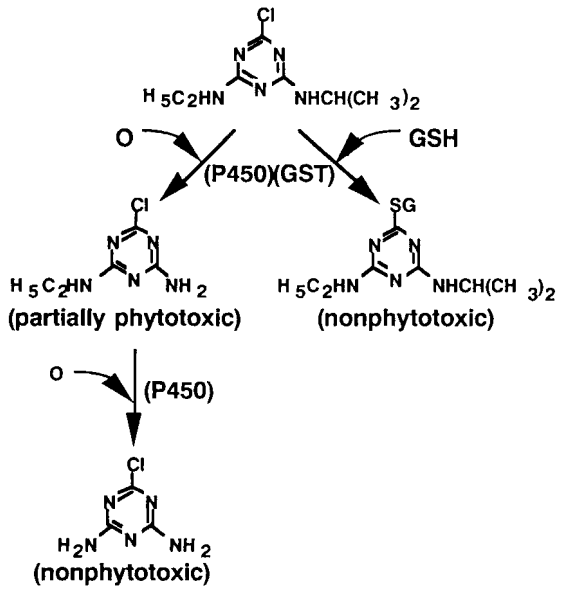

Figure 1. Metabolism of atrazine in maize.

2.3 Transfer of herbicide resistance genes into crops

Herbicide resistance genes were isolated for several herbicides of different modes of action. These genes coded for the target or detoxification enzymes of the corresponding herbicides (Table 1). 5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase encoded by the *aro*A locus is a target site of the herbicide glyphosate inhibition.[5] The enzyme catalyzes an essential step in the shikimate pathway common to

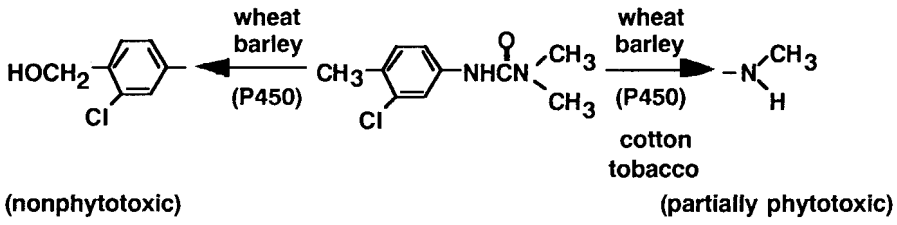

Figure 2. Metabolism of chlorotoluron in plants.

Table 1. Transfer of isolated herbicide resistance genes into plants

Herbicide		*Source of resistance gene*	*Enzyme*	*Transfer in plants*	*References*
Glyphosate	*aroA*	*Arabidopsis thaliana* Heynh	EPSPS	Arabidopsis	12
		A thaliana	EPSPS	Rapeseed	12, 13
		Petunia hybrida Vilm	EPSPS	Tobacco and others	12, 13
		Salmonella typhimurium Cast & Chalm	EPSPS	Tobacco and others	14
Chlorsulfuron	*csr*-1	*A thaliana*	ALS	Rice	15
		A thaliana	ALS	Rapeseed	15
		A thaliana	ALS	Canola	16
		A thaliana	ALS	Tobacco	17, 18
		Brassica napus L	ALS		19
		Nicotiana tabacum L	ALS	Tobacco	
Norflurazon	*crt*-1	*Erwinia uredovora* Dye	Phytoene desaturase	Tobacco	20
Glufosinate	*bar*	*S hygroscopicus*	PAT	Tomato	21
		S hygroscopicus	PAT	Tobacco	21, 22
		S hygroscopicus	PAT	Potato	21, 22
		S hygroscopicus	PAT	Soybean	23
		S hygroscopicus	PAT	Maize	23
		S hygroscopicus	PAT	Wheat	24, 25
		S hygroscopicus	PAT	Sugarbeet	26
			PAT	Rice	27, 28
		S viridochromogenes	PAT	Tobacco	29
		S viridochromogenes	PAT	Tomato	29
		S viridochromogenes	PAT	Rapeseed	29
		S viridochromogenes	PAT	Sugarbeet	29
		S viridochromogenes	PAT	Maize	29
		S viridochromogenes	PAT	Soybean	29
		S viridochromogenes	PAT	Wheat	29
Bromoxynil	*bxn*	*Klebsiella ozenae*	Nitrilase	Tobacco	30
		K ozenae	Nitrilase	Cotton	30
		K ozenae	Nitrilase	Clover	30
		K ozenae	Nitrilase	Rapeseed	30
2,4-D	*tfd*A	*Alcaligenes eutrophus*	Monooxygenase	Cotton	31
		A eutrophus	Monooxygenase	Tobacco	31, 32, 33
Dalapon		*Pseudomonas putida* (Tzer) Mig	Dehalogenase	*N. plumbaginifolia*	34
Cyanamide		*Myrothecium verrucosa*	Cyanamide hydratase	Tobacco	
Metolachlor		Maize	GST	Tobacco	10
Chlorotoluron & Atrazine		Rat	CYP1A1	Tobacco and Potato	11, 35, 36

aromatic amino acid biosynthesis. The over-expression of the enzyme in plants resulted in tolerance to the herbicide.[6,12] Acetolactate synthase (ALS) is an essential enzyme for many organisms, as it catalyzes the first step in the biosynthesis of the branched-chain amino acids valine, isoleucine, and leucine. The transgenic plants expressing the mutant ALS isolated from *Arabidopsis* and tobacco were tolerant to the sulfonylurea herbicide chlorsulfuron. The mutant enzyme has a single amino acid, changed from Trp to Leu, within a conserved region of ALS.[19] The herbicide bilanafos is converted into phosphinothricin in plants and thereby inhibits glutamine synthase. However, expression of phosphinothricin acetyltransferase (PAT) encoded by the *bar* gene in plants inactivated the herbicidal activity and resulted in tolerance of the herbicide (Fig 3).[7] The enzymes from *Streptomyces hygroscopicus* (Jensen) Waks & Henrici and *Streptomyces viridochromogenes* (Kransky) Waks & Henrici show a very high substrate specificity. Therefore, perfect selectivity was found in the transgenic plants expressing the variants of PAT.[23] Because traces of the PAT enzyme in transgenic cells are sufficient for inactivation of phosphinothricin, the corresponding gene from *S hygroscopicus*, first described as bilanafos-resistance (BAR) gene and the related gene from *S viridochromogenes*, first described as PAT, gene are powerful selectable marker genes. They enabled researchers to develop gene transfer methods for cereals where the widely used NTP II gene conferring kanamycin resistance was not efficient enough. 2,4-Dichlorophenoxyacetic acid (2,4-D)

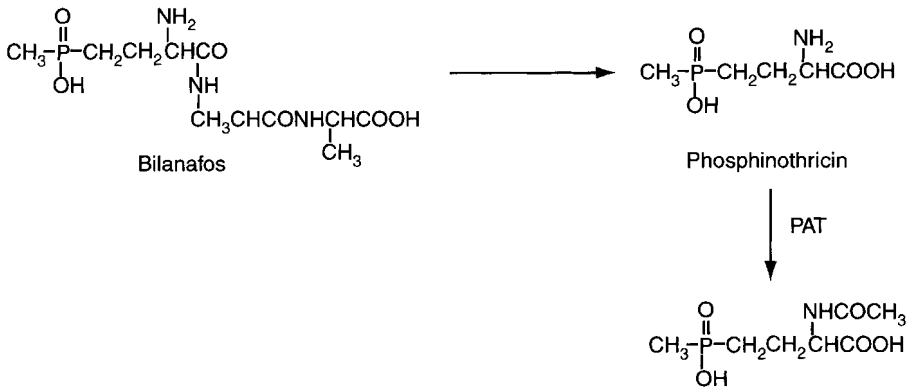

Figure 3. Inactivation of the herbicidally active phosphinothricin by *N*-acetylation with PAT.

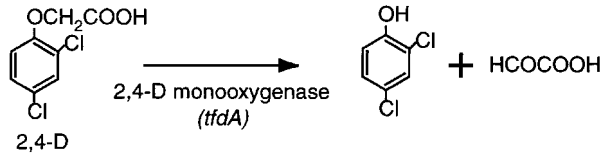

Figure 4. Metabolism of 2,4-D by a 2,4-D monooxygenase.

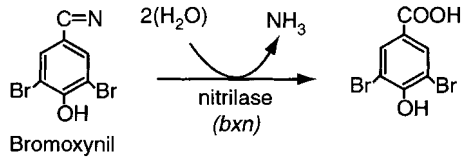

Figure 5. Metabolism of bromoxynil by a bromoxynil-specific nitrilase.

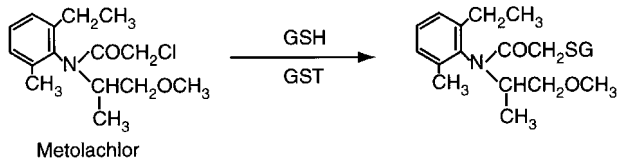

Figure 6. Glutathione conjugation of metolachlor.

mono-oxygenase encoded by the *tfd*A gene catalyzes the first step in the bacterial 2,4-D degradative pathway (Fig 4).[8] When the gene was expressed in plants under the control of either a constitutive or a light-inducible promoter, the transgenic plants tolerated elevated levels of foliar-applied 2,4-D.[31,32] The *bxn* gene encoded a specific nitrilase that converts bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) to its primary metabolite 3,5-dibromo-4-hydroxybenzoic acid (Fig 5). The transgenic plants expressing the enzyme tolerated high levels of a commercial formulation of bromoxynil.[9,30] Maize GST IV subunits expressed in transgenic tobacco plants formed active homodimers which catalyzed glutathione conjugation with metolachlor (Fig 6) and conferred tolerance to the herbicide.[10] Rat P4501A1 and Rat P4501A1/yeast P450 reductase(YR) fused enzyme, both of which metabolized the herbicides chlorotoluron and atrazine, were expressed in potato and tobacco plants, respectively. The transgenic potato and tobacco plants showed cross-tolerance to chlorotoluron and atrazine.[11,36]

3 CYTOCHROME P450 SPECIES FOR HERBICIDE TOLERANCE IN PLANTS

3.1 Plant P450 species metabolizing herbicides

P450 enzymes metabolizing herbicides in plants play an important role in herbicide selectivity and resistance. However, molecular information on these P450 species was quite limited, since it is difficult to identify a function of a specific P450 species in a large gene family. It was found that cultured tobacco S401 cells treated with 2,4-D metabolized chlorotoluron to give ring-methyl hydroxylated and *N*-demethylated metabolites, whereas the cells hardly produced these metabolites without 2,4-D treatment. Based on these results, we attempted to clone cDNAs for P450 species metabolizing chlorotoluron in cultured tobacco S401 cells treated with 2,4-D. As a result, four novel P450 cDNA clones were isolated. Based on their sequences, these were named as CYP71A11, CYP81B2, CYP81C1 and CYP81C2. These P450 species, when cloned, were found to be in type A, which are mostly related to the biosynthesis of phenylpropanoids and other secondary metabolites.[37]

Northern blot analysis with the cloned cDNAs revealed that both CYP71A11 and CYP81B2 were highly induced in the S401 cells with 2,4-D treatment. Therefore, both cDNA clones were expressed in the yeast *Saccharomyces cerevisiae* Meyer ex Hansen. Both CYP71A11 and CYP81B2 expressed in the yeast together with yeast P450 reductase or tobacco P450 reductase showed a higher 7-ethoxycoumarin *O*-deethylase activity than that of the control yeast cells. In addition, the yeast cells expressing both CYP71A11 and yeast P450 reductase exhibited enhanced ring-methyl hydroxylation and *N*-demethylation towards chlorotoluron, whereas the yeast cells expressing both CYP81B2 and tobacco P450 reductase showed a slightly enhanced ring-methyl hydroxylation. Therefore, both CYP71A11 and CYP81B2 were found to be involved in the metabolism of chlorotoluron in the S401 cells treated with 2,4-D, as shown in Fig 7.[38] Recently, it was reported that CYP73A1, CYP76B1, CYP81B1 from plants also metabolized chlortoluron,

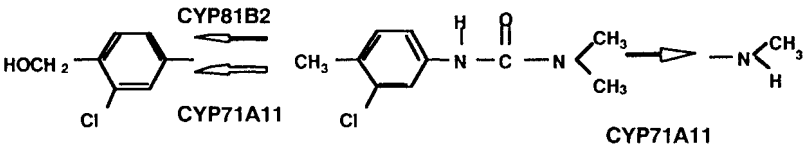

Figure 7. Metabolism of the herbicide chlorotoluron in cultured tobacco S401 cells treated with 2,4-D.

Table 2. Plant P450 species metabolizing herbicides

	Substrate	
P450	*Endogenous*	*Exogenous*
CYP71A11	—	Chlortoluron
CYP73A1	—	Chlortoluron
CYP76B1	—	Chlortoluron
CYP81B1	Fatty acids	Chlortoluron
CYP81B2	—	Chlortoluron
CYP86A1	Fatty acids	DCMU(?)

although fatty acids were endogenous substrates for CYP81B1 as listed in Table 2.[39,40]

3.2 Mammalian P450 species metabolizing herbicides

Commercial herbicides have been examined for metabolism in mammals from the standpoint of risk assessment prior to marketing. P450 monooxygenases in mammalian liver microsomes are well known as drug-metabolizing enzymes involved in oxidative metabolism of xenobiotics, including herbicides. However, it has yet to be determined which P450 species catalyze oxidative reactions of any given herbicide.

We have examined the expression of mammalian P450 species in yeast and the metabolism of herbicide chemicals in recombinant yeast strains. Chlorotoluron and atrazine were found to be metabolized through ring-methyl hydroxylation and *N*-demethylation, and *N*-dealkylation, respectively, by several mammalian P450 species, as shown in Fig 8. Human CYP1A1 catalyzed both ring-methyl hydroxylation and *N*-demethylation, as well as *N*-dealkylation of atrazine.[41,42] CYP1A2 and CYP2C19 catalyzed the same reactions. It was also found that the specific activity of human CYP1A1 expressed in the yeast towards chlorotoluron was higher than those of tobacco CYP81B2 and CYP71A11.

3.3 Genetic engineering of herbicide-tolerant plants

Since mammalian CYP1A1 (P4501A1) metabolized chlorotoluron via ring-methyl hydroxylation and *N*-demethylation, and atrazine via *N*-dealkylation, at higher rates than tobacco CYP81B2 and CYP71A11, and showed a broad substrate specificity towards xenobiotics, we attempted to express rat CYP1A1 cDNA under the control of CaMV 35S promoter and NOS terminator in potato plants and rat CYP1A1/yeast P450 reductase fused enzyme in tobacco plants (Fig 9). Both plants expressing the corresponding enzymes, which were mainly located on the micro-

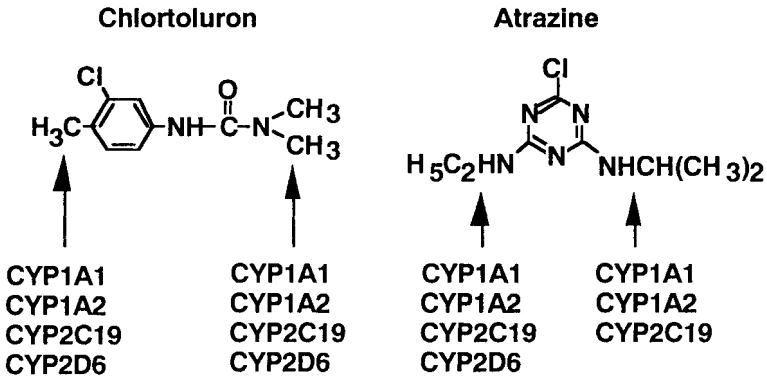

Figure 8. Metabolism of herbicides in mammalian P450 species.

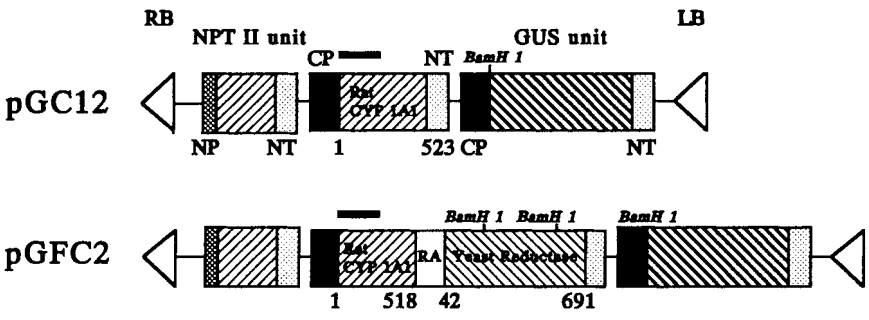

Figure 9. The structures of the expression plasmids pGC12 for rat CYP 1A1 cDNA and pGFC2 for rat CYP1A1 and yeast P450 reductase fused gene.

somes, exhibited a higher P450-dependent mono-oxygenase activity *in vitro* towards 7-ethoxycoumarin than did the control plants. The transgenic plants expressing rat CYP1A1 and its fused enzyme showed tolerance to chlorotoluron. [^{14}C]Chlorotoluron added to a nutrient solution was taken up by both transgenic and control plants similarly. Analysis of ^{14}C-metabolites revealed that the transgenic plants metabolized the herbicide more rapidly than did the control plants via both ring-methyl hydroxylation and *N*-demethylation. Enhanced ring-methyl hydroxylation in the transgenic plants appeared to be responsible for tolerance of the herbicide. These transgenic plants also exhibited tolerance to the herbicides atrazine and pyriminobac-methyl. Thus, it was found that the expression of single CYP1A1 species in plants conferred cross-resistance to herbicides with different structures and modes of herbicide action.[43–45]

4 RISK ASSESMENT OF HERBICIDE-TOLERANT CROPS

Before commercialization, transgenic plants are tested in the field. In a risk assessment, the safety of the gene and its gene product, the properties of the modified plants, the expression stability of the introduced gene and the safety of the modified plant must be considered. The expressed protein in glyphosate-tolerant soybeans, EPSP synthase from *Agrobacterium* sp strain CP4, was rapidly digested *in vitro* in simulated gastric and intestinal fluids, and was not toxic to acutely gavaged mice.[46] The feeding value of glyphosate-tolerant soybeans fed to rats, chickens, catfish and dairy cattle was not altered as compared with the susceptible ones.[47] When the bound pesticide residues from susceptible and tolerant soybeans treated with metribuzin and canola treated with atrazine were fed to rats, these residues were not accumulated in the body organs examined.[48] Also, monitoring of the transgene in glyphosate-tolerant soybeans by PCR revealed that the transgene was found in approximately 1.1% of the commercially available soybeans. This level was somewhat lower than an estimated value on the basis of the cultivation area of the glyphosate-tolerant soybean.[49]

Potential interactions of the modified plants with wild relatives and even the remote probability of undesired interaction with other organisms must be evaluated. If a herbicide detoxification gene is introduced into crops, the substrate specificity of a novel enzyme as well as the toxicological properties of herbicide metabolites must be evaluated. In the case of mammalian microsomal P450 species metabolizing xenobiotics, these are multiple enzymes and show broad and overlapping substrate specificity towards a number of foreign chemicals with different structures including natural products. For example, CYP1A2 and CYP2A6 metabolize caffeine and nicotine, respectively. Therefore, the metabolites of foreign chemicals, as well as the pattern of secondary metabolites in the transgenic plants with these enzymes, must be considered.

5 CONCLUDING REMARKS

P450 mono-oxygenases play an important role in herbicide selectivity and resistance in plants. The plant P450 species metabolizing the herbicide chlorotoluron in cultured tobacco S401 cells treated with 2,4-D were identified as CYP71A11 and CYP81B2. On the other hand, several mammalian P450 species including CYP1A1 were found to metabolize chlorotoluron as well as atrazine. So, it was suggested that, in plants and mammals, different species of P450 enzymes catalyzed the same metabolic reactions towards herbicide chemicals, although the specific activity of CYP1A1 towards chlorotoluron was higher than that of the plant enzymes.

The yeast and plant expression systems for P450 species metabolizing xenobiotics from plants and mammals were useful for comparative metabolism of the chemicals between plants and mammals. Particularly, the systems expressing human P450 species are important models for human metabolism of pesticides.

The expression of CYP1A1 in potato plants conferred cross-resistance to the herbicides chlorotoluron and atrazine. The transgenic plants expressing P450 species metabolizing xenobiotics appear to be useful for breeding crops with herbicide tolerance as well as low pesticide residues. These plants are also important for phytoremediation. However, it is essential to examine the substrate specificity of the introduced P450 species towards xenobiotics as well as plant secondary metabolites, and also to assess the safety of novel metabolites produced in transgenic plants.

ACKNOWLEDGEMENTS

This study was in part supported by the Program for Promotion of Basic Research Activities for Innovative Bioscience of the Bio-oriented Technology Research Advancement Institution of Japan.

REFERENCES

1 Gronwald JW, Resistance to photosystem-II-inhibiting herbicides, in *Herbicide Resistance in Plants*, ed by Powles SB and Holtum JAM, Lewis Publishers, Boca Raton, FL. pp 27–60 (1994).

2 Smeda RJ and Vaughn KC, Mechanisms of resistance to herbicides, in *Chemistry of Plant Protection:* **13** *Molecular Mechanisms of Resistance to Agrochemicals*, ed by Sjut V, Springer-Verlag, Berlin, Heidelberg. pp 79–123 (1997).

3 Donn G, Herbicide-resistant crops generated by biotechnology, in *Weed and Crop Resistance to Herbicides*, ed by De Prado R, Jorrin J and Garcia-Torres L, Kluwer Academic Publishers, Dordrecht. pp 217–227 (1997).

4 Hatzios KK, Regulation of enzymatic systems detoxifying xenobiotics in plants: A brief overview and directions for future research, in *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*, ed. by Hatzios KK, Kluwer Academic Publishers, Dordrecht. pp 1–5 (1997).

5 Padgette SR, Re DB, Barry GF, Eichholtz DE, Delannay X,

Fuchs RL, Kishore GM and Fraley RT, New weed control opportunities: Development of soybeans with a Roundup Ready™ gene, in *Herbicide-Resistant Crops*, ed by Duke SO, Lewis Publishers, Boca Raton, FL. pp 53–84 (1996).

6 Saari LL and Mauvais CJ, Sulfonylurea herbicide-resistant crops, in *Herbicide-Resistant Crops*, ed by Duke SO, Lewis Publishers, Boca Raton, FL. pp 53–84 (1996).

7 Vasil IK, Phosphinothricin-resistant crops, in *Herbicide-Resistant Crops*, ed by Duke SO, Lewis Publishers, Boca Raton, FL. pp 85–91 (1996).

8 Llewellyn D and Last D, Genetic engineering of crops for tolerance to 2,4-D, in *Herbicide-Resistant Crops* ed by Duke SO, Lewis Publishers, Boca Raton, FL. pp 159–174 (1996).

9 Stalker DM, Kiser JA, Baldwin G, Coulombe B and Houch CM, Cotton weed control using the BXN system, in *Herbicide-Resistant Crops*, ed by Duke SO, Lewis Publishers, Boca Raton, FL. pp 93–105 (1996).

10 Jepson I, Holt DC, Roussel V, Wright SY and Greenland AJ, Transgenic plant analysis as a tool for the study of maize glutathione *S*-transferases, in *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*, ed by Hatzios KK, Kluwer Academic Publishers, Dordrecht. pp 313–323 (1997).

11 Ohkawa H, Shiota N, Inui H, Sugiura M, Yabusaki Y, Ohkawa Y and Ishige T, Herbicide-resistant tobacco and potato plants expressing mammalian P450 monooxygenases, in *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*, ed by Hatzios KK, Kluwer Academic Publishers, Dordrecht. pp 307–312 (1997).

12 Klee HJ, Muskopf YM and Gosser CS, Cloning of an *Arabidopsis thaliana* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: Sequence analysis and manipulation to obtain glyphosate-tolerant plants. *Mol. Gen. Genet* **210**:437–442 (1987).

13 Kishore GM, Padgette SR and Fraley RT, History of herbicide-tolerant crops, methods of development and current state of art-emphasis on glyphosate tolerance. *Weed Technology* **6**:626–634 (1992).

14 Stalker DM, Hiatt WR and Comai L, A single amino acid substitution in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase confers resistance to the herbicide glyphosate. *J Biol Chem* **260**:4724–4728 (1985).

15 Li Z, Hayashimoto A and Murai N, A sulfonylurea herbicide resistance gene from *Arabidopsis thaliana* as new selectable marker for production of fertile transgenic rice plants. *Plant Physiol* **100**:662–668 (1992).

16 Miki BL, Labbe JH, Hattori J, Ouellet T, Gabard J, Sunohara G, Charest PJ and Iyer VN, Transformation of *Brassica napus* canola cultivars with *Arabidopsis thaliana* acetohydroxyacid synthase genes and analysis of herbicide resistance. *Theor Appl Genet* **80**:449–458 (1990).

17 Ott KH, Kwagh JG, Stockton GW, Sidorov V and Kakefuda G, Rational molecular design and genetic engineering of herbicide-resistant crops by structure modeling and site-directed mutagenesis of acetohydroxyacid synthase. *J Mol Biol* **263**:359–368 (1996).

18 Hattori J, Rutledge R, Labbe H, Brown D, Sunohara G and Miki B, Multiple resistance to sulfonylureas and imidazolinones conferred by an acetohydroxyacid synthase gene with separate mutations for selective resistance. *Mol Gen Genet* **232**:167–73 (1992).

19 Hattori J, Brown D, Mourad G, Labbe H, Ouellet T, Sunohara G, Rutledge R, King J and Miki B, An acetohydroxyacid synthase mutant reveals a single site involved in multiple herbicide resistance. *Mol Gen Genet* **246**:419–425 (1995).

20 Misawa N, Yamano S, Linden H, De Felipe MR, Lucas M, Ikenaga H and Sandmann G, Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crt*I in transgenic plants showing an increase of β -carotene biosynthesis activity and resistance to the bleaching herbicide norflurazon. *Plant Journal* **4**:833–840 (1993).

21 De Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, Gossele V, Rao Movva N, Thompson C, Van Montagu M and Leemans J, Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO Journal*. **6**:2513–2518 (1987).

22 Padegimas L, Shulga OA and Skriabin KG, Creation of transgenic plants *Nicotiana tabacum* and *Solanum tuberosum* resistant to the herbicide phosphinothricin. *Mol Biol (Mosk)* **28**:437–443 (1994).

23 Spencer TM, Gordon-Kamm WJ, Daines RJ, Start WG and Lemaux PG, Bialaphos selection of stable transformants from maize cell culture. *Theor Appl Genet* **79**:625–631 (1990).

24 Vasit V, Srivastava V, Castillo AM, Fromm ME and Vasil IK, Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. *Bio/Technology* **11**:1553–1558 (1993).

25 Nehara NS, Chibbar RN, Leung N, Caswell K, Mallard C, Steinhauer L, Baga M and Kartha KK, Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. *Plant Journal* **5**:285–297 (1994).

26 D'Halluin K, Bossut M, Bonne E, Mazur B, Leemans J and Botterman J, Transformation of sugarbeet and evaluation of herbicide resistance in transgenic plants. *Bio/Technology* **10**:309–314 (1992).

27 Datta SK, Datta K, Soltanifar N, Donn G and Potrykus I, Herbicide-resistant Indica rice plants from IRRI breeding line IR72 after PEG-mediated transformation of protoplasts. *Plant Mol Biol* **20**:619–629 (1992).

28 Rathore KS, Chowdhury VK and Hodges TK, Use of *bar* as a selectable marker gene and for the production of herbicide-resistant rice plants from protoplasts. *Plant Mol Biol* **21**:871–884 (1993).

29 Donn G and Eckes P, Basta-vertragliche Kulturpflanzen durch Ubertragung eines synthetischen Phosphinothricin-acetyl-transferase-gens. *Z PfKrankh Pfschutz, Sonderh* **XIII**:499–504 (1992).

30 Stalker DM, McBride KE and Malyj LD, Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. *Science* (*Washington*) **242**:419–423 (1988).

31 Bayley C, Trolinder N, Ray C, Morgan M, Quisenberry JE and Ow DW, Engineering 2,4-D resistance into cotton. *Theor Appl Genet* **83**:645–649 (1992).

32 Streber WR and Willmitzer L, Transgenic tobacco plants expressing a bacterial detoxifying enzyme are resistant to 2,4-D. *Biol/Technology* 7:811–815 (1989).

33 Lyon BR, Llewellyn DJ, Huppatz JL, Dennis ES and Peacock WJ, Expression of a bacterial gene in transgenic tobacco plants confers resistance to the herbicide 2,4-dichlorophenoxyacetic acid. *Plant Mol Biol.* **13**:533–540 (1989).

34 Wollaston VB, Snape A and Cannon F, A plant selectable marker gene based on the detoxification of the herbicide dalapon. *Plant Cell Reports* **11**:627–631 (1992).

35 Shiota N, Nagasawa A, Sakaki T, Yabusaki Y and Ohkawa H, Herbicide-resistant tobacco plants expressing the fused enzyme between rat cytochrome P4501A1 (CYP1A1) and yeast NADPH-cytochrome P450 oxidoreductase. *Plant Physiol*, **106**:17–23 (1994).

36 Inui H, Shiota N, Ishige T, Ohkawa Y and Ohkawa H, Herbicide metabolism and resistance of transgenic potato plants expressing rat cytochrome P4501A1. *Breeding Science* **48**:135–143 (1998).

37 Durst F and Nelson DR, Diversity and evolution of plant P450 and P450 reductases. *Drug Metabol Drug Interact* **12**:189–205 (1995).

38 Yamada T, Genetic engineering studies on cytochrome P450 monooxygenases in higher plants. PhD Thesis, Kobe University (1998).

39 Durst F, Benveniste I, Lesot A, Pinot F, Salaun JP and Werck-Reichhart D, Regulation of cytochrome P450 monooxygenases in plants. In *BRAIN Seminar, Cytochrome P450 and plant genetic*

engineering ed by Ohkawa H and Ohkawa Y, BRAIN, Tokyo. pp 1–4 (1997).

40 Durst F, Salaun JP, Werck-Reichhart D and Zimmerlin F, Induction of plant cytochrome P450, in *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*, ed by Hatzios KK, Kluwer Academic Publishers, Dordrecht. pp 19–34 (1997).

41 Shiota N, Inui H and Ohkawa H, Metabolism of the herbicide chlortoluron in transgenic tobacco plants expressing the fused enzyme between rat cytochrome P4501A1 and yeast NADPH-cytochrome P450 oxidoreductase. *Pestic Biochem Physiol* **54**:190–198 (1996).

42 Shiota N and Ohkawa H, Expression of mammalian cytochtome P450 monooxygenases related to drug metabolism in transgenic tobacco plants, in *BRAIN Seminar, Cytochrome P450 and plant genetic engineering* ed by Ohkawa H and Ohkawa Y, BRAIN, Tokyo. pp 31–34 (1997).

43 Ohkawa H, Shiota N, Inui H, Sugiura M, Yabusaki Y, Ohkawa Y and Ishige T, Herbicide-resistant tobacco and potato plants expressing mammalian P450 monooxygenases, in *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*, ed by Hatzios KK, Kluwer Academic Publishers, Dordrecht. pp 307–312 (1997).

44 Ohkawa H, Imaishi H, Shiota N, Inui H and Ohkawa Y, Molecular mechanisms of herbicide resistance. *Rev Toxicol.* **2**:245–252 (1998).

45 Inui H, Ueyama Y, Shiota N, Ohkawa Y and Ohkawa H, Herbicide metabolism and cross-tolerance in transgenic potato plants expressing human CYP1A1. *Pestic. Biochem Physiol* **64**:33–46 (1999).

46 Harrison LA, Bailey MR, Naylor MW, Ream JE, Hammond BG, Nida DL, Burnette BL, Nickson TE, Mitsky TA, Taylor ML, Fuchs RL and Padgette SR, The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from Agrobacterium sp strain CP4, is rapidly digested *in vitro* and is not toxic to acutely gavaged mice. *J Nutr* **126**:728–740 (1996).

47 Hammond BG, Vicini JL, Hartnell GF, Naylor MW, Knight CD, Robinson EH, Fuchs RL and Padgette SR, The feeding value of soybeans fed to rats, chickens, catfish and dairy cattle is not altered by genetic incorporation of glyphosate tolerance. *J Nutr* **126**:717–727 (1996).

48 Mathew R, Kacew S and Khan SU, Bioavailability in rats of bound pesticide residues from tolerant or susceptible varieties of soybean and canola treated with metribuzin or atrazine. *Chemosphere* **36**:589–596 (1998).

49 Shirai N, Momma K, Ozawa S, Hashimoto W, Kito M, Utsumi S and Murata K, Safety assessment of genetically engineered food: detection and monitoring of glyphosate-tolerant soybeans. *Biosci Biotechnol Biochem* **62**:1461–1464 (1998).