Genetic Engineering Studies on Herbicide-Resistant Transgenic Potato Plants Expressing Mammalian Cytochrome P450 Monooxygenases

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(Degree)
博士（農学）

(Date of Degree)
1999-03-31

(Date of Publication)
2007-10-03

(Resource Type)
doctoral thesis

(Report Number)
甲1945

(JaLCDOI)
https://doi.org/10.11501/3156346

(URL)
https://hdl.handle.net/20.500.14094/D1001945

博士論文

GENETIC ENGINEERING STUDIES ON HERBICIDE-RESISTANT TRANSGENIC POTATO PLANTS EXPRESSING MAMMALIAN CYTOCHROME P450 MONOOXYGENASES

哺乳動物チトクローム P450 モノオキシゲナーゼを発現した除草剤耐性バレイショに関する遺伝子工学的研究

平成 11 年 1 月

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GENETIC ENGINEERING STUDIES ON HERBICIDE-RESISTANT TRANSGENIC POTATO PLANTS EXPRESSING MAMMALIAN CYTOCHROME P450 MONOOXYGENASES

A dissertation for partial fulfillment of a Doctoral Degree at the Graduate School of Science and Technology, Kobe University

January, 1999

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CONTENTS

ABBREVIATIONS

AC	Acetochlor
AT	Atrazine
CaMV	Cauliflower mosaic virus
CSPD	Disodium 3-(4-methoxyspirol{1,2-dioxetane-3,2'-(5'-chloro) tricyclo[3.3.1.1.^{3,7}] decan}-4-yl) phenyl phosphate
CT	Chlortoluron
DCMU	Diuron
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
F	Fusion expression of P450 species with yeast reductase
GSH	Glutathione
GST	Glutathione *S*-transferase
GUS	ß-Glucronidase
HPLC	High-performance liquid chromatography
KPb	Potassium phosphate buffer
LC/MS	Liquid chromatography/ mass spectrometry
MC	Metolachlor
MT	Methabenzthiazuron
NADPH	Nicotinamide adenine dinucleotide phosphate
NPT II	Neomycin phosphotransferase II
MS	Murashige and Skoog
4-MU	4-Methylumbelliferone
Nos-P	Nopaline synthase promoter
Nos-T	Nopaline synthase terminator
NR	Norflurazon
PC	Pyributicarb

PCR	Polymerase chain reaction
S	Single expression of P450 species
SDS	Sodium lauryl sulfate
T	Triple expression of P450 species
TLC	Thin layer chromatography
UTP	Uridine 5′-triphosphate
YR	Yeast NADPH-cytochrome P450 oxidoreductase

CHAPTER 1
GENERAL INTRODUCTION

Plant protection

Crop production is mainly damaged by many environmental factors including attacks by insects, diseases and weeds, resulted in heavy loss of agricultural products every year(TABLE 1-1)(1,2). For the maintenance of crop productivity and quality as well as efficient agricultural operation, a number of agricultural chemicals including insecticides, fungicides and herbicides are utilized. Since the damage by weeds is the largest in crop productivity among the pest attacks, the herbicides account for 50% of the total agricultural chemicals used(FIGURE 1-1)(3). However, the use of agricultural chemicals caused adverse effects on environmental and food contamination as well as development of insecticide-, fungicide- and herbicide-resistant strains. In order to overcome these adverse effects of the agrochemicals, one of the most important technologies is to generate and utilize resistant crops to the pests. Particularly, herbicides resistant crops are the most important in the field of plant protection, since the use of herbicides is the largest among agricultural chemicals.

Herbicide-resistant transgenic plants

There are several strategies to generate herbicide-resistant crops. One is manipulation of a target enzyme toward herbicides(TABLE 1-2)(4) and the other is introduction of a herbicide-detoxifying enzyme into crop plants(TABLE 1-3)(4). The gene of the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase(EPSPS) for the herbicide glyphosate was cloned from bacteria and plants, and expressed in plants. As a result, these transgenic plants showed glyphosate-tolerance.

TABLE 1-1 Estimated percentage losses of potential crop yield[a]

Crop	South America	Africa	Asia
Wheat	31	42	30
Rice	28	36	57
Maize	44	75	42
Sugar cane	44	67	71
Potatoes	44	62	49
Vegetables and pulses	30	39	36
Coffee	47	56	43
Cocoa	48	52	38
Soya beans	32	42	40
Copra	34	30	50
Cotton	42	45	36

[a]Source: Edwards *et al.*(1986). Reproduced by kind permission of publisher.

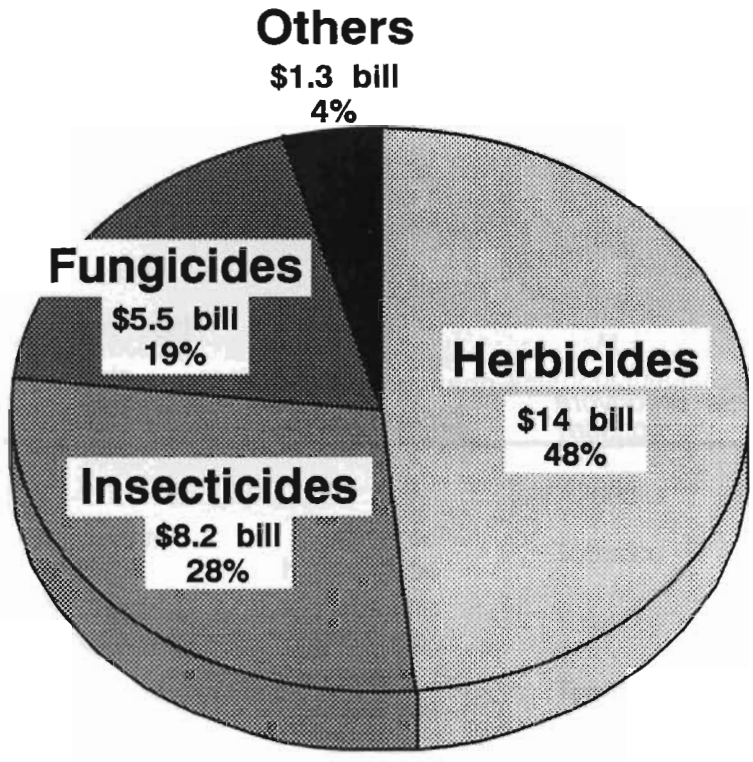

FIGURE 1-1 World agrochemical market sales-1995

TABLE 1-2 Transfer of isolated herbicide-resistance genes into plants with manipulation of target enzymes

Herbicide	Source of Resistance Gene	Mode of Resistance	Transfer in Plants	Evaluation in Breeding Programmes	References
Glyphosate	*Salmonella typhymurium*	Altered EPSPS overexpression	Tobacco and others	yes	5
	Escherichia coli	Overexpression			6, 7
	Petunia hybrida	Overexpression	Tobacco and others	yes	7, 8, 9, 10
	Arabidopsis thaliana	Overexpressed natural and mutated gene	Rapeseed	yes	
		Overexpression of EPSPS			11, 12
	Agrobacterium tumefaciens		Soybean	yes	11, 12
			Rapeseed		11, 12
Chlorsulfuron	*Nicotiana tabacum*	ALS mutated gene	Tobacco		
	Arabidopsis thaliana	ALS mutated gene	Tobacco		13
			Rice		14
			Rapeseed		15
Norflurazon	*Erwinia uredovora*	Enhanced carotenoic biosynthesis	Tobacco		16

TABLE 1-3 Transfer of isolated herbicides-resistance genes into plants with introduction of herbicide-detoxifying enzymes

Herbicide	Source of Resistance Gene	Mode of Resistance	Transfer in Plants	Evaluation in Breeding Programmes	References
Glufosinate	*Streptomyces hygroscopicus*	Expression of PAT	Tobacco		17
			Tomato		18
			Potato		18
			Rapeseed	yes	18
			Sugarbeet	yes	19
			Maize	yes	20
			Soybean	yes	
			Wheat		21, 22
	Streptomyces viridochromogenes	Expression of PAT	Tobacco		
			Tomato		
			Potato		
			Rapeseed	yes	23
			Sugarbeet	yes	
			Maize	yes	23, 24
			Soybean	yes	
			Wheat		
Bromoxynil	*Klebsiella ozenae*	Nitrilase	Cotton	yes	25, 26
			Clover		
			Rapeseed		
Dalapon	*Pseudomonas putida*	Dehalogenase	*N. plumbaginifolia*		27
2,4-D	*Alcaligenes eutrophus*	Monooxygenase	Tobacco		28
			Cotton		29
Phenmedipham	*Arthrobacter oxidans*	Carbamate hydroxylase	Tobacco		30
Cyanamide	*Myrothecium verrucosa*	Cyanamide hydratase	Tobacco		
Metolachlor	*Zea mays*	Glutathione *S*-transferase	Tobacco		31
Chlortoluron	rat	P450 monooxygenase	Tobacco		32
			Potato		33
DCMU	rat	P450 monooxygenase	Potato		33

The mutant genes encoding an insensitive acetolactate synthase(ALS) of *Nicotiana tabacum* and *Arabidopsis thaliana* genes were introduced into tobacco, rice and rapeseed, which obtained chlorsulfuron-resistance. On the other hand, detoxifying enzymes including phosphinothricin acethyltransferase(*bar*), nitrilase, dehalogenase, monooxygenase, carbamate hydroxylase, cyanamide hydratase, glutathione *S*-transferase, P450 monooxygenase were each introduced into several plant species. As a result, these transgenic plants showed herbicide-tolerance.

Cytochrome P450 monooxygenases metabolizing xenobiotics in higher plants

Cytochrome P450 monooxygenases play an important role in the oxidative metabolism of endogenous and exogenous lipophilic compounds. The enzyme system located on the microsomes is consist of a number of cytochrome P450(P450 or CYP) species and a generic NADPH-cytochrome P450 oxidoreductase(P450 reductase). Estimates from current genome projects imply that the number of P450 genes exceeds 200 in *Arabidopsis thaliana*. Over 80 P450 sequences from higher plants were currently known, but the physiological functions for about 20 genes have been identified. Important P450 enzymes whose characterization remains elusive or poorly understood are related to the biosynthesis of sterols, glucosinolates, phenylpropanoids, salicylic acid, jasmonic acid, brassinosteroids and alkaloids, summerized in a previous report(34,35). P450 Nomenclature Committee has assigned names to a P450 gene or enzyme based on decisions about the family or subfamily, if the deduced amino acid sequences of any

newly discovered P450 cDNA or gene were sent(36). A P450 protein sequence from one gene family is usually defined as having ≤40% amino acid identity to a P450 protein from any other family. Namely, the P450 protein sequences within a given gene family, are >40% identical. Mammalian P450 sequences within the same subfamily are always >55% identical.

P450-dependent metabolism of herbicides in plant microsomes has been reported so far(37,38). These are listed in TABLE 1-4. Whether these reactions are P450-dependent was examined by incubation of microsomes with carbon monoxide, P450 inhibitors and without NADPH. The P450 genes cloned and identified as a herbicide-metabolizing P450 were listed in TABLE 1-5. These P450 species were cloned from etiolated plant samples and then heterologously expressed in the yeast *Saccharomyces cerevisiae* or *Escherichia coli*. The P450 fractions from each of the recombinant strains showed herbicide metabolism toward CT. Furthermore, CYP73A1(72) and CYP76B1(73) heterologously expressed showed xenobiotic metabolism toward the standard substrates for mammalian xenobiotic metabolizing P450 species. However, molecular information on these P450 species related to herbicide metabolism was quite limited(75).

The biotransformation of xenobiotics in higher plants are categorized into three phases known as Phase I(conversion), Phase II(conjugation) and Phase III(conpartmentation)(FIGURE 1-2). Cytochrome P450 monooxygenases and esterases which catalyze oxidative and hydrolytic reactions involve in Phase I. These enzymes increase polarity or susceptibility to further processing. In Phase II, glutathione-, glucosyl- and

TABLE 1-4 P450-dependent oxidation of herbicides characterized in plant microsomes

herbicide	P450 reaction	source	reference
acetochlor	-[a]	maize	39
alachlor	*O*-demethylation	mung bean	40
atrazine	*N*-deethylation, *N*-deisopropylation	tulip	41
bentazon	aryl-hydroxylation	maize	42, 43
		rice	44
		sorgum	43, 45, 46
		soybean	47
chlorimuron	hydroxylation	maize	48, 49
chlorsulfuron	aryl-hydroxylation	maize	50
		mung bean	50
		sorghum	50
		wheat	51
chlortoluron	*N*-demethylation, hydroxylation	maize	52
		wheat	53
diclofop	aryl-hydroxylation	wheat	51, 54, 55, 56
flumetsulam	hydroxylation, aryl-hydroxylation	barley	57
		maize	39, 57
		wheat	57
imazethapyr	hydroxylation	maize	58, 59
isoproturon	*N*-demethylation, hydroxylation	marine macroalgae	60
linuron	*N*-demethylation	maize	61
		wheat	51, 62
metolachlor	*O*-demethylation	maize	61
		mung bean	40
		sorghum	63
monuron	*N*-demethylation	cotton	64
nicosulfuron	hydroxylation	maize	43, 59, 61, 65
primisulfuron	aryl-hydroxylation	maize	43, 61, 66
		cupglass	43
prosulfuron	aryl-hydroxylation, *O*-demethylation	avocado	67
		barley	67
		maize	61, 67
		oat	67
		rice	67
		sorghum	67
		wheat	67
triasulfuron	aryl-hydroxylation	maize	61, 68
		wheat	51, 69

[a] not identified.

TABLE 1-5 Plant cytochrome P450 monooxygenases related to herbicide metabolism

P450	source of plant	herbicide metabolized	reference
CYP71A11	tobacco	chlortoluron	70
CYP71B1	field pennycress	chlortoluron	71
CYP73A1	Jerusalem artichoke	chlortoluron	72
CYP76B1	Jerusalem artichoke	chlortoluron	73
		isoproturon	73
CYP81B1	Jerusalem artichoke	chlortoluron	74
CYP81B2	tobacco	chlortoluron	70

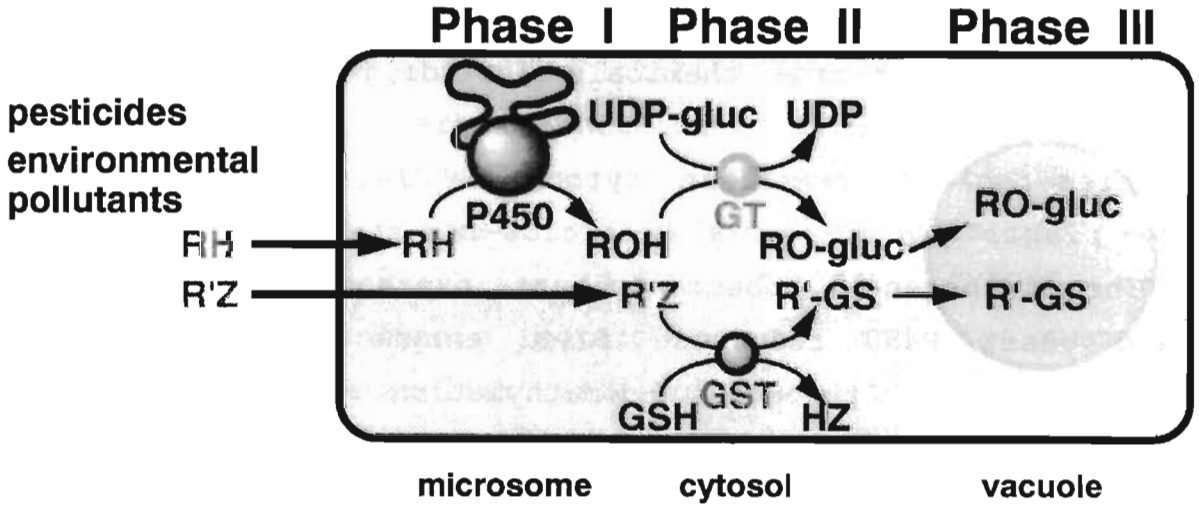

FIGURE 1-2 Schematic representation of xenobiotic metabolism in a plant cell
P450; cytochrome P450 monooxygenase, GT; glucosyl transferase, GST; glutathione *S*-transferase

malonyl-conjugations are occurred to xenobiotics directly or indirectly, resulting in increase of water solubility. Conpartmentation in Phase III is responsible for membrane-bound ATP-dependent transporters which export xenobiotics conjugated into vacuole or other organs. Detoxifying enzymes of xenobiotics in Phase I and II are related with herbicide selectivity and resistance in plants.

On the other hand, there are a number of P450 species metabolizing xenobiotics in the microsomes of mammalian livers. These P450 species showed a broad and overlapping substrate-specificity each other toward lipophilic xenobiotics including herbicides. Especially, CYP1, CYP2 and CYP3 families are known as xenobiotic metabolizing enzymes in mammals. In the case of human P450 species, eleven species cover more than 90% of P450-dependent xenobiotic metabolism in human livers(76). Particularly, it was found that human CYP1A1 plays an important role in the metabolism of a number of herbicide chemicals including CT.

Expression of mammalian cytochrome P450 monooxygenases in plants to confer a herbicide-resistance

The transgenic tobacco plants expressing rat CYP1A1 and yeast P450 reductase fused enzyme metabolized the herbicide CT through *N*-demethylation and ring-methyl hydroxylation, giving rise to resistance to the herbicide(32,77).

Objective of this study

We attempted to generate transgenic potato plants expressing mammalian cytochrome P450 monooxygenases by the Agrobacterium-transformation system, and to examine

for herbicide tolerance of these transgenic potato plants toward several herbicides with different structures and modes of action. Potatoes(*Solanum tuberosum*) are widely cultivated and consumed in both developing and developed countries. In addition, the transformation method with microtuber discs from sterilely grown potato plants was well established, and its transformation efficiency is high(78).

In chapter 2, it was attempted to express rat CYP1A1 in transgenic potato plants and to examine for herbicide-metabolism and tolerance toward the phenylurea herbicides CT and DCMU which inhibit photosynthesis. The reason why transgenic potato plants expressing rat CYP1A1 show tolerance toward CT will be revealed.

In chapter 3, it was attempted to express human CYP1A1 in transgenic potato plants and to test herbicide-metabolism and tolerance in these plants toward the phenylurea herbicide CT, the triazine herbicide AT, which also inhibits photosynthesis, and the pyrimidinyl carboxy herbicide PM which inhibits ALS, since human CYP1A1 metabolized many herbicides.

In chapter 4, based on previous results it was attempted to co-express human CYP1A1, CYP2B6 and CYP2C19 in transgenic potato plants and to examine for herbicide-metabolism and tolerance toward a large number of herbicides which are metabolized by three P450 species. Furthermore, the cooperation of two or three P450 species in the metabolism of herbicides will be investigated.

These studies may discover a novel approach for generation of herbicide-tolerant and low pesticide-residual crops as well as phytoremediation of

contaminants in the environment.

CHAPTER 2
HERBICIDE METABOLISM AND RESISTANCE OF TRANSGENIC POTATO PLANTS EXPRESSING RAT CYP1A1

INTRODUCTION

Transgenic tobacco plants expressing rat CYP1A1 and CYP1A1/YR fused enzyme were generated. These metabolized the herbicide CT mainly through *N*-demethylation and ring-methyl hydroxylation, and showed tolerance toward the herbicide(32,77). Then, it was attempted to express rat CYP1A1 and CYP1A1/YR fused enzyme in transgenic potato plants. Potatoes are an important dicot food crop in not only developed but also developing countries. Also, technology for regeneration and transformation systems has been well established in commercial varieties(78). Introduction of rat CYP1A1 cDNA and/or its fused gene with YR into potato plants may give rise to the herbicide resistance in it as with tobacco plants.

MATERIALS & METHODS

Plant materials

Solanum tuberosum cv. MayQueen was sterilely grown as a shoot culture on modified MS medium(79) in plant boxes under fluorescent light(16h of light) at 20°C. Potato microtubers produced *in vitro* were used for plant transformation. Regenerated potato plants were grown in a growth chamber under fluorescent light(8h of light) for *in vivo* herbicide-tolerant tests, and specially mixed soil and an artificial fertilizer were used.

Plant transformation

The construction of the expression plasmids pGC12 for rat CYP1A1 cDNA and pGFC2 for rat CYP1A1 and yeast reductase fused enzyme gene has been reported by Shiota *et al.*(1994)(FIGURE 2-1). These plasmids were each introduced into *Agrobacterium tumefaciens* strains LBA4404 and C58C1 by the freeze-thaw method(80). Transformation of potato microtuber discs with *A. tumefaciens* was previously described(FIGURE 2-2)(78). Shoots regenerated from calli on the microtuber discs were selected on the modified MS medium containing 100mg/l kanamycin and 300mg/l cefotaxime for three times. Then, shoots were used for further analysis.

GUS assay

Whole potato plants and microtubers were used for histochemical staining of GUS activity as reported(81). Potato tissues were also used for GUS assay with the fluorescence method(82). Fluorescent intensity was

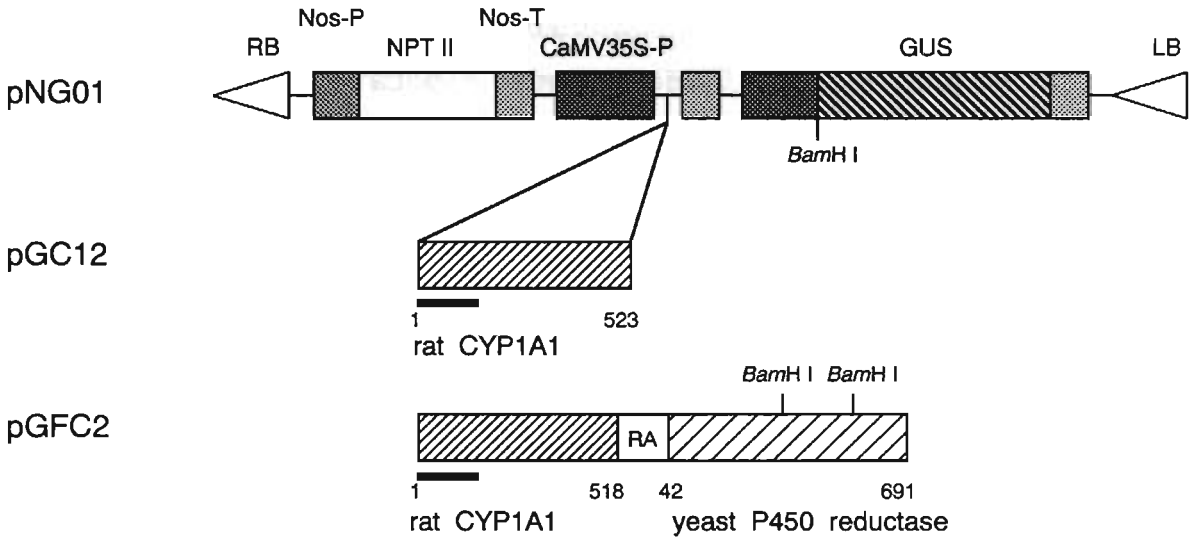

FIGURE 2-1 The structure of the expression plasmids pGC12 for rat CYP1A1 cDNA and pGFC2 for rat CYP1A1 and yeast reductase fused gene
R and A represent for amino acid residues derived from the synthesized DNA linker. Numbers below the coding regions indicate the number of amino acid residues as counted from the amino terminus of the corresponding proteins. The probes labeled with DIG-UTP used in southern blot analysis are also showed at the upper side of both expression plasmids with a bold line.

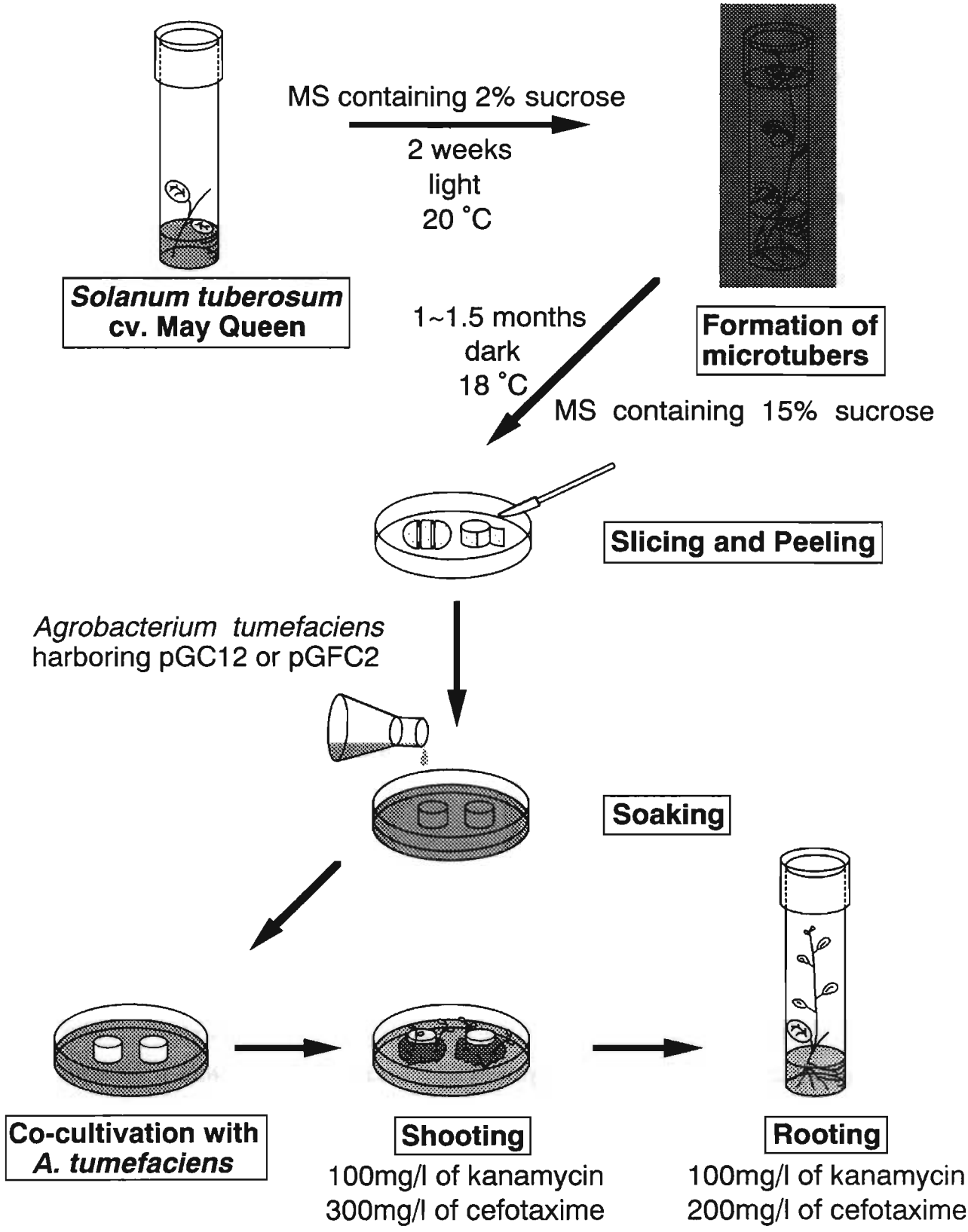

FIGURE 2-2 A schematic procedure for infection of potato microtubers with *Agrobacterium tumefaciens* having one of the expression plasmids

measured in a fluorometry F-3010(Hitachi Co. Ltd., Tokyo, Japan) under at excitation 365nm and emission 455nm. The GUS activity was expressed as production of 4-MU as a standard.

Southern blot analysis

Genomic DNA preparations from potato plants were each digested with *Bam*H I and then electrophoretically separated on an 0.7% agarose gel. Blotting was performed with the standard method(83). Probes were prepared by PCR method in the reaction mixture containing rat CYP1A1 specific primers, (5′-ATGCCTTCTGTGTATGGATT-3′ and 5′-ACGTCTGCCAAAGCATATGG-3′), pGC12 as a template and DIG-labeled UTP(Boehringer Mannheim Co., Mannheim, Germany). For detection with the DIG system, CSPD(Tropix Inc., Massachusetts, USA) was used as a substrate for alkaline phosphatase. Then, membranes were exposed to an X-ray film for 4h(84).

Northern blot analysis

mRNA was extracted from potato tissues by the use of a QuickPrep *Micro* mRNA Purification Kit(Pharmacia Biotech., Uppsala, Sweden). mRNA samples of 1μg each were applied to formaldehyde-denatured 1.2% gel for RNA electrophoresis. DIG-labeled riboprobes were prepared by T7 RNA polymerase and P450 cDNAs cloned into vectors which contain promoter for T7/SP6 RNA polymerase(*in vitro* transcription system in DIG RNA Labeling Kit(Boehringer Mannheim Co., Mannheim, Germany)). Detection of mRNA bands was as described in the method of Southern blot analysis.

Western blot analysis

A potato microsomal fraction was prepared by the method reported previously(32). Protein quantitation was carried out by the method of Lowry *et al.*(85). Microsomal fractions of 20μg protein were each applied to SDS-PAGE on a 10% running gel. After electroblotting to a PVDF-Plus membrane(Micron Separations, Westboro, MA), protein bands were detected by the use of primary polyclonal antibodies against rat CYP1A1(Daiichi Pure Chemicals Co., LTD., Tokyo, Japan) and secondary antibody conjugated with alkaline phosphatase(BioMakor, Israel).

7-Ethoxycoumarin O-deethylase and cytochrome c oxidoreductase assays

7-Ethoxycoumarin *O*-deethylase(ECOD)(86) and cytochrome c oxidoreductase(CCOR)(87) activities of potato microsomal fractions were measured as described previously with a slight modification. In the measurement of CCOR activity, the absorbance at 550nm was monitored at 30°C and a molar absorption coefficiency of $21mM^{-1}cm^{-1}$ for horse heart cytochrome c was used for determination of the enzyme activity.

Chemicals

[^{14}C]-Ring-labeled CT[*N*-(3-chloro-4-methylphenyl)-*N*,*N*-dimethylurea](sp. act. 2.99MBq/mg, radiochemical purity >98%), cold CT, demethylated CT[*N*-(3-chloro-4-methylphenyl)-*N*-methylurea], ring-methyl hydroxylated CT[*N*-(3-chloro-4-hydroxyphenyl)-*N*,*N*-dimethylurea], demethylated ring-methyl hydroxylated CT[*N*-(3-chloro-4-hydroxyphenyl)-*N*-methylurea], carboxylated CT[*N*-(3-chloro-4-carboxyphenyl)-*N*,*N*-dimethylurea] and didemethylated CT[*N*-(3-chloro-4-methylphenyl)urea] were provided by

Novartis Crop Protection, Inc.(Basel, Switzerland). DCMU[3-(3,4-dichlorophenyl)-1,1-dimethylurea] was purchased from Nacalai Tesque Inc.(Kyoto, Japan).

Herbicide metabolism in transgenic potato plants

Sterilely grown potato plants in 10cm high were used for *in vitro* herbicide metabolism. Application of [^{14}C]CT to a nutrient solution at the concentration of 20μM, extraction from plants and TLC analysis of metabolites were carried out according to the methods reported previously(77,88). TLC plates(Merck, Darmstadt, Germany) were developed in chloroform/ethanol, 9/1(v/v). The plant materials treated with [^{14}C]CT were incubated for 0, 0.5, 1, 2 and 8 days.

In vivo herbicide resistance tests

In vivo herbicide-tolerance tests were carried out by spraying the herbicide CT or DCMU in a growth chamber. CT of 10μmol or DCMU of 2μmol in water containing 0.02% Tween 20 and 0.015% sticker(spray adjuvant) was sprayed to a plant per pot 3 weeks after transferred to pots. Control plants were also sprayed with the same solution with and without a herbicide. Day length in a growth chamber was 8h. Photograph was taken when phenotypic changes were observed. Potato tubers were harvested 3 weeks after spraying a herbicide and then weighed. This experiment was carried out with three independent replicates.

RESULTS

Potato transformation

Potato microtuber discs were infected with *A. tumefaciens* strain LBA4404 having the expression plasmid pGC12 for rat CYP1A1 cDNA or the expression plasmid pGFC2 for rat CYP1A1/YR fused enzyme gene. Six potato plants(GC) for pGC12 and twenty plants(GFC) for pGFC2 were obtained from the kanamycin resistant calli. Most of the selected resistant plants showed normal phenotypes in morphology. However, slow growth and shrunken leaves were found in both S1187 and F1185.

The selected resistant potato plants were assayed for GUS activity with both histochemical staining and fluorescence methods. In the histochemical staining, 2 of 6 GC plants and 12 of 20 GFC plants showed blue color signals in leaves, stems, roots as well as microtubers, whereas no untransformed control plants showed blue color signals(FIGURE 2-3). Particularly, the leaves and microtubers of the transgenic potato plants showed a higher GUS activity than that of the other tissues. With the fluorescence method, the transgenic plants showed 23 to 443-fold higher activities than that of the control plants(TABLE 2-1). These results were almost agreed with those of the histochemical staining. PCR analysis with rat CYP1A1 and YR specific primers showed that 2 GC plants and 11 GFC plants contained the corresponding genes(data not shown). Then, these plants were examined for tolerance to the herbicide CT(10μmol/pot).

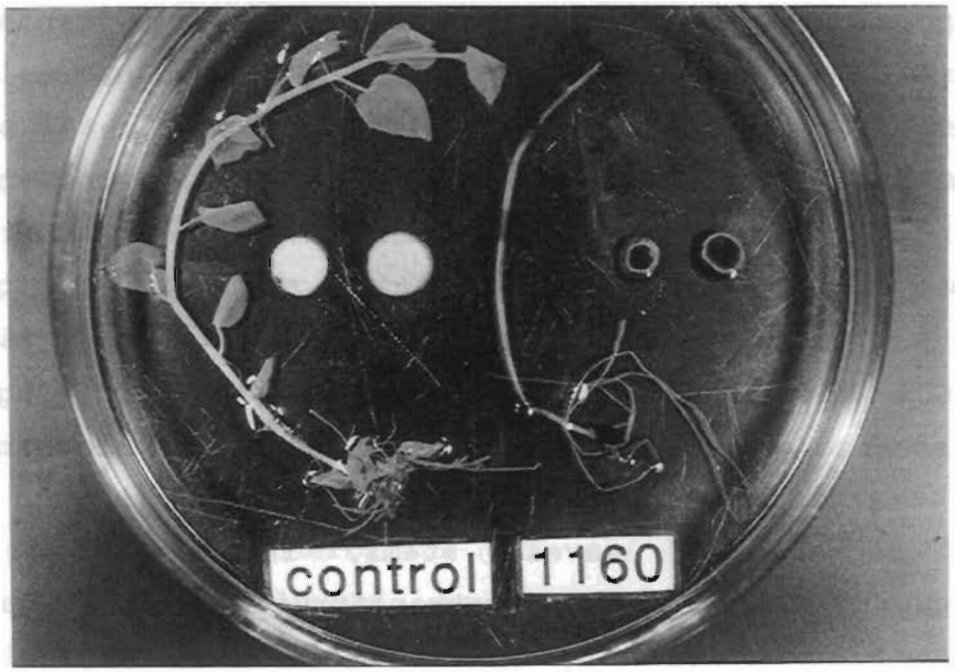

FIGURE 2-3 Histochemical staining for GUS of the control and S1160 carrying rat CYP1A1 cDNA

TABLE 2-1 Comparison of GUS, 7-ethoxycoumarin *O*-deethylase(ECOD) and cytochrome c oxidoreductase(CCOR) activities in the transgenic potato plants

transgenic plant	GUS activity			ECOD activity		CCOR activity	
	histochemical staining[a]	Fluorescence[b]	relative activity	7-hydroxycoumarin produced[c]	relative activity	reduced cytochrome c[c]	relative activity
Control	-	6.4±3.1	1	1.1±0.1	1	39.4±4.1	1
S1160	+++	779.3±86.2	122	2.4±0.3	2.2	59.5±9.8	1.5
F1155	+++	946.8±223.7	148	1.8±0.2	1.6	105.3±49.6	2.7
F1156	++	1347.8±198.8	211	1.5±0.4	1.4	67.7±10.2	1.7
F1157	++	507.1±100.7	79	1.7±0.4	1.5	61.5±27.2	1.6
F1165	+++	1245.1±155.3	195	2.4±0.7	2.2	96.9±21.2	2.5
F1166	++	145.3±57.3	23	2.1±0.3	1.9	51.9±4.5	1.3
F1167	++	145.0±35.0	23	2.6±0.3	2.4	97.2±10.1	2.5
F1179	+++	450.6±59.3	70	1.9±0.8	1.7	70.1±15.5	1.8
F1180	+++	691.0±238.6	108	2.7±0.4	2.5	137.0±28.2	3.5
F1182	+++	465.6±120.0	73	1.9±0.3	1.7	100.4±11.6	2.5
F1185	+++	2833.4±409.2	443	3.8±0.2	3.5	113.4±20.8	2.9

[a] -: negative, +: low, ++: high, +++: very high

[b] 4-methylumbelliferone(4-MU) pmol/min/mg protein

[c] ECOD and CCOR activities are assayed with the substrates 7-ethoxycoumarin and cytochrome c, and indicated as picomoles per minutes per mg protein of microsomal fractions with sample standard deviations.

Southern blot analysis

Southern blot analysis was carried out to detect CYP1A1 cDNA gene or CYP1A1/YR fused gene in the plant genomes. The genomic DNA preparations from the GUS-positive plants were digested with *Bam*H I. After blotting to a membrane followed by hybridization with a DIG-labeled probe, one of the GC plants showed the presence of two bands and ten of the GFC plants contained one to five bands. On the other hand, the control plant did not show any corresponding bands(FIGURE 2-4). The GFC plants F1166 and F1167 showed the same pattern of five bands. S1187, F1181 and F1184 plants showed no corresponding bands. The GFC plant F1185 with three bands showed the highest GUS activity, while both F1166 and F1167 with five bands showed a lower GUS activity than that of F1185. All transgenic plants were listed in TABLE 2-1

Northern blot analysis

Northern blot analysis was examined for mRNA extracts from transgenic plants and a part of results was shown in FIGURE 2-5(A). The GC plant S1160 carrying rat CYP1A1 cDNA showed a distinct mRNA band corresponding to 1.6 kb-CYP1A1 cDNA, although the size of mRNA with poly(A) seemed to be slightly longer. Fairly low level of mRNA corresponding to 3.5 kb-CYP1A1/YR fused gene was found in the GFC plant F1167. Also, shorter mRNA bands in length as compared with the predicted molecular weight of CYP1A1/YR fused mRNA band were observed in F1167. The levels of the fused enzyme-mRNA in the other 9 plants were lower than that of F1167. The level of P450-mRNA in the GC plant S1160 was 5 times higher than that of the

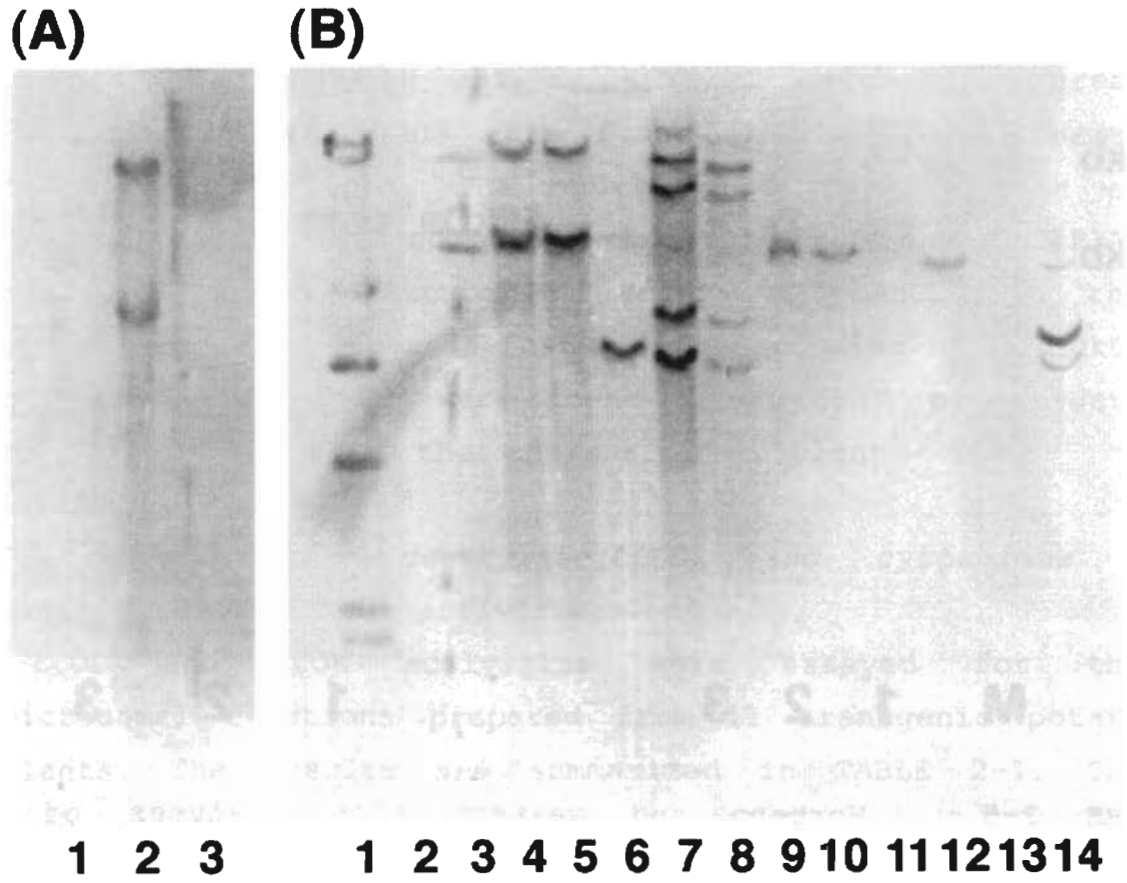

FIGURE 2-4 Southern blot analysis of the regenerated potato plants selected by the PCR amplification (A): lane 1, control; lane 2, S1160; lane 3, S1187. (B): lane 1, λ-*Hin*d III marker; lane 2, control; lanes 3 to 14, F1155~F1157, F1165~F1167, F1179~F1182, F1184 and F1185, respectively.

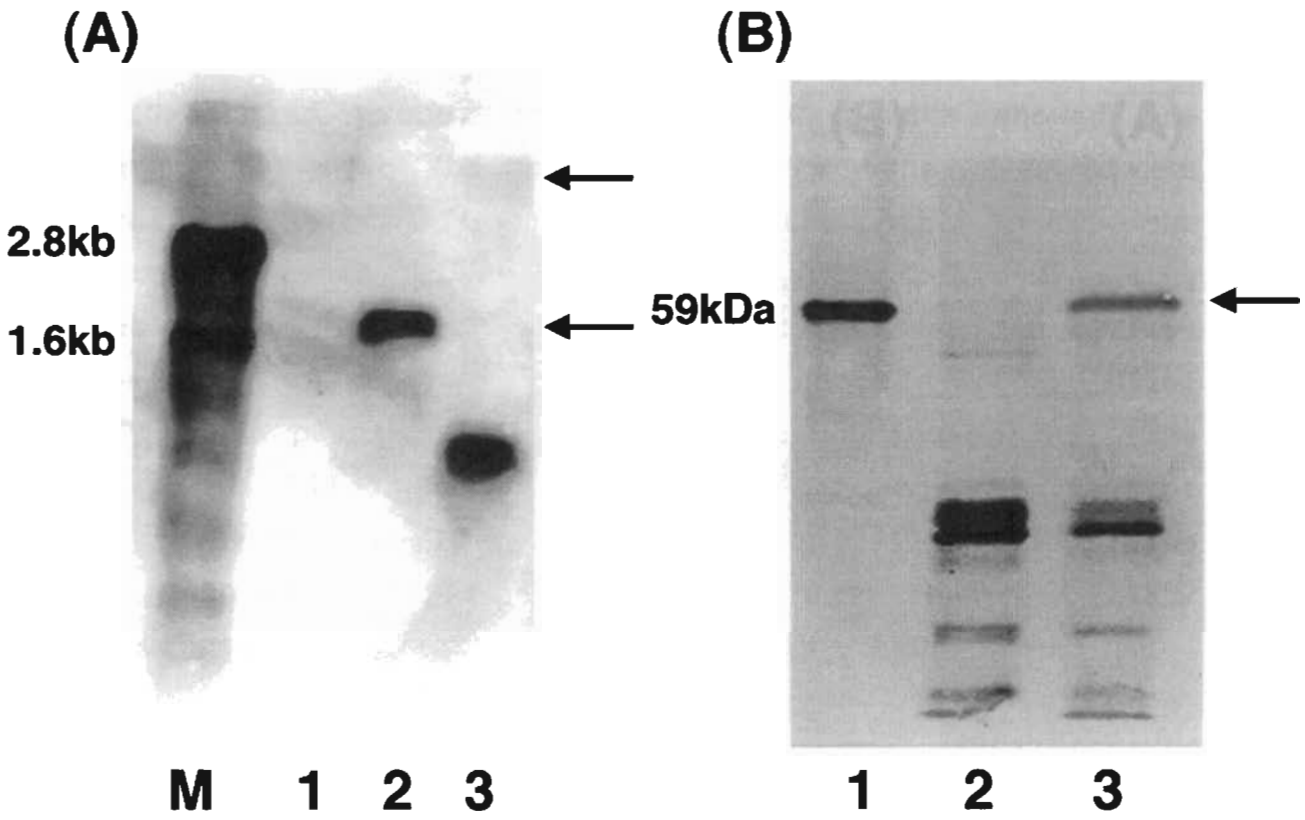

FIGURE 2-5 Nortern and western blot analyses of the transgenic plants
(A): Upper and lower bold arrows indicate mRNA corresponding to CYP1A1/YR fused gene and CYP1A1 cDNA, respectively. M, lanes 1 to 3 represent for RNA molecular weight marker I(Boehringer Mannheim Co., Mannheim, Germany), control, S1160 and F1167, respectively. (B): Lane 1, the microsomal fraction expressing rat CYP1A1 of the recombinant yeast AH22/ pAMC1; lane 2, the untransformed control plant; lane 3, S1160 expressing rat CYP1A1.

fused enzyme-mRNA.

Western blot analysis

Microsomal fractions prepared from the transgenic plants carrying rat CYP1A1 cDNA were analyzed by immunoblotting with anti-rat CYP1A1 antibody. In the GC plant S1160, the microsomes contained a 59kDa protein reacted with anti-rat CYP1A1 antibody, whereas no corresponding bands were found in the untransformed plant(FIGURE 2-5(B)). The mobility of this protein on SDS-PAGE was corresponding to that of rat CYP1A1 produced in the recombinant yeast AH22/pAMC1. On the other hand, no protein bands corresponding to 130kDa of the fused enzyme were found in any GFC plants(data not shown) as with the untransformed plant.

7-Ethoxycoumarin O-deethylase(ECOD) and cytochrome c oxidoreductase(CCOR) activities

ECOD and CCOR activities were assayed for the microsomal fractions prepared from 11 transgenic potato plants. The results are summarized in TABLE 2-1. The ECOD activity of the GC plant S1160 was 2.2 times higher than that of the control plant. Also, the GFC plants showed 1.4 to 3.5 times higher activity than that of control. The GFC plant F1185 showed the highest ECOD activity. The activity of F1166 and F1167 was less than that of F1185. These values were almost agreed with the GUS activity. In the CCOR activity, the GC plant S1160 showed 1.5 times higher activity than that of the control, although the activity did not seem to be a significant increase as compared with the control. The GFC plants were 1.3 to 3.5 times higher than that of the control. The

highest activity was found in the GFC plant F1180 and the lowest activity was in F1166.

Herbicide metabolism in the transgenic potato plants

The metabolism of [^{14}C]CT was examined in S1160. The whole plants were harvested at 0, 0.5, 1, 2 and 8 days after application of the herbicide chemical to a nutrient solution at a concentration of 20μM. The methanol extracts from whole plants were each subjected to TLC analysis. CT was found to be rapidly taken up into both control and S1160 plants within half a day and metabolized to give *N*-demethylated(DM), ring-methyl hydroxylated(OH), and *N*-demethylated ring-methyl hydroxylated(DMOH) metabolites(FIGURE 2-6). The Rf values of these metabolites were identical to those of the standard reference compounds. Each of these metabolites was also confirmed by the TLC co-chromatography with the standard compounds, as reported previously(88). The time courses of the amounts of CT and its metabolites in the plants are showed in FIGURE 2-7. The level of CT in the control was slightly higher than that of S1160 plant. The amount of DM in the control plant was also higher 2 and 8 days after treatment as compared with S1160 plant. On the other hand, the metabolite OH in S1160 plant accounted for 12% of the total after one day and then decreased to 5% after 8 days, although the amount of OH was fairly low in the control plant. The metabolite DMOH in S1160 plant was also greater than that of the control. The COOH and unknown metabolites continued to increase over 8 days in S1160 plant as compared with the control. On the other hand, in the nutrient solution DM was found

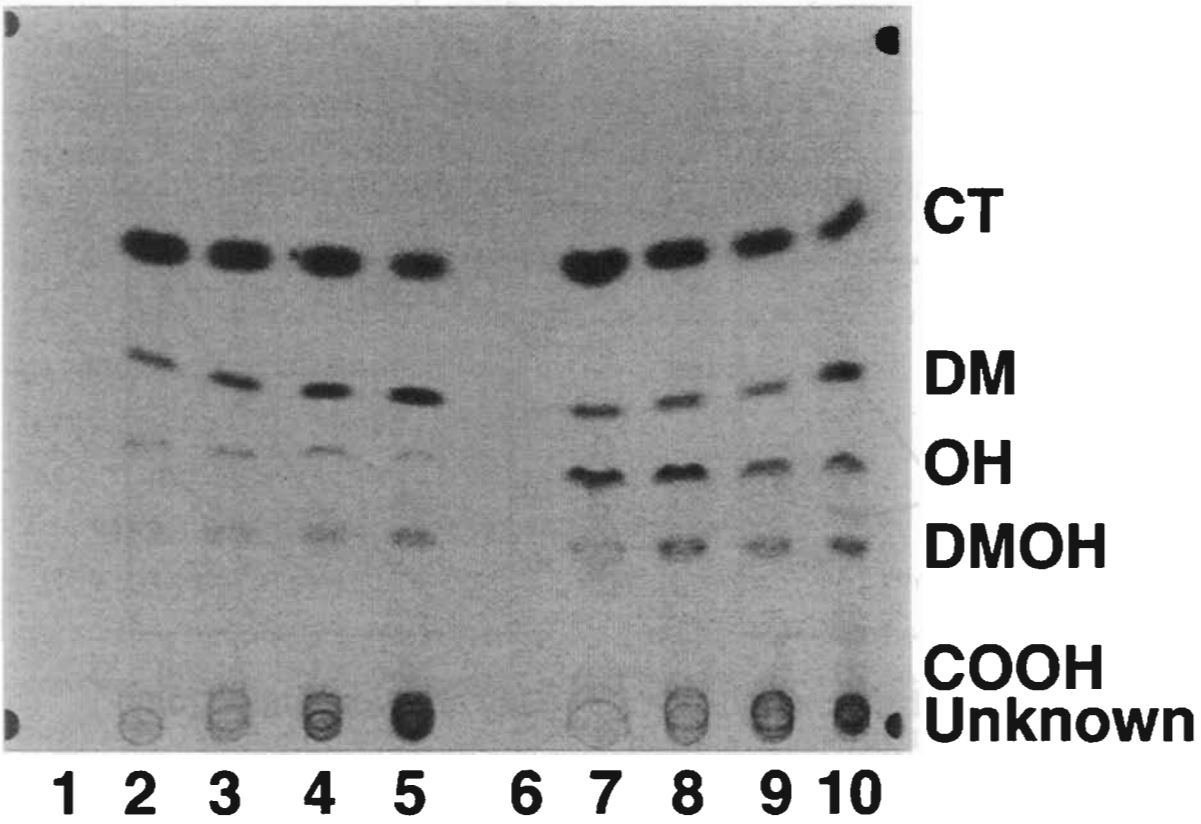

FIGURE 2-6 TLC analysis of methanol extracts from the potato plants treated with [^{14}C]chlortoluron
CT, DM, OH, DMOH and COOH indicate chlortoluron, *N*-demethylated, ring-methyl hydroxylated, *N*-demethylated ring-methyl hydroxylated and 4-carboxyphenyl metabolites. Lanes 1 to 5 are methanol extracts from the untransformed control plants and lanes 6 to 10 are from S1160 plants. Lane 1 and 6, 2 and 7, 3 and 8, 4 and 9, and 5 and 10 indicate incubation for 0, 0.5, 1, 2 and 8 days, respectively.

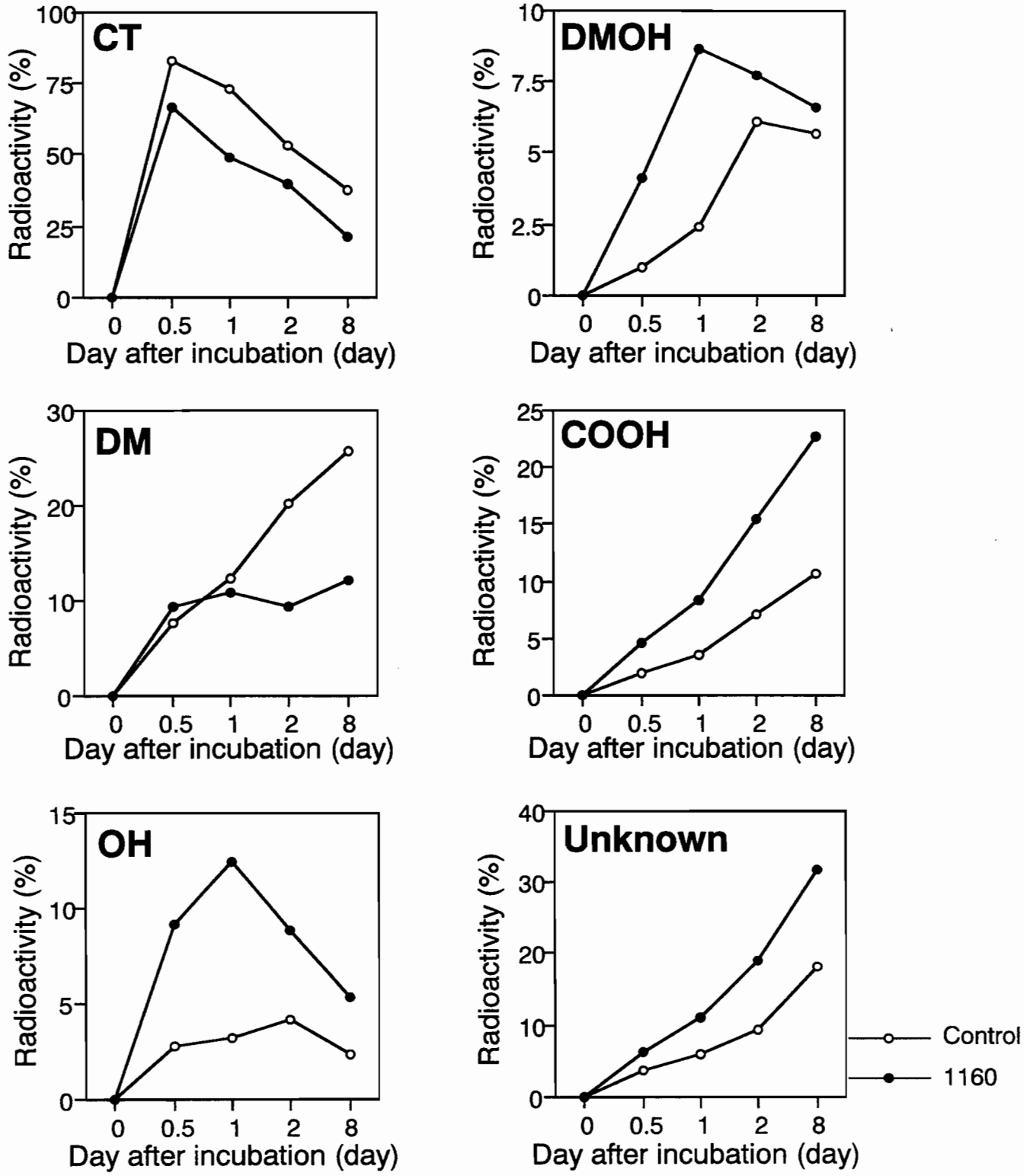

FIGURE 2-7 Time courses of the amounts of [^{14}C] chlortoluron and its metabolites in both control and S1160 potato plants

These values represent for the percentage of each metabolite in the methanol extracts of both control and S1160 plants at the same sampling day based on the measurement by scanning in a Bio Imaging Analyzer BAS2000. CT, DM, OH, DMOH and COOH were described above. These values are the average of three experiments performed independently.

with S1160 plant to a greater extent, but to a lesser extent in the control plant(data not shown). Based on these results, it was found that S1160 metabolized CT more rapidly than the control plant to yield the major metabolites OH, DMOH, COOH and unknowns, although the levels of CT and DM were higher in the control than in S1160. The F1167 was also examined for the metabolism of [^{14}C]CT as with S1160(data not shown). The same metabolites as found in S1160 were also found, although the amounts were slightly different among the metabolites detected as compared with those in S1160.

In vivo herbicide resistance tests

The herbicides CT and DCMU were sprayed for the control and transgenic potato plants at the concentration of 10μmol and 2μmol, respectively(FIGURE 2-8). Although the control plants started to wither by the spray of CT after 8 days with a typical appearance for treatment of photosynthesis-inhibitors, and completely died 14 days, S1160 expressing rat CYP1A1 did not show any phytotoxic changes. On the other hand, F1167 expressing the CYP1A1/YR fused enzyme did not show tolerance to the herbicide CT. In the DCMU treatment, the control plants started to wither after 9 days, while both S1160 and F1167 did not show any severe phytotoxic changes after 13 days.

It was attempted to use a point system for evaluation of the degree of leaf-damages as shown in FIGURE 2-9. The S1160 plant showed the highest tolerance toward both CT and DCMU. The GFC plants F1155, F1180 and F1185 appeared to show higher points by CT treatment. In addition, the production of

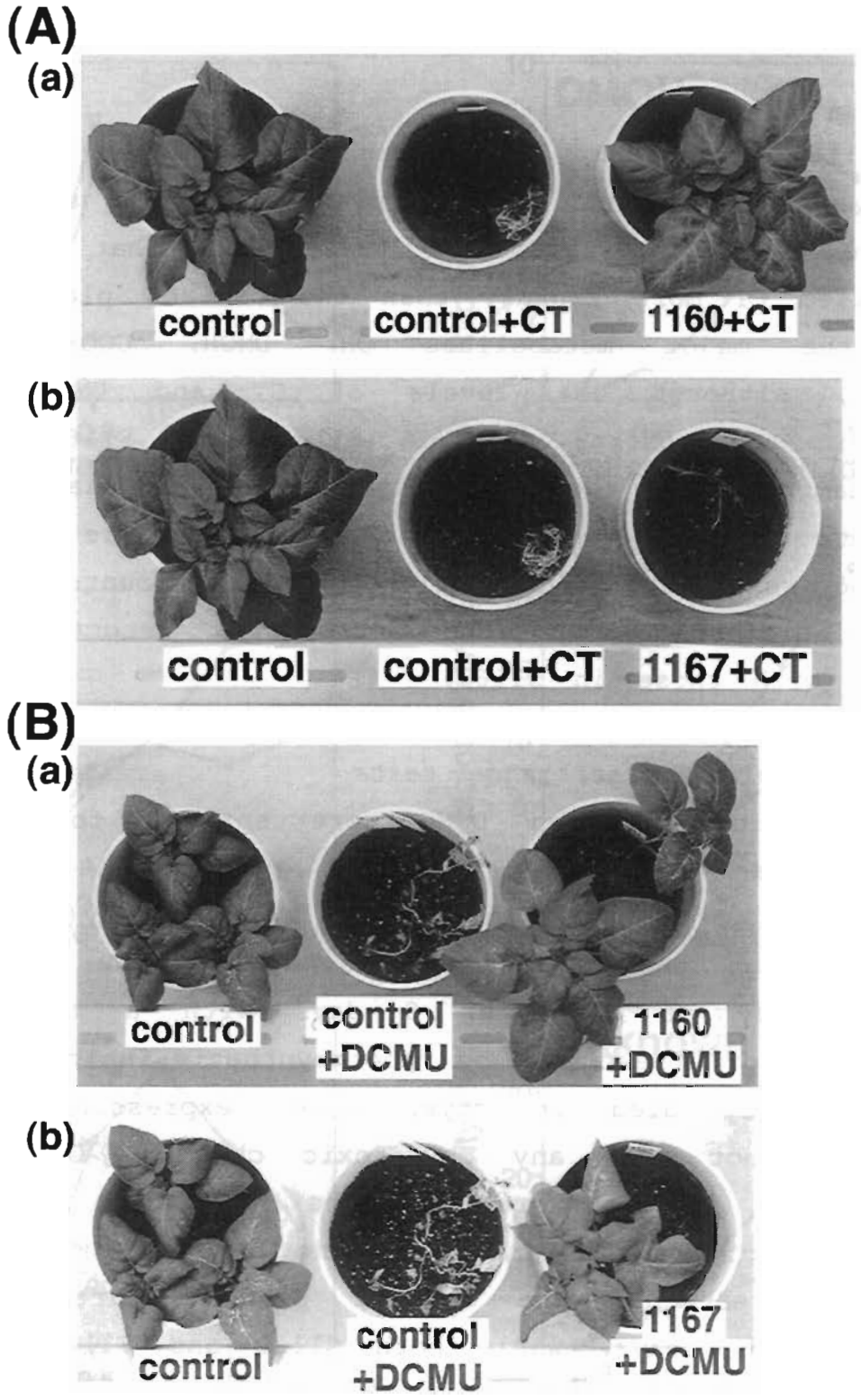

FIGURE 2-8 *In vivo* herbicide-tolerant tests toward the herbicides chlortoluron(A) and DCMU(B) in the transgenic potato plants
The herbicides CT and DCMU were each sprayed at the concentration of 10μmol and 2μmol, respectively. Sprayed herbicide-solution contains 0.015% stickers(spray adjuvants) and 0.02% Tween 20. The control plant was sprayed with the herbicide-solutions with and without herbicide. The transgenic potato plants expressing rat CYP1A1 alone(a) and expressing rat CYP1A1/yeast reductase fused enzyme(b) were also examined.

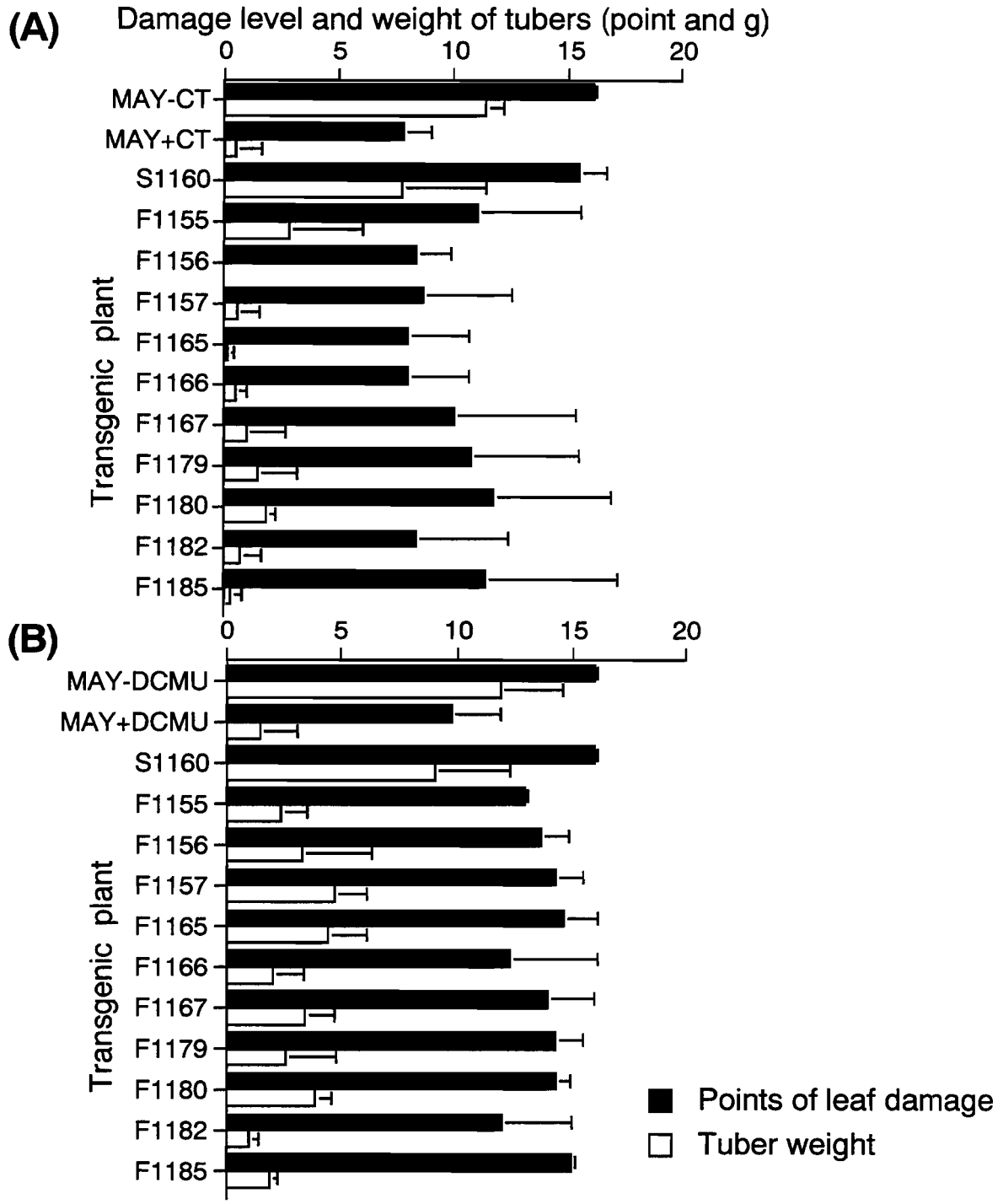

FIGURE 2-9 The levels of leaf damage and tuber weight in the transgenic potato plants with and without treatment of the herbicides chlortoluron(CT)(A) and DCMU(B)
The point system was utilized for evaluation of leaf damage. Points 4, 3, 2, 1 and 0 represent that a plant is living vigously, a part of plant leaf is withering or chlorotic, withering and chlorotic, a plant is almost withering, and withering completely, respectively. These values are indicated with the total points of different four days observed. MAY-herbicide or MAY+herbicide represent for MayQueen with spraying water containing only methanol for dissolving herbicide, MayQueen with spraying water containing each herbicide, respectively. Tubers were harvested 3 weeks after spraying a herbicide. The data represent the results of three independent experiments with standard deviations.

potato tubers in the pot cultivation of the transgenic plants was examined. The results are also shown in FIGURE 2-9. The amount of tubers produced with S1160 was the largest among the transgenic plants treated with CT. Most of the GFC plants produced smaller amounts of tubers as compared with S1160 plant. With DCMU treatment, the GFC plants showed higher tolerance as compared with CT treatment. The DCMU-treated transgenic plants also produced a smaller amount of tubers as compared with the control without DCMU treatment. However, S1160 plants produced a larger amount of tubers as compared with the control treated with DCMU. The tubers produced *in vivo* showed nearly the same size, shape and color on the surface and content as those of the control(data not shown).

DISCUSSION

It was attempted to express rat CYP1A1 cDNA and rat CYP1A1/YR fused enzyme gene in the transgenic potato plants. CYP1A1 is one of the most abundant P450 species related to drug metabolism in rat livers and has been successfully expressed in transgenic tobacco plants, which metabolized and exhibited resistance to the herbicide CT. In the case of potato, microtubers were used for transformation. This method was highly efficient as compared with the methods of leaf discs(89), stems(90,91) and callus(92). As a result, one transgenic plants for CYP1A1 cDNA and ten plants for the fused enzyme gene were obtained. In the previous report, when a rabbit liver P450 cDNA was integrated into tobacco plants, the transformants showed marked phenotypic changes, notably a tendency rapidly to senesce caused by accumulation of a degradative metabolite of nicotine alkaloids(93). However, such phenotypes were not observed in transgenic tobacco plant(32) as well as in the transgenic potato plants in the present study except for the GFC plants F1182 and F1185 which showed a dwarf phenotype. The GUS and ECOD activities of the transgenic potato plants were 300~400-fold and 1.4~3.5-fold higher than those of the control potato plant, respectively, whereas 800~1000-fold and 3.4~11.0-fold higher in the transgenic tobacco plants as compared with those of the control(32). The reason why the transgenic potato plants showed such lower activities than the transgenic tobacco plants was not known yet. The different genus in the same family may cause these differences in GUS and ECOD activities. CaMV

35S promoter was widely used for the constitutive expression in a whole plant(94). In the present study, the GUS activity was highly expressed in leaves and microtubers, particularly in pith but not in cortex.

Southern blot analysis revealed that the transgenic plants containing three bands of the P450 gene showed the highest activities of both GUS and P450 monooxygenase. However, in other cases the plants having multiple bands showed a low activity. The high activity of the expressed enzymes in plants may be related to the copy number or the stability of the enzyme in the plant cells.

Northern blot analysis was performed in order to confirm the transcription of the integrated genes. The GC plant S1160 produced a high level of P450-mRNA. On the other hand, in the all GFC plants including F1167 the level of CYP1A1/YR fused enzyme-mRNA was very low. In addition, small sizes of mRNAs were detected, although these seemed to be degradation products. In transgenic tobacco plants expressing rat CYP1A1/YR fused enzyme, small sizes of mRNAs were not found(data not shown). The reason is not clear.

Western blot analysis was examined in order to confirm the translation of these mRNAs. The CYP1A1 enzyme in the GC plant S1160 was detected in the microsomes by the use of anti-rat CYP1A1 antibody, although no corresponding bands were found in the GFC plants(data not shown). Since the mRNA level of the fused enzyme was so low as compared with that of the S1160 plant, it might result in low levels of the fused protein. However, 7-ethoxycoumarin *O*-deethylase activity was nearly the same between the plants S1160 and F1167. As reported previously(95), the CYP1A1/YR

fused enzyme showed a high activity in comparison with CYP1A1 alone when expressed in the yeast, since the electron transfer was efficiently performed from the yeast reductase part to the CYP1A1 part.

The metabolism of [^{14}C]CT was examined in the transgenic potato plants. The metabolites, *N*-demethylated(DM), ring-methyl hydroxylated(OH), *N*-demethylated ring-methyl hydroxylated(DMOH) and 4-carboxyphenyl(COOH) CT, unknown ones were found in plants and only DM was in medium(data not shown). These same results were also found in the transgenic tobacco plants(77). Namely, after uptake of the herbicide CT, CT was metabolized in the control plants to yield DM which is still phytotoxic, and in the transgenic plants to yield OH which is almost non-phytotoxic. The OH metabolite in the transgenic plant was further metabolized to COOH which is less toxic. In addition, DM was also metabolized to DMOH. These metabolites were conjugated with glucose in the tobacco plants(77). Similar metabolic pathways for CT were found in rat(96) and Japanese quail(97) livers. In wheat(98), barley(99) and other crops tolerant toward CT(100), which was metabolized through *N*-demethylated and ring-methyl hydroxylation. These studies have reported that at least two distinct enzymatic systems may participate in metabolism of CT, since 1-aminobenzotriazole, P450 enzyme inactivator, inhibited the conversion of CT to OH in spite of non-inhibition of the conversion of CT to DM(98,101,102). On the other hand, *in vitro* metabolism of CT in germinating wheat also suggested that ring-methyl hydroxylation of CT was involved(52). The ring-methyl hydroxylation seemed to be responsible for the

tolerance to CT. Thus, introduction of rat CYP1A1 into potato plants was found to enhance ring-methyl hydroxylation and resulted in tolerance to the herbicide.

In vivo herbicide-tolerance toward the herbicides CT and DCMU was tested by spraying each herbicide. The S1160 was the most tolerant toward both herbicides. But this slightly decreased the production of tubers on the treatment with CT as compared with that of the control, suggesting that photosynthesis may be affected by the herbicide CT, although the growth of shoot was well comparable to the control. Although S1160, and F1165, F1166, F1167 and F1180 showed nearly the same level of the ECOD activity, the tolerance to both herbicides was higher in the GC plant than in the GFC plants. After spraying the herbicide, in both S1160 and F1167 plants the endogenous P450 reductase was slightly induced within two days(data not shown). This was supported by the results of Reichhart *et al.*(103). Therefore, the induced endogenous reductase may be more effective in the GC plant to increase the monooxygenase activity than in the GFC plants, since the fused enzyme had a sufficient reductase moiety for the function of the P450 moiety. The levels of tolerance to DCMU were higher than to CT in most of the transgenic plants. This may be related to a lower concentration of DCMU treated as compared with CT.

We did not analyze alkaloids produced in tubers. However, the alkaloid pattern may be changed in the transgenic plants.

The present study seemed to be useful for production of transgenic plants metabolizing herbicide residues as

well as environmental pollutants, since P450 species related to drug metabolism show a broad and overlapped substrate specificity each other. These transgenic plants are expected to be also cross-resistant toward many herbicides with different structures and different modes of action.

CHAPTER 3
HERBICIDE METABOLISM AND CROSS-TOLERANCE IN TRANSGENIC POTATO PLANTS EXPRESSING HUMAN CYP1A1

INTRODUCTION

Pesticide chemicals in the market had been already tested before release into the market about the safety of the chemicals and their metabolites toward human health and the environment. On the tests, experimental animals were used for these chemicals. Accordingly, it is important to extrapolate the animal data to human health for their safety assessment. However, there is a species difference in the metabolism and toxicity between human and experimental animals, showing that kinds and amounts of the metabolites generated in human are diverse with experimental animals. The metabolites of pesticides in the transgenic potato plants expressing rat CYP1A1 may be different from them produced in transgenic plants expressing human CYP1A1. On the other hand, the metabolites produced in plants expressing human CYP1A1 are the same metabolites generated in human liver. These 'human-type' metabolites seem to be safer to human health. Therefore, the transgenic plants expressing human P450 species may be a human model system for pesticide metabolism. It is possible that the transgenic plants expressing human P450 species take place of this evaluation system. In addition, transgenic potato plants expressing human CYP1A1 metabolize herbicide chemicals to exhibit the herbicide resistance. These plants also metabolize not only herbicides but also other environmental chemicals. Therefore, these may be useful for phytoremediation. Hence, it was attempted to produce transgenic potato plants expressing human CYP1A1.

MATERIALS & METHODS

Plasmid construction and potato transformation

Human CYP1A1 cDNA was cloned from a cDNA library of human liver as reported previously(104). The vector pNG01 with both NPT II and GUS expression units, and pUTR121H with both NPT II expression unit and alfalfa mosaic virus 5′-untranslated region under the CaMV 35S promoter were used in the present study(94,105). The expression plasmids pIH1A1, pIHFAR and pHF1A1 were each constructed by the insertion of human CYP1A1 cDNA and human CYP1A1/yeast P450 reductase fused enzyme gene between CaMV 35S promoter and Nos-T of the vectors pNG01 and pUTR121H according to the procedures reported previously(FIGURE 3-1)(32,104). Potato transformation was carried out as reported previously(78). Regenerated plants from callus on microtuber discs infected with *Agrobacterium tumefaciens* strains LBA4404 and C58C1 having each of the recombinant plasmids were selected as a kanamycin-resistant and then subjected to PCR analysis and herbicide-tolerance tests. Transgenic plants cultivated in pots were sprayed with 10μmol/pot of the herbicide CT and observed damage levels. CT-tolerant plants selected were used for further analyses.

Southern, northern and western blot analyses

Genomic DNA(15μg) and mRNA(0.7μg) extracted from whole potato plants cultured on a modified MS medium for 2 weeks were used for southern and northern blot analyses, respectively. A probe for southern hybridization was prepared by PCR using human CYP1A1 specific primers(5′-GCCAAGCTTTCTAACAATGC-3′ and 5′-

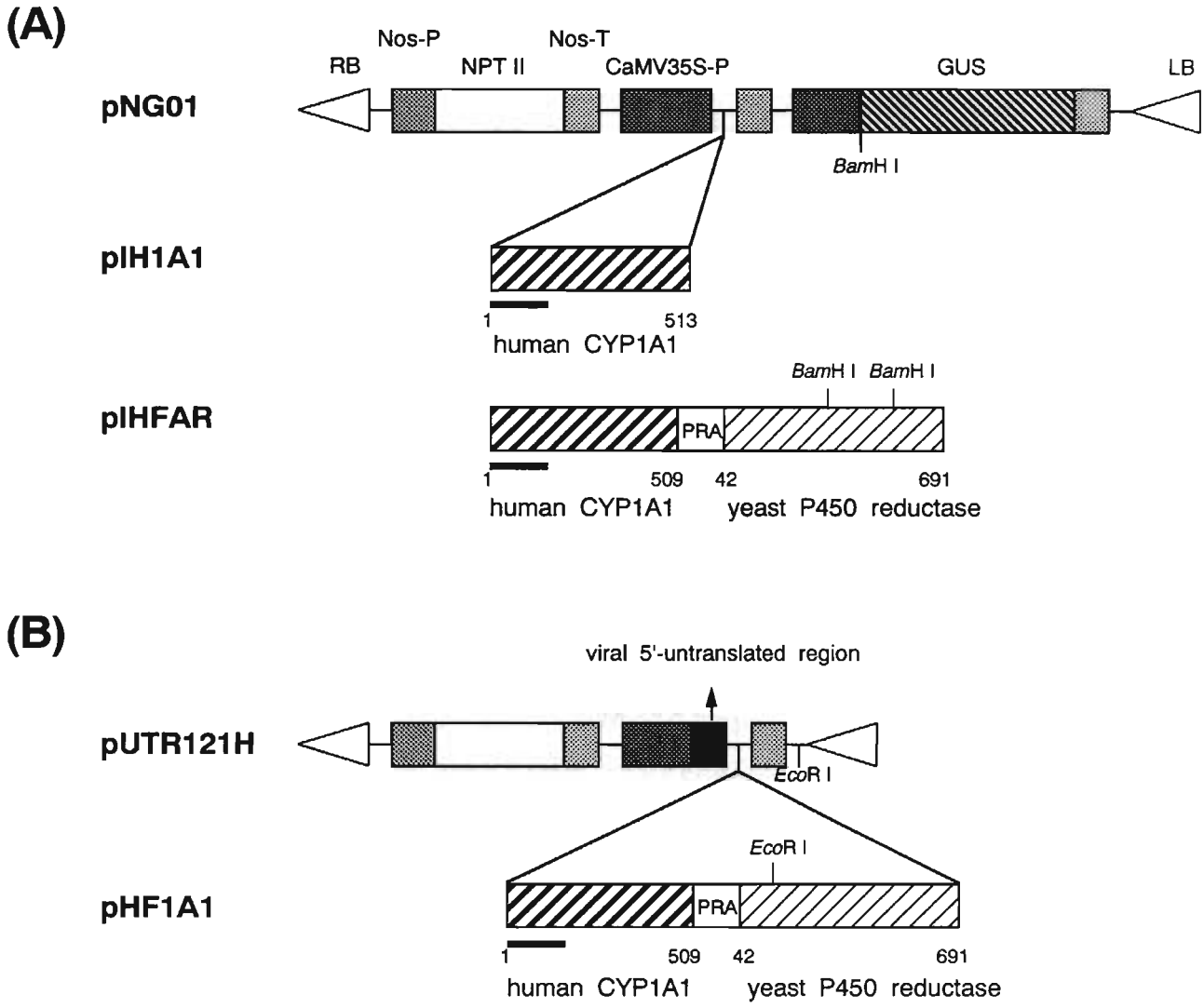

FIGURE 3-1 The structure of the constructed expression plasmids for human CYP1A1 and human CYP1A1/yeast P450 reductase fused enzyme using the vectors pNG01(A) and pUTR121H(B)
R and A represent for amino acid residues derived from the synthesized DNA linker. Numbers below the coding regions indicate the number of amino acid residues as counted from the amino terminus of the corresponding proteins. The probes labeled with DIG-UTP used in southern blot analysis are also shown at the lower side of both expression plasmids with a bold line.

AAGGACATGCTCTGACCATT-3′. A riboprobe for northern hybridization was made by a DIG RNA Labeling Kit. Detections were performed by the use of a DIG detection system, as described above.

Preparation of a microsomal fraction and western blot analysis were performed as reported previously with modifications(32). Twenty and forty micrograms of microsomal fractions of transgenic plants expressing human CYP1A1 and human CYP1A1/YR fused proteins were used for western blot analysis, respectively. Anti-rat CYP1A1 antibodies were used as primary polyclonal antibodies.

Assays of 7-ethoxycoumarin O-deethylase(ECOD) and cytochrome c oxidoreductase(CCOR)

ECOD and CCOR activities in the microsomal fractions of potato plants were measured as described in CHAPTER 2.

Chemicals

[^{14}C]-Ring-labeled AT[6-chloro-*N*-ethyl-*N*-(1-methylethyl)-1,3,5-triazine-2,4-diamine](sp. act. 1.99MBq/mg, radiochemical purity 99.0%) were provided by Novartis Crop Protection, Inc. AT, deisopropylated AT(2-amino-4-ethylamino-6-chloro-1,3,5-triazine; DI), deisopropylated deethylated AT(6-chloro-2,4-diamino-1,3,5-triazine; DIDE) were purchased from Riedel-de Haën AG(Seelze, Germany). PM(methyl 2-[(4,6-dimethoxypyrimidin-2-yl)oxy]-6-[1-(methoxyimino)ethyl]benzoate) was synthesized from methyl 6-[1-(methoxyimino)ethyl]salicylate and 4,6-dimethoxy-2-methanesulfonylpyrimidine, as reported previously(106,107).

Metabolism of herbicide chemicals in the yeast microsomes expressing human CYP1A1

The microsomes of the yeast *Saccharomyces cerevisiae* strain expressing human CYP1A1 at a level of 60pmol/mg microsomal protein was obtained from Sumitomo Chemical Co. Ltd., Takarazuka, Hyogo, Japan. The herbicide chemicals CT, AT and PM were each added at 10nmol to the reaction mixtures containing KPb(pH7.4), glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADPH and yeast microsomal protein equivalent to 25pmol of human CYP1A1 and incubated at 37°C. The parent and its metabolites were then analyzed in a high performanced liquid chromatography(HPLC; Model L-6200, Hitachi, Tokyo, Japan)(32).

Metabolism of [^{14}C]CT and [^{14}C]AT

Transgenic potato plants grown sterilely on a modified MS medium were placed in a nutrient solution containing [^{14}C]CT or [^{14}C]AT at the concentrations of 20μM and 5μM, respectively and sampled on 0, 0.5, 1, 2, and 8 days. Then, extracts from plants with a mixture of methanol and water(9:1, v/v) were analyzed by TLC using a mixture of chloroform and ethanol(9:1, v/v) for CT metabolites, and chloroform for AT metabolites as a developing solvent. Silica gel-precoated TLC plates and silanized silica gel plates were used for analysis of CT and AT metabolites, respectively. Radioactivity was measured in a FLA-2000 Bio Imaging Analyzer(Fuji Photo Film Co. Ltd., Tokyo, Japan).

Tolerance tests to herbicides

In a CT tolerance test, 0μmol to 17.6μmol of

solutions of CT in a spray-mixture containing 0.02% Tween 20 and 0.015% spreader were each sprayed to potato plants cultivated in pots. After twelve days, damage levels were evaluated by a damage point system as shown in FIGURE 3-5.

In AT and PM tolerance tests, 2μmol of AT in a spray-water and 1μmol of PM in a spray-water were each sprayed on potato plants cultivated in pots, and placed under light conditions for 8 hours at 20°C. After twelve days for AT-spray and forty-seven days for PM-spray, potato plants were observed.

RESULTS

Generation of transgenic potato plants

We constructed expression plasmids for human CYP1A1 and its fused enzyme with YR. The structures of the expression plasmids pIH1A1 for human CYP1A1 constructed from the vector pNG01, pIHFAR and pHF1A1 for human CYP1A1/YR fused enzyme from the vectors pNG01 and pUTR121H are shown in FIGURE 3-1. Potato microtuber discs were infected with both *A. tumefaciens* strains LBA4404 and C58C1 harboring each of the expression plasmids pIH1A1, pIHFAR and pHF1A1. Then, kanamycin-resistant potato shoots regenerated from callus were selected. Twelve plants transformed with pIH1A1 for human CYP1A1, twenty-nine plants transformed with pIHFAR and twenty-three plants transformed with pHF1A1 for human CYP1A1/YR fused enzyme were obtained. These plants were subjected to further selection by PCR analysis. As a result, ten plants for pIH1A1, twenty-three for pIHFAR and eighteen for pHF1A1 were selected. Then, these selected plants were examined for tolerance to the herbicide CT. As a result, eight plants for pIH1A1, nine for pIHFAR and twelve for pHF1A1 were tolerant against the herbicide. Then, based on western blot analysis, eight CT-tolerant plants for pIH1A1, two for pIHFAR and seven for pHF1A1 were selected. Finally the selected plants producing a relatively large amount of the corresponding P450 proteins were designated as S1384 with pIH1A1, F1386 with pIHFAR and F1515 with pHF1A1. These transgenic plants were subjected to further analyses.

Southern, northern and western blot analyses of the transgenic potato plants

The selected transgenic potato plants S1384, F1386 and F1515 were each analyzed by southern blotting to confirm the integration of T-DNA into genomic DNA. FIGURE 3-2(A) shows that two to three bands were hybridized with the DNA probe corresponding to human CYP1A1 in these transgenic plants, whereas no bands were found in the control plants. Therefore, the integration of the corresponding P450 genes into the genomic DNA was ascertained in these transgenic plants.

In northern blot analysis shown in FIGURE 3-2(B), S1384 contained a large amount of 1.9kb-mRNA corresponding to human CYP1A1 cDNA in a poly(A) RNA fraction prepared from the whole plants. Both F1386 and F1515 contained the corresponding fused enzyme mRNA bands detected with the DIG-labeled riboprobe of human CYP1A1 cDNA, although amounts of these mRNAs were lower than the mRNA band in S1384. No hybridized bands were found in the control plant. Therefore, it was found that the integrated genes were transcribed into the corresponding P450-mRNAs.

Western blot analysis revealed that S1384 and F1515 contained positive bands corresponding to human CYP1A1 and the fused enzyme proteins with 55kDa and 130kDa, respectively as shown in FIGURE 3-3. On the other hand, the control and S1378 plants which were not tolerant to CT as well as F1386 plants did not show the corresponding positive bands. Therefore, it was found that the P450-mRNAs were translated into the corresponding P450 proteins in both S1384 and F1515 plants.

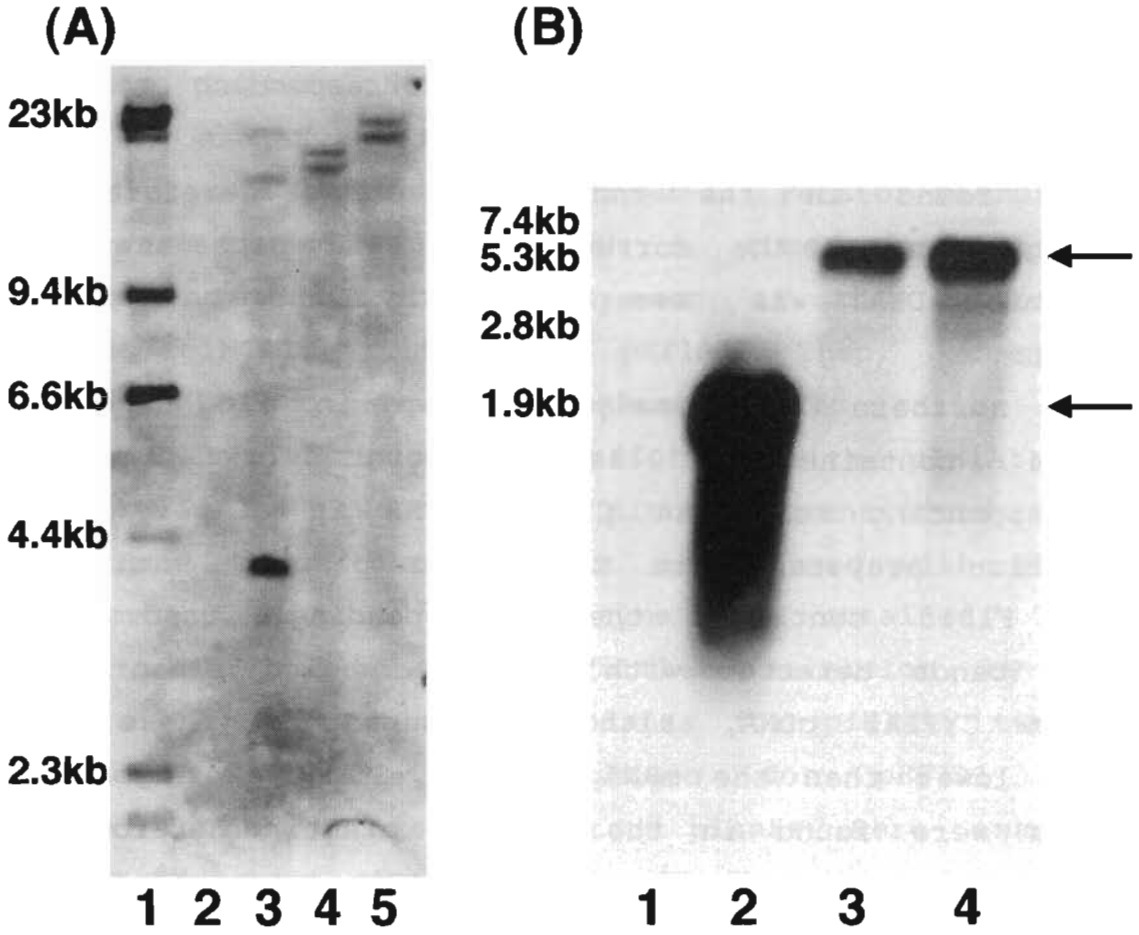

FIGURE 3-2 Southern(A) and northern(B) blot analyses of the transgenic potato plants
(A): Lane 1 to 5 show λ-*Hin*d III marker, untransformed control, S1384, F1386 and F1515, respectively. (B): Lane 1 to 4 represent control, S1384, F1386 and F1515, respectively.

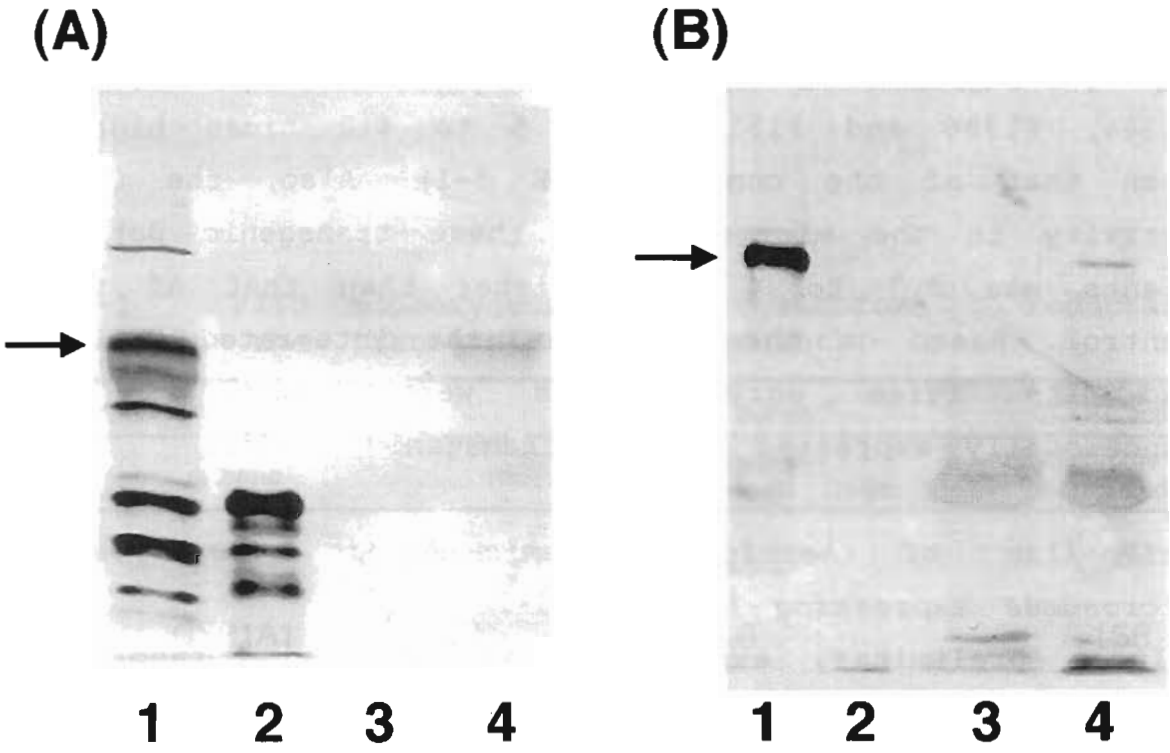

FIGURE 3-3 Western blot analysis of the microsomal fractions from the transgenic potato plants expressing human CYP1A1(A) and human CYP1A1/yeast reductase fused enzyme(B)
(A): Lane 1 to 4 show positive control derived from yeast microsomal fraction expressing human CYP1A1 and untransformed control, S1378 sensitive to chlortoluron-spraying and S1384 tolerant, respectively. (B): Lane 1 to 4 represent positive control from yeast microsomal fraction expressing human CYP1A1/yeast P450 reductase fused enzyme, untransformed control, F1386 and F1515, respectively.

ECOD and CCOR activities of the transgenic potato plants

The ECOD activity in the microsomal fraction of the transgenic potato plants was assayed. The activity of S1384, F1386 and F1515 was 3.5 to 4.2 times higher than that of the control(TABLE 3-1). Also, the CCOR activity in the microsomes of these transgenic potato plants was 3.7 to 4 times higher than that of the control. Based on these results, the integrated CYP1A1 and its fused enzyme genes were found to be functionally expressed in the transgenic plants.

Metabolism of herbicide chemicals in the yeast microsomes expressing human CYP1A1

In a preliminary experiment, we examined metabolism of a number of herbicide chemicals in an *in vitro* system consisting of the yeast microsomes expressing human CYP1A1(32). It was found that the herbicides CT, AT and PM were metabolized by human CYP1A1. About 85%, 13% and 30% of CT, AT and PM of 10nmol were metabolized for 1 hour at 37°C by 25pmol of the P450 species in the presence of 50nmol of NADPH. Two major metabolites of CT were identified by HPLC co-chromatography with the authentic standards as *N*-demethylated and ring-methyl hydroxylated CT, respectively. Also, two metabolites of AT were similarly identified as deethylated and deisopropylated AT. Moreover, two metabolites of PM were detected by HPLC(data not shown). However, these were not identified yet, since authentic standards were not available. Based on these results, it was found that human CYP1A1 metabolized the herbicides CT, AT and

TABLE 3-1 P450 monooxygenase and cytochrome c reductase activities in the transgenic potato plants

Expression		Transformant	ECOD activity [a]	CCOR activity [b]
vector	enzyme		7-Hydroxycoumarin formed	Cytochrome c reduced
-	-	control	1.1	39
pNG01	CYP1A1	S1384	3.8	156
pNG01	CYP1A1/ YR	F1386	4.6	186
pUTR121H	CYP1A1/ YR	F1515	4.2	143

These values represent for the average of three independent experiments.

[a] ECOD activity, 7-Ethoxycoumarin *O*-deethylase activity(pmol/min/mg microsomal protein)

[b] CCOR activity, Cytochrome c oxidoreductase activity(pmol/min/mg microsomal protein)

PM.

Metabolism of [^{14}C]CT and [^{14}C]AT in the transgenic potato plants

Metabolism of [^{14}C]CT was assayed for the transgenic potato plants as shown in TABLE 3-2. When 100nmol of [^{14}C]CT was applied into a nutrient solution, [^{14}C]CT was rapidly taken up into the plants and then decreased to the levels of 1.9, 2.1 and 2.3nmol of CT/plant during 2 days with S1384, F1386 and F1515, respectively, whereas the control contained 12.1nmol of CT/plant. From TABLE 3-2 it can be inferred that S1384 is 6.4 times faster than the control in the metabolism of the herbicide CT. [^{14}C]Metabolites were extracted from the plants and analyzed by TLC. The major metabolites produced during 2 days in S1384, F1386 and F1515 were identified by TLC co-chromatography with the authentic compounds as DM, which were 6.3, 4.9 and 3.7 times lower than those in the control, respectively. Thus, the transgenic potato plants S1384 expressing human CYP1A1 seemed to be found to metabolize CT mainly through *N*-demethylation and ring-methyl hydroxylation to yield relatively non-phytotoxic metabolites. The total ^{14}C-recovery in plants of applied [^{14}C]CT in the control, S1384, F1386 and F1515 was 21.5%, 4.7%, 3.4% and 7.4%, respectively.

When [^{14}C]AT was applied into a nutrient solution at 25nmol, [^{14}C]AT was rapidly taken up into the plants. [^{14}C]Metabolites were extracted from the plants and analyzed by TLC(FIGURE 3-4). Four metabolites were found and two of them were identified as deisopropylated(DI) and deisopropylated deethylated(DIDE)

TABLE 3-2 Metabolism of [^{14}C]-chlortoluron in the transgenic potato plants

CT/ metabolite[a]	metabolite produced(nmol/plant/2days) [b]			
	control	S1384	F1386	F1515
CT	12.1±4.2	1.9±0.3	2.1±0.2	2.3±0.6
DM	4.4±1.8	0.7±0.2	0.9±0.1	1.2±0.4
OH	0.8±0.7	0.5±0.1	0.1±0.1	0.5±0.2
DMOH	1.1±0.6	0.2±0.1	<0.1	0.3±0.2
COOH	1.2±0.4	0.4±0.1	<0.1	0.8±0.4
UK	1.9±0.6	1.0±0.3	0.3±0.1	2.3±0.7

[a] CT, DM, OH, DMOH, COOH and UK indicate chlortoluron, demethylated chlortoluron, ring-methyl hydroxylated cholrtoluron, demethylated ring-methyl hydroxylated chlortoluron, carboxylated chlortoluron and unknown metabolites, respectively.

[b] These values are the average of three independent experiments.

[c] These values are the average of total amounts of each day.

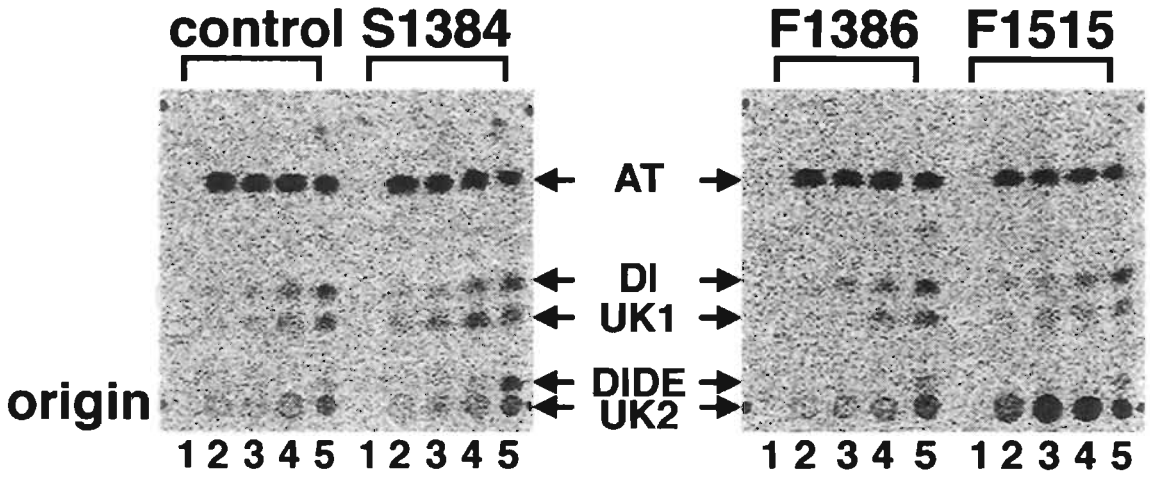

FIGURE 3-4 TLC analysis of [^{14}C]atrazine and its metabolites produced in the transgenic potato plants
Lane 1 to 5 show 0, 0.5, 1, 2, 8 days after incubation. AT, DI, UK1, DIDE and UK2 represent atrazine, deisopropylated atrazine, unknown metabolite 1, deisopropylated deethylated atrazine and unknown metabolite 2, respectively.

metabolites by TLC co-chromatography with the authentic standards in S1384, F1386 and F1515 as well as in the control plant. However, deethylated AT(DE) was not found in these transgenic and control plants. Unknown 1(UK1) and unknown 2(UK2) were not identified, since authentic standards were not available. The amount of DIDE which is non-phytotoxic was 5 and 3 times higher in S1384 and F1515 than the control during 8 days(TABLE 3-3). UK2 at the origin of the TLC plate appeared to be higher in the transgenic plants particularly in F1515 as compared with the control. Thus, the transgenic plants expressing human CYP1A1 and its fused enzyme with YR were found to metabolize AT to a higher extent as compared with that of the control mainly through *N*-deisopropylation and *N*-deisopropyl-deethylation to yield non-phytotoxic metabolites. The total ^{14}C-recovery in plants of applied [^{14}C]AT in the control, S1384, F1386 and F1515 was 21.6%, 26.4%, 20.4% and 57.2%, respectively.

Herbicide-tolerance tests

The tolerance to the herbicide CT was assayed for S1384, F1386 and F1515 planted in pots for twelve days after spraying as shown in FIGURE 3-5(B). S1384 was the most tolerant among the transgenic potato plants examined and was slightly damaged at 17.6μmol of CT/pot, although the control, F1386 and F1515 plants were damaged even at 10μmol of CT/pot. The CT concentration giving damage 2 in the transgenic plants S1384, F1386 and F1515 was more than 5, 1.2 and 1.8 times higher than that of the control, respectively.

The S1384 plant was also tolerant to AT at 2μmol/pot whereas the control on spraying, F1386 and

TABLE 3-3 Metabolism of [^{14}C]atrazine in the transgenic potato plants

AT/ metabolite[a]	metabolite produced(nmol/plant/8days)[b]			
	control	S1384	F1386	F1515
AT	3.0±1.3	2.5±0.9	2.6±0.5	5.4±3.8
DI	0.8±0.4	1.1±0.2	0.8±0.4	1.5±0.8
UK1	0.6±0.1	0.8±0.2	0.6±0.2	1.1±0.8
DIDE	0.2±0.1	1.0±0.3	0.3±0.1	0.6±0.1
UK2	0.8±0.3	1.2±0.3	0.8±0.3	5.7±4.3

[a] AT, DI, UK1, DIDE and UK2 indicate atrazine, deisopropylated atrazine, unknown metabolite 1, deisopropylated deethylated atrazine and unknown metabolite 2, respectively.

[b] These values represent for the average of three independent experiments.

[c] These values are the average of total amounts of each day.

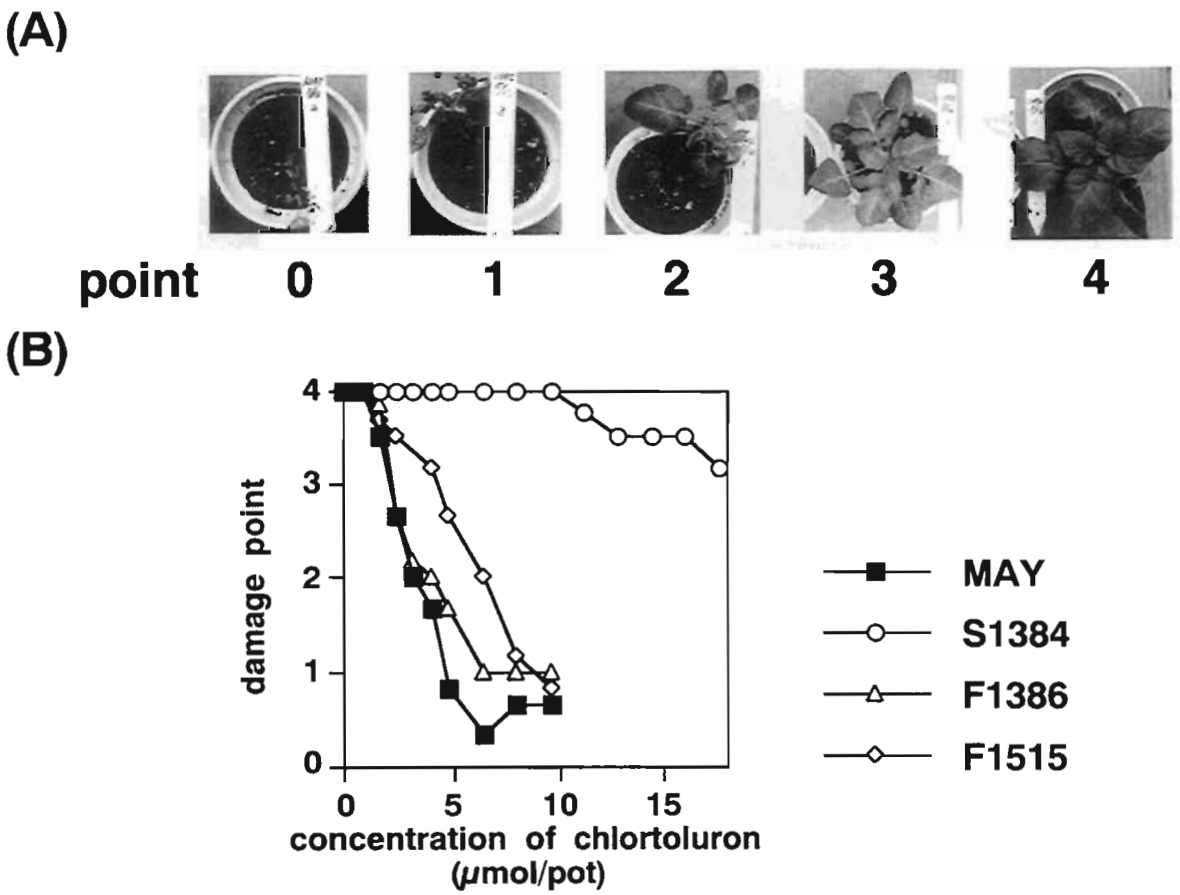

FIGURE 3-5 Tolerance of the transgenic potato plants to the herbicide chlortoluron
(A): Damage levels were observed with the point system. Point 4 shows the highest and point 0 shows the lowest tolerance toward chlortoluron. (B): Different amounts of the herbicide chlortoluron were sprayed, and the transgenic plants were observed after twelve days. These values are the average of three independent experiments.

F1515 were damaged at the same amount of AT(FIGURE 3-6(A)). In addition, these three transgenic plants assayed showed tolerance to the herbicide PM at 1μmol/pot, although the control had died at the same concentration(FIGURE 3-6(B)). Thus, the transgenic potato plants expressing human CYP1A1 were found to show cross-tolerance to the herbicides CT, AT and PM, which are different in their chemical structures and herbicide modes of action, whereas the plants expressing the fused enzyme was slightly tolerant against PM.

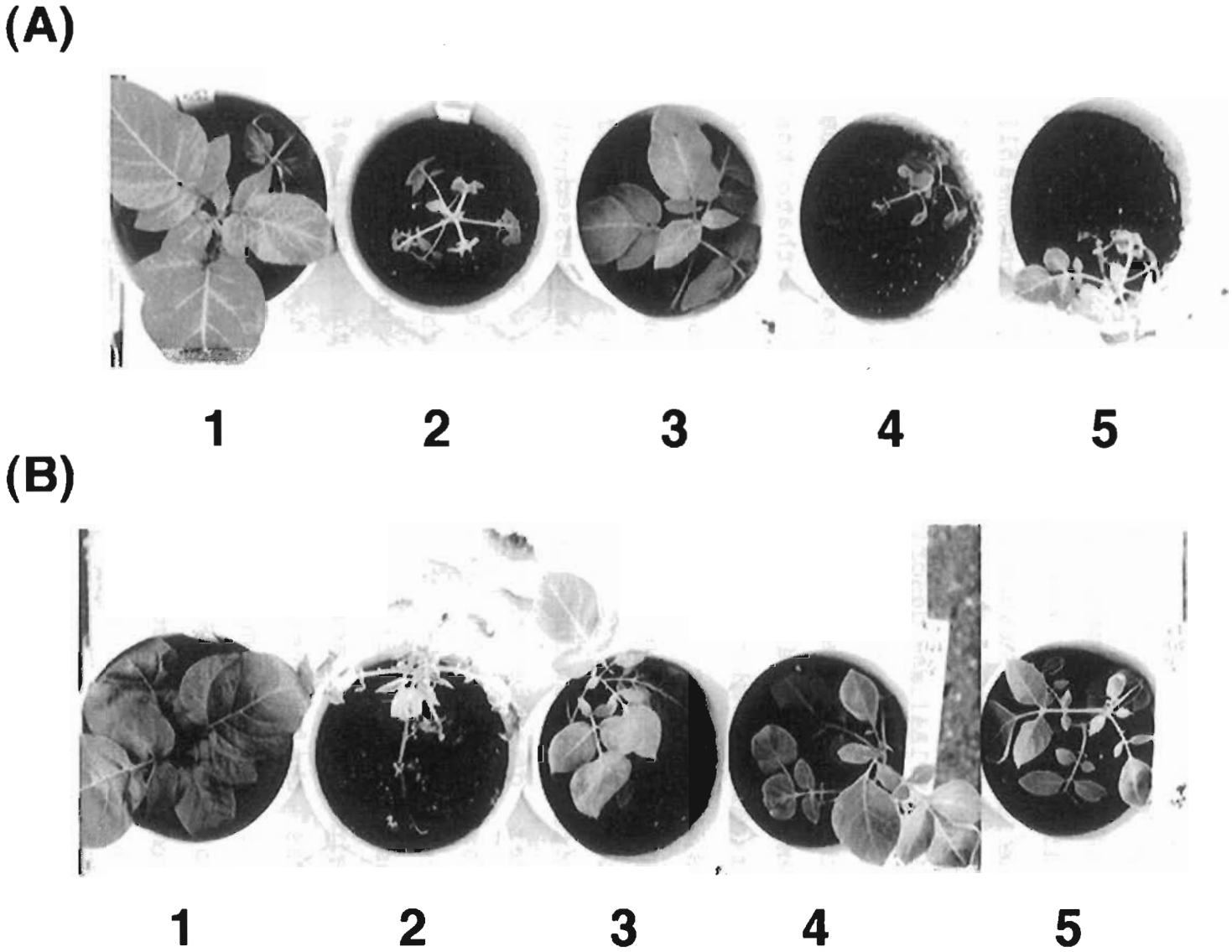

FIGURE 3-6 Tolerance to the herbicides atrazine(A) and pyriminobac-methyl(B) of the transgenic potato plants
Lane 1, untransformed control without herbicide; lane 2, untransformed control plants with herbicide; lane 3 to 5, transgenic plants S1384, F1386 and F1515 with herbicide. (A): The herbicide atrazine was sprayed at 2µmol/pot on plants cultivated in pots. (B): The herbicide pyriminobac-methyl was sprayed at 1µmol/pot.

DISCUSSION

The transgenic potato plants S1384 expressing human CYP1A1, and both F1386 and F1515 expressing human CYP1A1/YR fused enzyme were generated by Agrobacterium-transformation of microtuber discs. Although the ECOD activity of S1384 was nearly the same as those of F1386 and F1515 plants, the S1384 plants metabolized the herbicide CT more rapidly than F1386 and F1515 plants, as found in the transgenic potato plants expressing rat CYP1A1 as described in CHAPTER 2. Also, the herbicide-tolerance toward CT of S1384 with CYP1A1 was higher than the other transgenic plants expressing the fused enzyme. These findings suggested that the expression of CYP1A1 alone was suitable for transgenic potato plants, while in the transgenic tobacco plants, the fused enzyme between CYP1A1 and YR was superior to CYP1A1 alone(32).

It was so far found that human CYP1A1 expressed in the microsomes of the recombinant yeast metabolized the herbicides CT and AT which inhibit the electron transfer in photosynthesis as well as the herbicide PM which is an inhibitor of the biosynthesis of branched chain amino acids, although the rate of metabolism was the highest with CT, followed by PM and AT(data not shown). As the results, the transgenic potato plant S1384 expressing human CYP1A1 was found to exhibit cross-tolerance to these three herbicides.

In wheat, barley and maize which were resistant to the herbicide CT, CT was metabolized mainly through ring-methyl hydroxylation and *N*-demethylation, although in the susceptible plants including tobacco, the

herbicide was metabolized through *N*-demethylation but not through ring-methyl hydroxylation(99). Mougin *et al.* reported that in wheat, ring-methyl hydroxylation of CT was catalyzed by a P450 species(53). Therefore, the P450 species mediated ring-methyl hydroxylation of CT seemed to be important for the herbicide selectivity and resistance. The S1384 plant expressing human CYP1A1 metabolized the herbicide mainly through ring-methyl hydroxylation and *N*-demethylation, as shown in FIGURE 3-7(A). The amount of the partially phytotoxic metabolite DM was smaller in S1384 than in the control. Probably, DM was further metabolized in S1384 to form non-phytotoxic metabolites such as DMOH and/or its conjugates with glucose. These were also agreed with the results of the transgenic potato plants expressing rat CYP1A1 in CHAPTER 2.

It has been reported that in AT-resistant plants, the major metabolic pathway was GSH conjugation as found in sorghum(108,109). It was also reported that *N*-dealkylation was less contributed to resistance toward triazines as compared with GSH conjugation(110). However, accumulation of the didealkylated metabolite DIDE seemed to be important for tolerance toward AT in the transgenic potato plant S1384 expressing human CYP1A1, since it seemed to metabolize [^{14}C]AT through deisopropylation and then deethylation to yield DIDE, as shown in FIGURE 3-7(B), which was 5 times higher than the control, whereas the production of DIDE in F1386 plant was nearly the same as that of the control. Therefore, higher tolerance of S1384 toward AT than F1386 and F1515 seemed to be due to the ability of the transgenic potato plants to form DIDE.

It was reported that the production of the

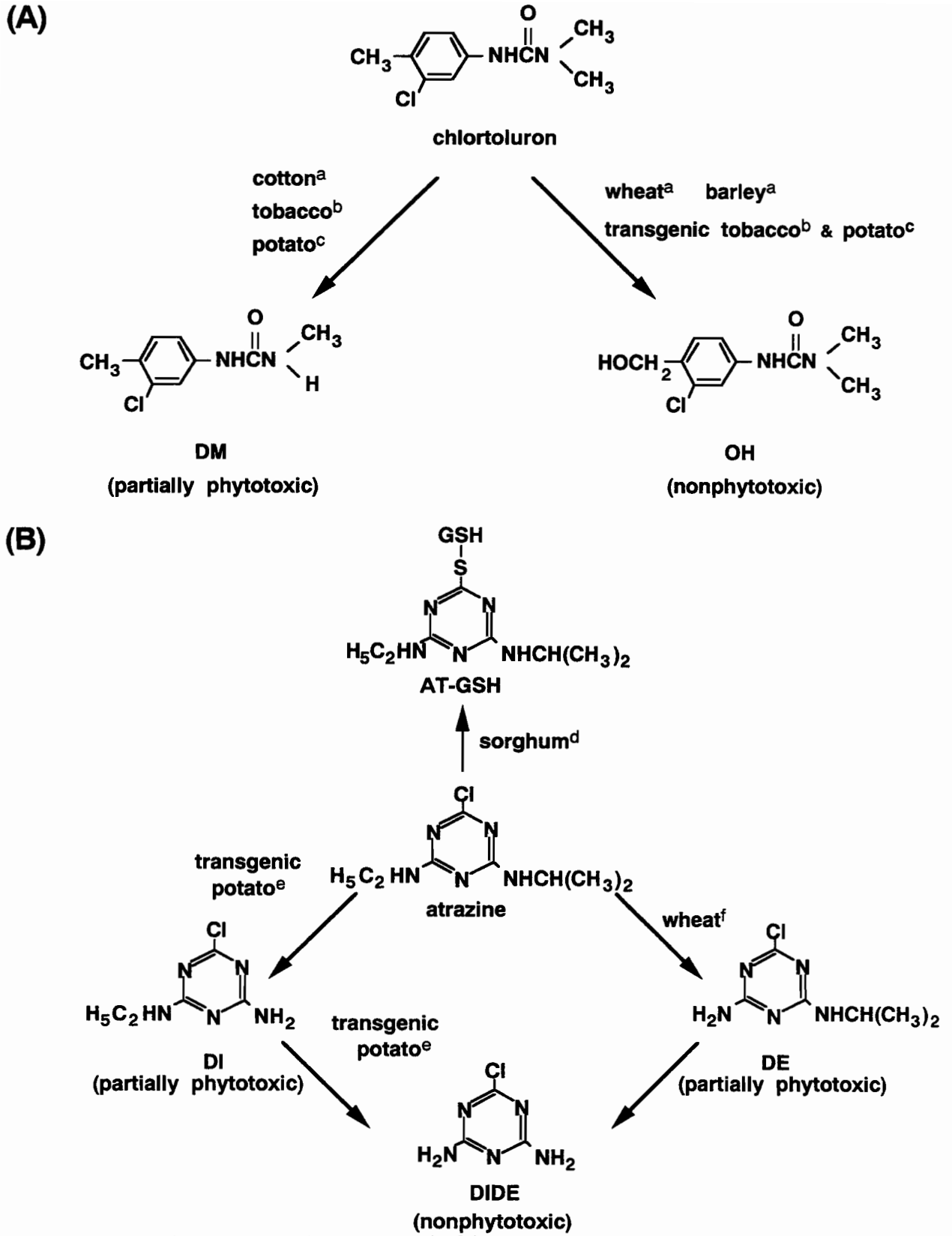

FIGURE 3-7 Major metabolic pathways for the herbicides chlortoluron(A) and atrazine(B) in higher plants (A): DM and OH represent for *N*-demethylated and ring-methyl hydroxylated metabolites, respectively. (B): GSH, AT, DI, DE and DIDE represent for glutathione, atrazine, deisopropylated, deethylated and deisopropylated deethylated metabolites, respectively. The superscripts of a to f show references for Ryan *et al.*(1981), Shiota *et al.*(1996), Inui *et al.*(1998), Lamoureux *et al.*(1973), the present study and Edwards *et al.*(1989), respectively.

metabolite DE was 3.5 times higher than that of DI in the microsomes of human liver(111). Also, in the urine of human exposed to AT, the main metabolite was DIDE, followed by DI and then DE(112). On the other hand, the amount of DI in the microsomal fraction of recombinant yeast expressing human CYP1A1 was 2 times larger than that of DE(data not shown). Based on these results, in human AT seemed to be transformed into DIDE through DE mainly. Therefore, CYP1A1 may be less contributed to the metabolism of AT in human.

It was suggested that 11 human P450 species in the liver microsomes maybe involved in more than 90% of xenobiotic metabolism in human(76). Thus, expression of each or a combination of these P450 species in plants may be useful as a model system for human metabolism of agrochemicals, since it is important for clarification of species-difference in the metabolism of agrochemicals between human and experimental animals or plants.

The transgenic plants S1384, F1386 and F1515 showed tolerance to the herbicide PM. This may be attributable to a small dose level of PM as compared with those of CT and AT. Probably, human CYP1A1 and its fused enzyme may metabolize PM to a higher extent than AT, as found in the metabolism of the herbicide in the yeast microsomes expressing human CYP1A1.

Since mammalian P450 species related to xenobiotic metabolism show a broad and overlapped substrate specificity each other, it was suggested that the transgenic plants expressing one or more of these P450 species may be tolerant toward a number of herbicides with different structures and herbicide modes of action. These transgenic plants expressing

P450 species also seem to metabolize not only herbicide residues but also insecticides as well as fungicides used on the same crop. Moreover, these transgenic plants expressing human P450 species may be useful for phytoremediation of pesticide residues and environmental pollutants(113,114).

CHAPTER 4
HERBICIDE-TOLERANCE AND METABOLISM IN TRANSGENIC POTATO PLANTS CO-EXPRESSING HUMAN CYP1A1, CYP2B6 AND CYP2C19

INTRODUCTION

There are a number of P450 species metabolizing xenobiotics in the microsomes of human liver. So far, it was reported that 11 P450 species in human liver cover 90% of P450-dependent metabolism of drugs(76). These P450 species are CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. Each of P450 species metabolizes several chemicals with different chemical structures, whereas one chemical is metabolized by several P450 species in the different subfamilies and families. Thus, it was found that P450 species metabolizing xenobiotics show a broad and overlapping substrate specificity. Accordingly, these enzymes metabolize a large number of unknown chemicals. Therefore, it was attempted to co-express three P450 species human CYP1A1, CYP2B6 and CYP2C19 in transgenic potato plants. These belong to different gene families and subfamilies. The produced transgenic plants may play an important role on phytoremediation of environmental contaminants as well as a human model system for pesticide metabolism. So far, a single expression of rat and human CYP1A1 described in CHAPTERS 2 and 3 provides higher tolerance toward the herbicides than the expression of the corresponding fused enzyme with YR. In this chapter, a single expression of three human P450 species was also demonstrated.

MATERIALS & METHODS

Plant materials, plasmid construction and plant transformation

Human CYP1A1 cDNA was cloned from a cDNA library of human liver as described in CHAPTER 3. Human CYP2B6 and CYP2C19 cDNAs were provided by Sumitomo Chemical Co., Ltd.(Hyogo, Japan). The vector pSNTLX with multi cloning sites and NPT II expression unit near the left border was constructed in this study(FIGURE 4-1(A)), and the vector pUTR121H was reported in CHAPTER 3. The expression plasmids pUHA1, pUHB6 and pUHC19 were each constructed by the insertion of each of human CYP1A1, CYP2B6 and CYP2C19 cDNAs, respectively, into pUTR121H(FIGURE 4-1(B)). The plasmid pIKBAC for the co-expression of CYP1A1, CYP2B6 and CYP2C19 was also constructed by the insertion of each of three expression units. The expression plasmids pSXA1, pXXB6 and pSSC19 derived from pUHA1, pUHB6 and pSSA1 were constructed by the ligation of *Sal* I or *Xho* I DNA linker(FIGURE 4-1(C)(D)(E)). Then, the pSXA1 fragment digested with *Sal* I and *Xho* I was subcloned into *Sal* I and *Xho* I sites of pSNTLX(FIGURE 4-1(F)). The pXXB6 fragment digested with *Xho* I was also integrated into pIKA1 cut by *Sal* I. Finally, the pIKBA13 constructed was digested with *Xho* I, and the pSSC19 fragment cut by *Sal* I was subcloned into it. Potato transformation was each carried out as reported previously. Kanamycin-resistant plants were selected and analyzed for further studies.

PCR analysis of transgenic potato plants

A genomic DNA prepared from each of kanamycin-

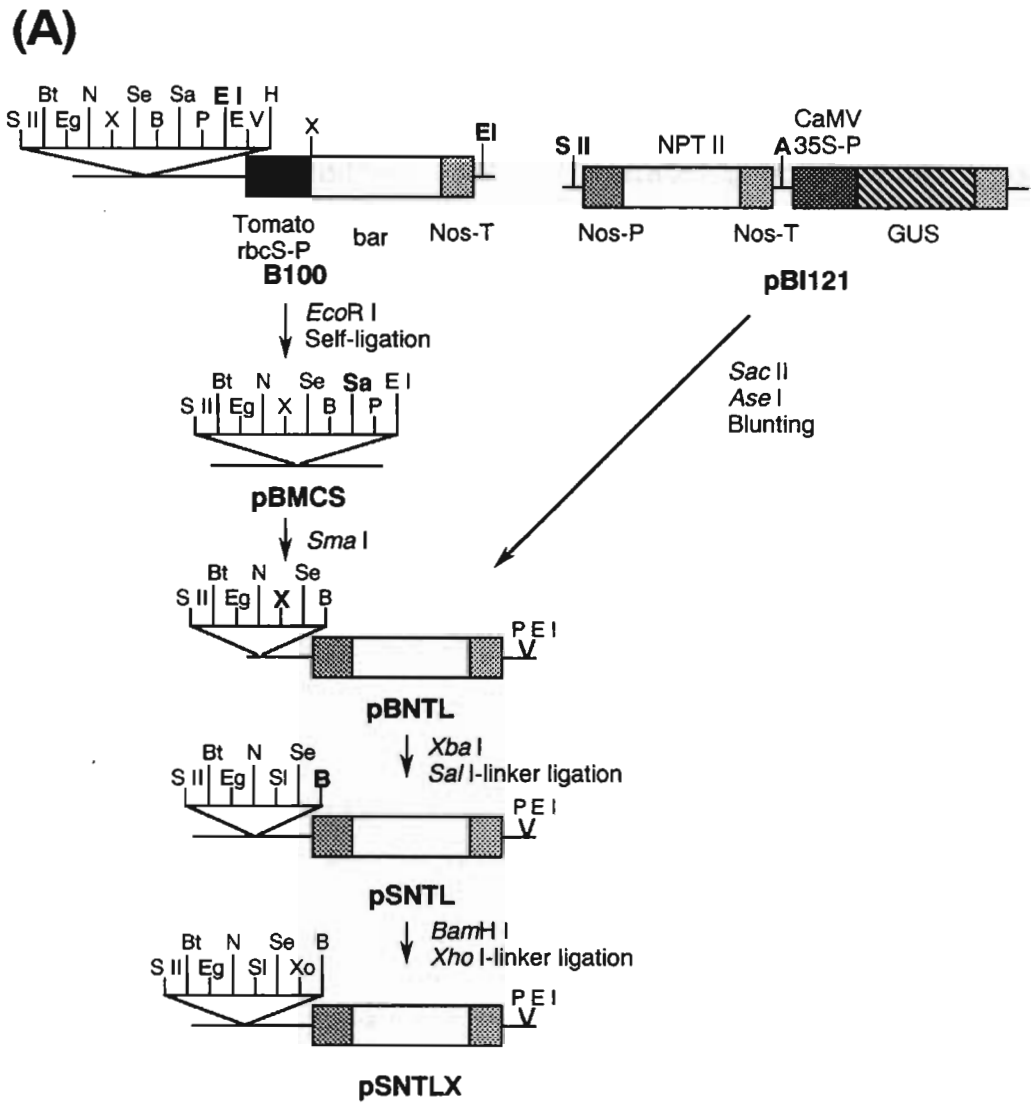

FIGURE 4-1 Schematic procedures for construction of the expression plasmids pSNTLX(A), pUHA1(B), pUHB6(B), pUHC19(B)(E), pSXA1(C), pSSA1(C), pXXB6 (D), pSSC19(E) and pIKBAC(F)
S II, Bt, Eg, N, X, Se, B, Sa, P, E I, E V, H, A, Xo, S l and D III represent for the digestion sites of restriction enzymes *Sac* II, *Bst*X I, *Eag* I, *Not* I, *Xba* I, *Spe* I, *Bam*H I, *Sma* I, *Pst* I, *Eco*R I, *Eco*R V, *Hin*d III, *Ase* I, *Xho* I, *Sal* I and *Dra* III, respectively. Bold and dotted lines represent for PCR probes of southern blot and the length of genomic DNA fragments predicted, respectively.

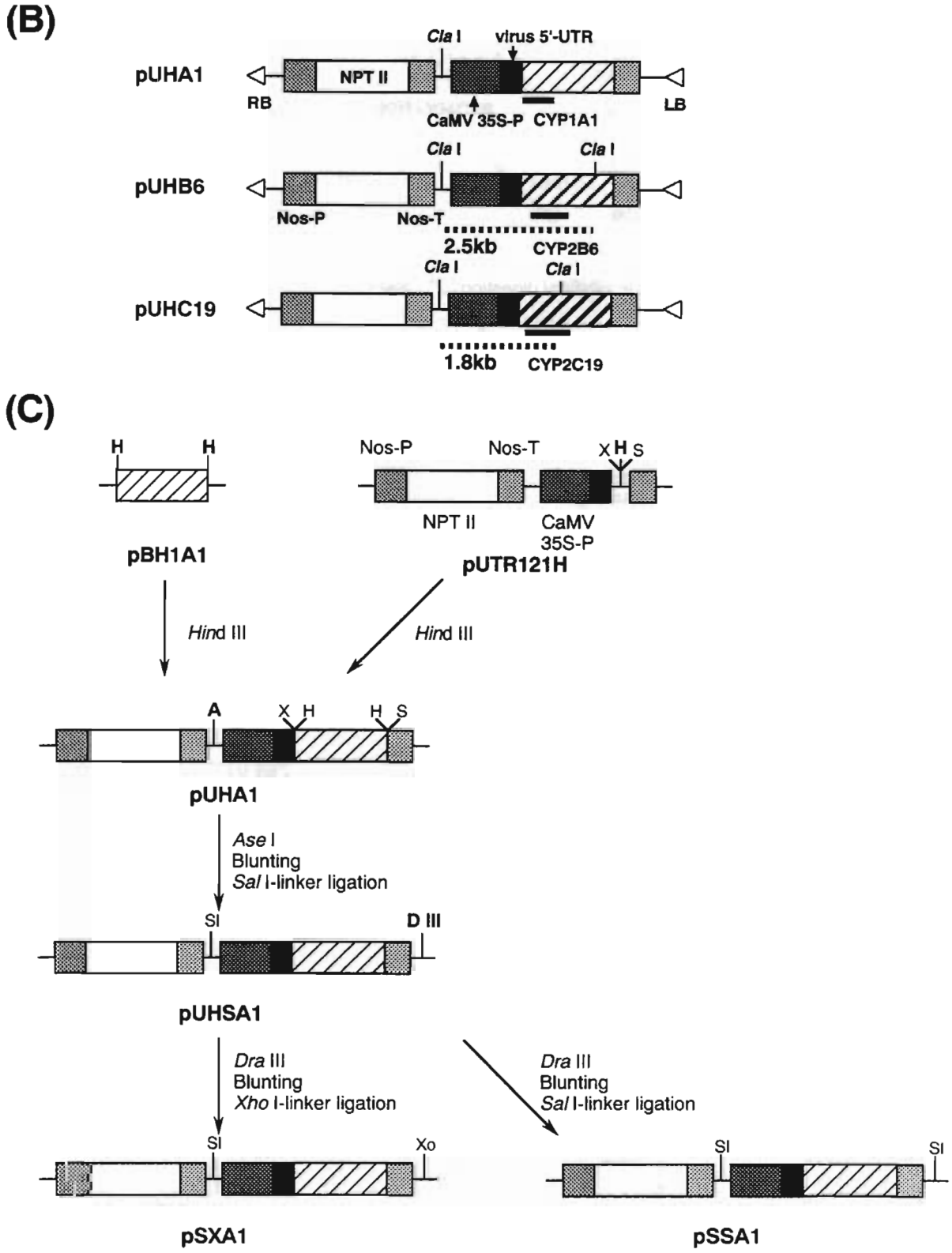

FIGURE 4-1 Continued

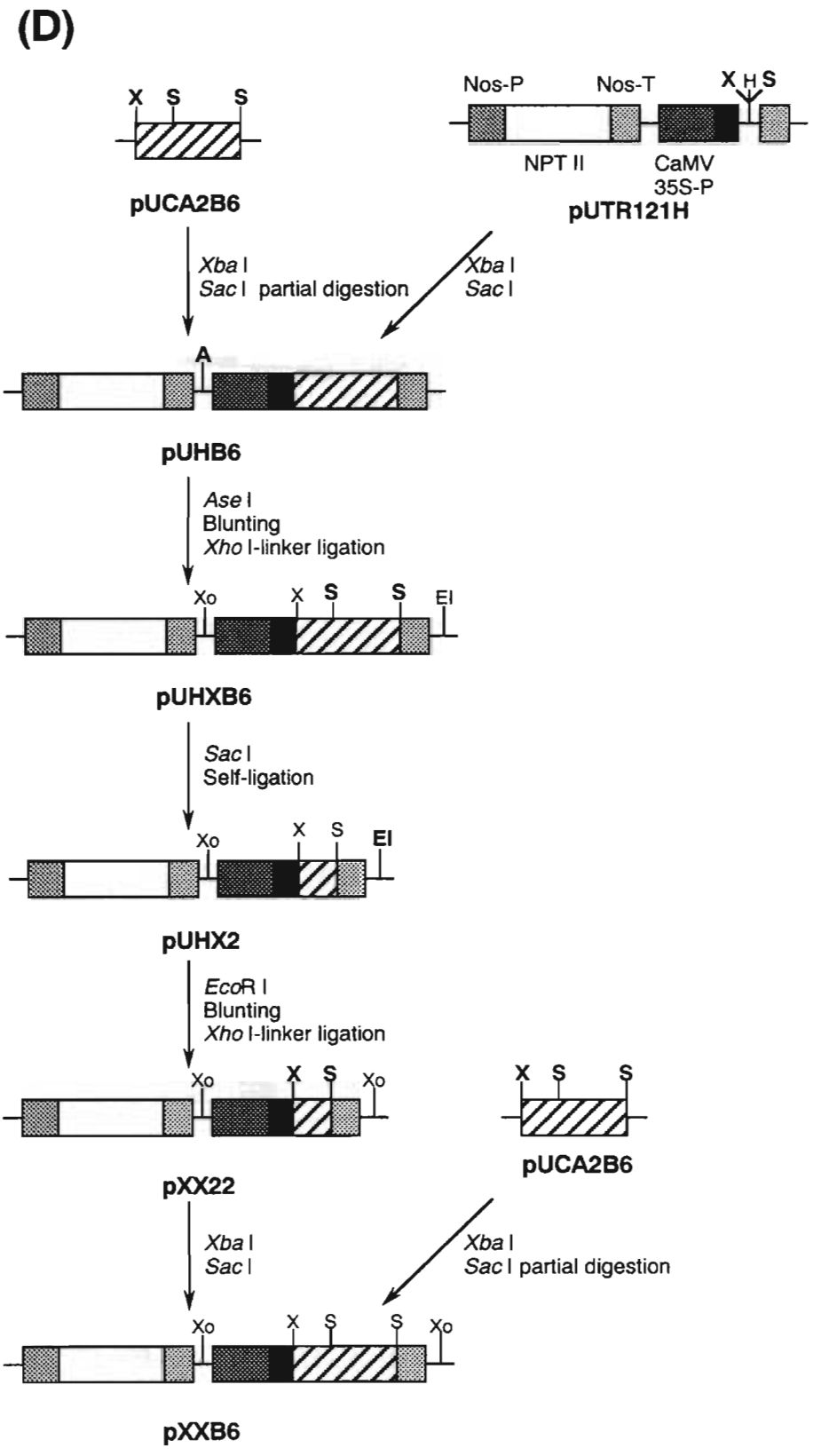

FIGURE 4-1 Continued

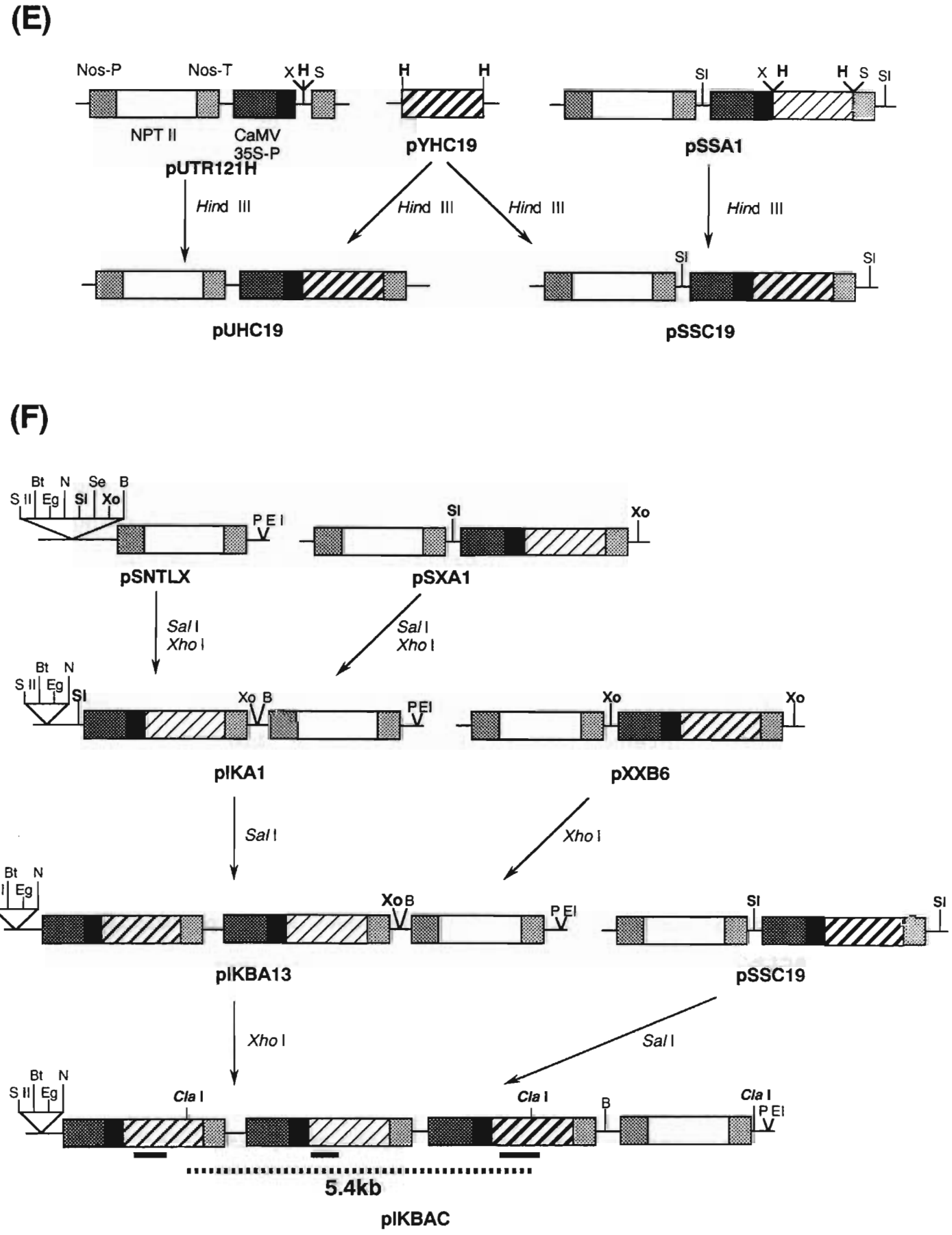

FIGURE 4-1 Continued

resistant plants transformed with *pIKBAC* was used for PCR analysis. PCR primers specific for CYP1A1 5′-GCCAAGCTTTCTAACAATGC-3' and 5'- AAGGACATGCTCTGACCATT-3'; for CYP2B6 5′-GACTCTTGCTACTCCTGGTT-3′ and 5′-CGAATACAGAGCTGATGAGT-3′; for CYP2C19 5′-TCCTTGTGCTCTGTCTCTCA-3′ and 5′-CCATCGATTCTTGGTGTTCT-3′ were used. The annealing temperatures were 55°C, 53°C and 55°C, respectively.

ECOD activity of transgenic potato plants

A 0.1g preparation of leaves from each of kanamycin-resistant plants transformed with pIKBAC, pUHA1 and pUHB6 was incubated in liquid MS medium containing 40μM of 7-ethoxycoumarin for 3 days in the 16h light conditions. Leaves were ground in 0.1M of KPb(pH7.4) and then centrifugated. Chloroform was added to the supernatant, and mixed. Then, the solution of 0.01N NaOH and 0.1M NaCl was added to the lower phase. Fluorescent intensity was measured in a fluorometry F-3010 at excitation 366nm and emission 452nm.

Western blot analysis of transgenic potato plants

A microsomal fraction(20μg) prepared from each of kanamycin-resistant plants transformed with pUHC19 was extracted and analyzed by western blotting as described in previous chapter. Immunodetection with anti-human CYP2C9 antibody was performed for detection of CYP2C19 protein.

Southern, northern and western blot analyses

Genomic DNA(15μg), mRNA(1μg) and microsomal(20μg) fractions prepared from whole potato plants were used. Probes used for southern hybridization were prepared

as with PCR based on human CYP1A1, CYP2B6 and CYP2C19 cDNA sequences described above. A genomic DNA was digested with *Cla* I. Riboprobes for northern hybridization were made with an *in vitro* transcription system using pSHA19, pSHB19 and pSPC19 plasmids derived from pSPT19 in DIG RNA Labeling Kit(SP6/T7). Southern and northern detections were performed by the use of a DIG detection system.

A microsomal fraction prepared from each of transgenic plants was electrophoresed on SDS-PAGE and then immunoblotted by anti-rat CYP1A1 antibody, anti-human CYP2B6 antibody and anti-human CYP2C9 antibody which were provided by Sumitomo Chemical Co., Ltd.(Hyogo, Japan), respectively. Anti-goat IgG for CYP1A1 and anti-rabbit IgG for CYP2B6 and CYP2C19 were used as a secondary antibody.

7-Ethoxyresorufin O-deethylase(EROD) activity in the microsomal fractions

A reaction mixture(1ml) containing 0.1M of KPb(pH7.4), 10μM of 7-ethoxyresorufin, 3mM of NADPH and 1mg of microsomal fraction was incubated at 37°C for 1 hour. A equal volume of cold acetone was added to the reaction mixture sampled, and then it was left on ice for 10 minutes. After centrifugation, the supernatant was measured at excitation of 550nm and emission of 586nm in a fluorometry F-3010.

Chemicals

[^{14}C]-Ring-labeled PC[*O*-[3-(1,1-dimethylethyl)phenyl](6-methoxy-2-pyridinyl)methylcarbamothioate](sp. act. 2.1MBq/mg, radiochemical purity >99.5%) and cold PC were provided

by Dainippon Ink and Chemicals Inc.(Tokyo, Japan). MT[*N*-2-benzothiazolyl-*N,N'*-dimethylurea], AC[2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide], MC[2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide] and NR[4-chloro-5-(methylamino)-2-[3-(trifluoromethyl)phenyl]-3(2*H*)-pyridazinone] were purchased from Riedel-de Haën AG(Seelze, Germany). *m-tert*-Butylphenol was purchased from Wako Pure Chemical Industries, Ltd.(Osaka, Japan).

Metabolism of herbicide chemicals in the yeast microsomes expressing each of human CYP1A1, CYP2B6 and CYP2C19

The herbicide chemicals AC, AT, CT, MT, MC, NR and PC were each incubated in the reaction mixture containing yeast microsomal protein at 37°C as reported in CHAPTER 3. The extract was analyzed by HPLC(Model L-6200, Hitachi, Tokyo, Japan) for the parent and its metabolites.

Metabolism of [^{14}C]CT, [^{14}C]AT and [^{14}C]PC

Metabolism of [^{14}C]CT and [^{14}C]AT metabolisms was carried out as described in CHAPTERS 2 and 3.

On metabolism of [^{14}C]PC, transgenic plants were incubated in a nutrient solution containing 10μM of [^{14}C]PC, and then its metabolites were extracted with a mixture of methanol and water(7:3, v/v)(115). After shaking for 30 minutes, the supernatants were filtrated, and the residues were washed with methanol. Total extracts were dried and dissolved again in a mixture of methanol and water(7:3, v/v). Silica gel TLC plates and a solvent system of benzene and ethyl acetate(9:1, v/v) were used for TLC of its

metabolites. Radioactivity was measured in a FLA-2000 Bio Imaging Analyzer(Fuji Photo Film Co. Ltd., Tokyo, Japan).

Tolerance tests to herbicides

The herbicides AC, AT, CT, MT, MC and NR were each sprayed to potato plants cultivated in pots at the amount of 20μmol, 1.2μmol, 17.6μmol, 10μmol, 30μmol, 12μmol, respectively dissolved in water containing 0.02% Tween 20 and 0.015% spreader.

The herbicide PC was added to modified MS medium at 10μM, and transgenic plants were incubated for 23 days.

RESULTS

Selection of transgenic potato plants

Twenty-seven PCR-positive shoots were selected from thirty-five kanamycin-resistant shoots transformed with pIKBAC. Based on ECOD activity of leaf samples, two transgenic plants T1977 and T1979 with the highest ECOD activity were selected. T1978 with ECOD activity similar to the control level was also used as a control.

Four transgenic plants S1965 and S1966 transformed with pUHA1 and S1971 and S1972 with pUHB6 were selected on the base of ECOD activity in their leaves from ninety-one and eighty kanamycin-resistant shoots, respectively. On the other hand, S1967 for pUHA1 and S1973 for pUHB6 with the lowest ECOD activity in kanamycin-resistant shoots were also used for western blot analysis.

Seventy-five plants expressing CYP2C19 were selected on the base of western blot analysis from eighty-two kanamycin-resistant shoots. Of these, two transgenic plants S1974 and S1975 expressing the highest amount of CYP2C19 protein were selected. Also, S1976 without P450 protein was used as a control.

Southern, northern and western blot analyses

Southern blot analysis with three different probes specific for CYP1A1, CYP2B6 and CYP2C19 showed that T-DNA containing three P450 cDNAs was integrated into genomic DNA of T1977 plant(FIGURE 4-2). The predicted two 5.4kb-bands were detected in genomic DNA from T1977 probed with CYP1A1 and CYP2C19 fragments. Also, positive bands were detected with the CYP2B6 probe.

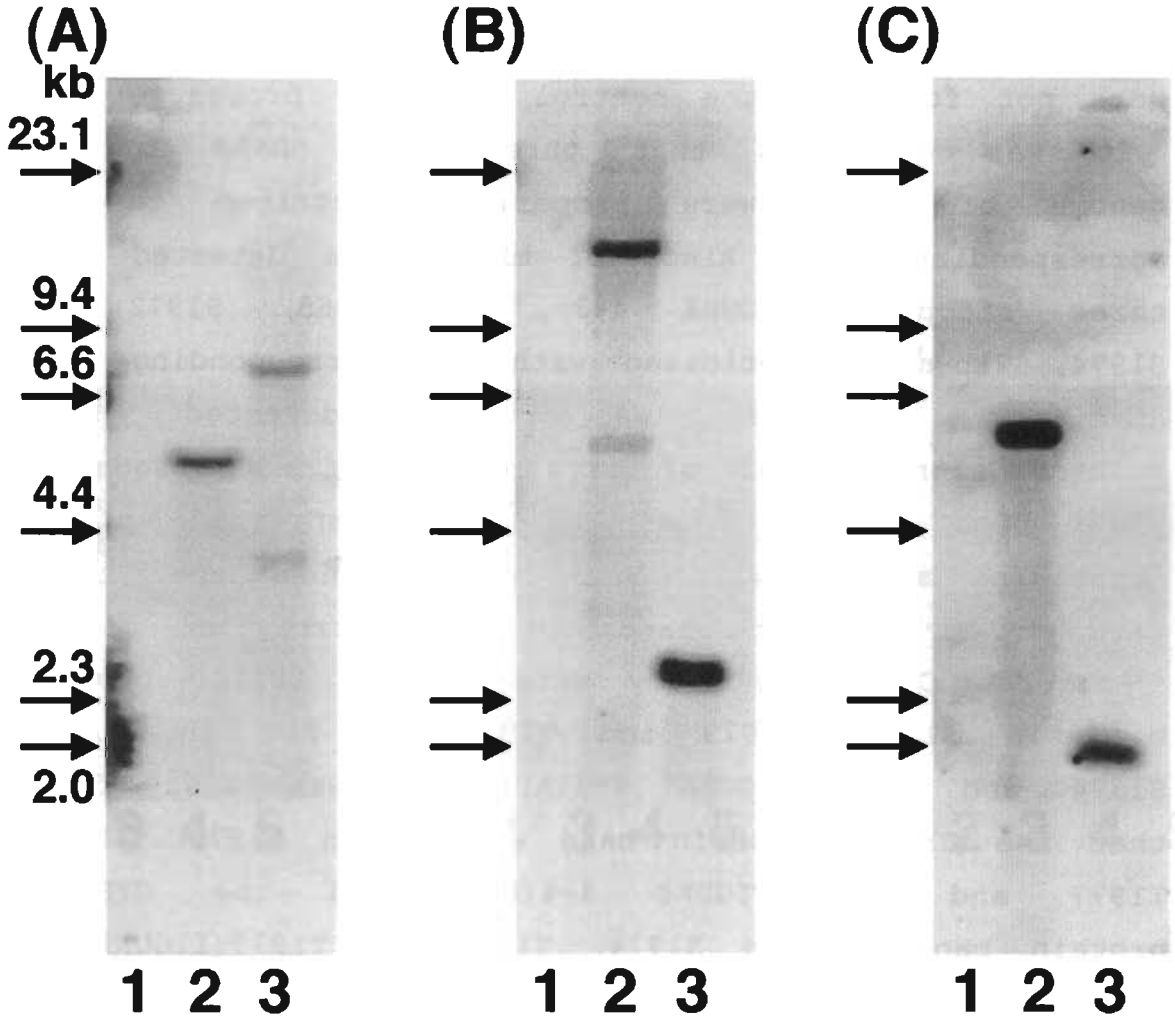

FIGURE 4-2 Southern blot analysis of the transgenic potato plants using human CYP1A1(A), CYP2B6(B) and CYP2C19 probes(C)
(A): Lane 1 to 3 show untransformed control, T1977 and S1965, respectively. (B): Lane 1 to 3 show untransformed control, T1977 and S1972, respectively. (C): Lane 1 to 3 show untransformed control, T1977 and S1974, respectively.

Furthermore, S1972 and S1974 showed 2.5kb- and 1.8kb-positive bands with the probes correspondent to parts of CYP2B6 and CYP2C19. It was also observed that S1965 contained a part of CYP1A1 cDNA in a genome. However, positive bands for integration of P450 cDNA were not found in the control with any probes.

It was confirmed that three P450 cDNAs in the genome of T1977 were properly transcribed to the corresponding three kinds of mRNA bands detected with three riboprobes(FIGURE 4-3). In S1965, S1972 and S1974, 2kb-mRNA hybridized with the corresponding P450 specific riboprobe was also detected. The transcriptional level of each of three mRNA bands in T1977 was higher than in the transgenic plants expressing single P450 cDNA. On the other hand, there were no corresponding bands in the control.

Anti-rat CYP1A1 antibody detected the CYP1A1 protein band in S1965, T1979 and T1977 but not in S1972, S1974 and T1978(FIGURE 4-4(A)). It was also found that the CYP2B6 protein band existed in S1972, T1979, T1977 and T1978(FIGURE 4-4(B)), and the CYP2C19 protein band was in S1974, T1979 and T1977(FIGURE 4-4(C)). In the control, there were no corresponding bands reactive with each anti-P450 antibody.

EROD activity

The standard substrate 7-ethoxyresorufin for CYP1A1 was incubated with the microsomal fraction prepared from the transgenic plants. The EROD activity of T1977 and S1965 expressing CYP1A1 were 2.92 and 5.78pmol/min/mg microsomal protein, respectively(TABLE 4-1). These values were 27 and 53 times higher than that of the control, respectively. The EROD activity

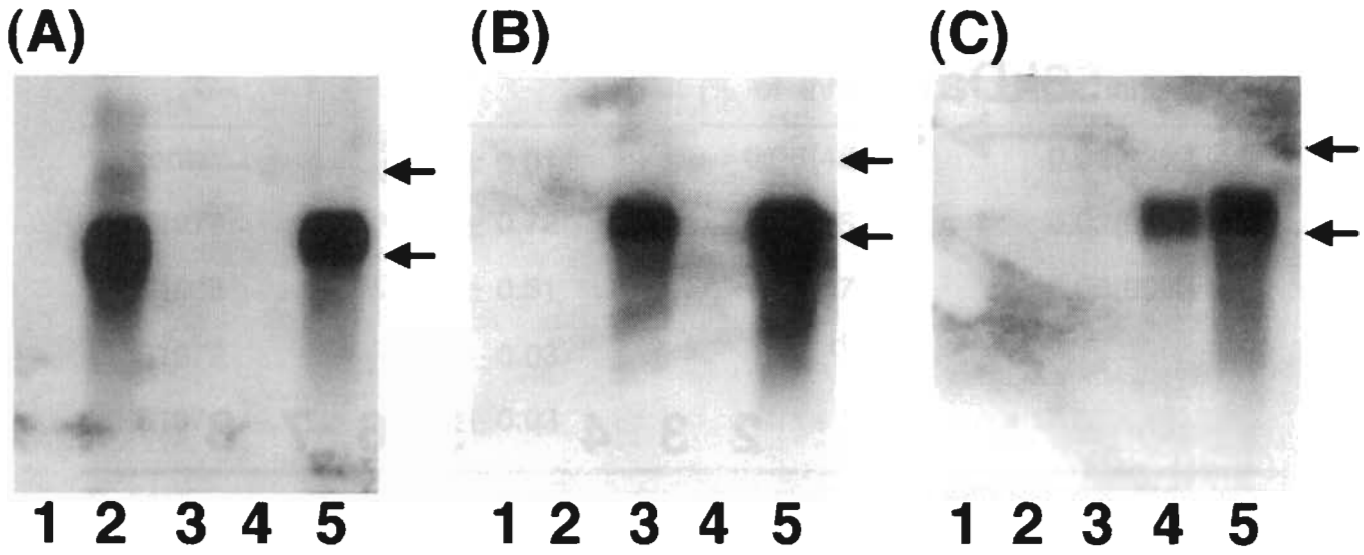

FIGURE 4-3 Northern blot analysis of the transgenic potato plants using human CYP1A1(A), CYP2B6(B) and CYP2C19 riboprobes(C)
Lane 1 to 5 show untransformed control, S1965, S1972, S1974 and T1977, respectively. Upper and lower arrows show 3.4kb and 1.8kb-length, respectively.

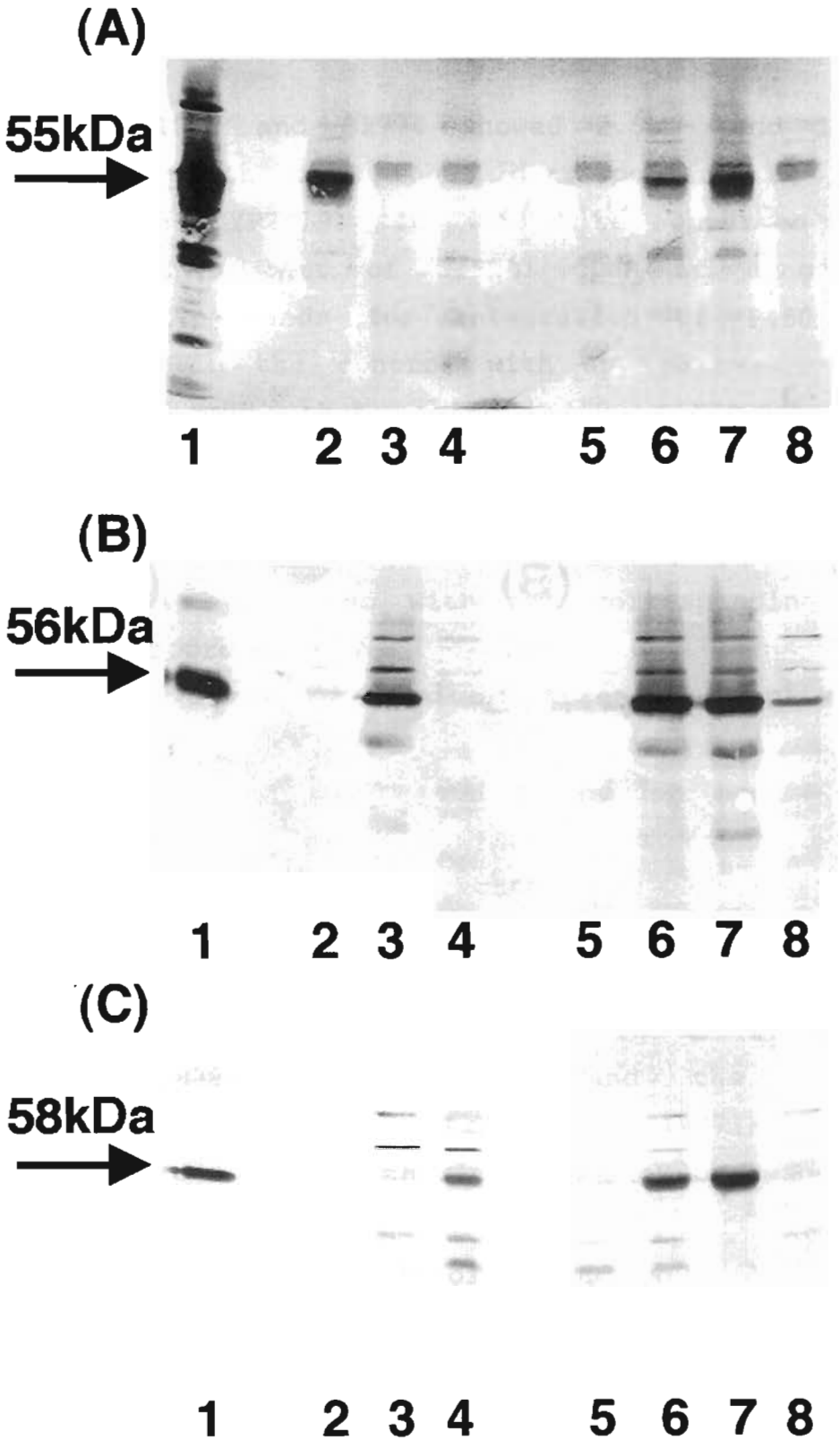

FIGURE 4-4 Western blot analysis of the transgenic potato plants using anti-rat CYP1A1(A), anti-human CYP2B6(B) and anti-human CYP2C9 antibodies (C)
Lane 1 to 8 show microsomal fractions expressing human CYP1A1(A), CYP2B6(B) or CYP2C19(C), S1965, S1972, S1974, untransformed control, T1979, T1977 and T1978, respectively.

TABLE 4-1 P450-dependent 7-ethoxyresolufin *O*-deethylase activity in the microsomal fraction from transgenic potato plants

transformant	EROD activity[a]	CO[b] (% of inhibition)	-NADPH[c] (% of inhibition)
control	0.11 ± 0.01	0.06(45%)	0.02(82%)
T1977	2.92 ± 0.72	0.69(76%)	0.02(99%)
S1965	5.78 ± 0.51	1.33(77%)	0.06(99%)
S1972	0.12 ± 0.03	-[d]	-
S1974	0.14 ± 0.03	-	-

[a] EROD activity, 7-Ethoxyresolufin *O*-deethylase activity(pmol/min/mg protein)
These values represent for the average of three independent experiments.

[b] The reaction mixtures were incubated with carbon monoxide.

[c] The reaction mixtures were incubated without NADPH.

[d] not determined

was strongly inhibited by the aeration of carbon monoxide and no addition of NADPH into the reaction mixtures. No EROD activity was found in S1972 and S1974.

Metabolism of herbicide chemicals in the yeast microsomes each expressing human CYP1A1, CYP2B6 and CYP2C19

In order to identify P450 species metabolizing herbicides, it was attempted to metabolize seven herbicide chemicals in the microsomal fraction of the recombinant yeast strains each expressing human CYP1A1, CYP2B6 and CYP2C19. It was revealed that human CYP1A1 and CYP2C19 metabolized the herbicide chemicals AT, CT, MT and NR. Furthermore, human CYP2C19 also metabolized AC, MC and PC. On the other hand, human CYP2B6 metabolized AC and MC(data not shown).

AT metabolism also showed that CYP2C19 metabolized AT through deisopropylation and deethylation as with human CYP1A1. Moreover, it was found that PC was metabolized by CYP2C19 to yield *m-tert*-butylphenol with P450-dependence(FIGURE 4-10).

Metabolism of [^{14}C]CT, [^{14}C]AT and [^{14}C]PC

The metabolites demethylated CT(DM), ring-methyl hydroxylated CT(OH), didemethylated CT(DDM), demethylated ring-methyl hydroxylated CT(DMOH), unknown metabolite 1(UK1), carboxylated CT(COOH) and unknown metabolite 2(UK2) were detected(TABLE 4-2). The amount of CT remained in T1977 and S1965 was much lower than the control in 12h. The partially phytotoxic metabolite DM in the control was increased time-dependently, but was decreased in T1977 and S1965.

TABLE 4-2 Metabolism of [^{14}C]-chlortoluron in the transgenic potato plants

CT/ metabolite [a]	metabolite produced(nmol/plant) [b]																								
	control(day)					T1977(day)					S1965(day)					S1972(day)					S1974(day)				
	0	0.5	1	2	8	0	0.5	1	2	8	0	0.5	1	2	8	0	0.5	1	2	8	0	0.5	1	2	8
CT	<0.1	8.5	9.9	12.1	18.0	<0.1	1.0	0.6	0.4	0.1	<0.1	1.3	1.1	0.7	0.2	<0.1	3.9	3.2	2.5	2.0	<0.1	5.1	4.6	4.2	3.1
DM	<0.1	0.6	1.4	4.4	11.1	<0.1	0.4	0.2	0.2	0.2	<0.1	0.4	0.5	0.3	0.2	<0.1	0.3	0.5	0.8	1.5	<0.1	0.7	1.1	1.6	2.2
OH	<0.1	0.2	0.3	0.8	0.8	<0.1	0.6	0.5	0.3	0.2	<0.1	0.8	1.1	0.6	0.4	<0.1	<0.1	0.1	0.2	0.2	<0.1	<0.1	0.1	0.2	0.2
DDM	-[c]	-	-	-	-	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DMOH	<0.1	<0.1	0.2	1.1	1.9	<0.1	0.6	0.4	0.3	0.3	<0.1	0.4	0.7	0.5	0.3	<0.1	<0.1	<0.1	0.1	0.5	<0.1	<0.1	<0.1	0.1	0.8
UK1	--	-	-	-	-	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	0.2	0.2	<0.1	<0.1	<0.1	<0.1	0.2	<0.1	<0.1	<0.1	<0.1	<0.1
COOH	<0.1	0.1	0.3	1.2	4.0	<0.1	0.7	0.9	1.1	2.1	<0.1	0.7	1.6	1.9	2.7	<0.1	<0.1	<0.1	0.1	1.1	<0.1	<0.1	0.1	0.1	0.5
UK2	<0.1	0.3	0.6	1.9	6.4	<0.1	1.2	1.6	2.3	5.0	<0.1	1.3	3.1	4.0	7.4	<0.1	0.1	0.3	0.5	4.5	<0.1	0.3	0.5	1.0	4.9

[a] CT, DM, OH, DDM, DMOH, UK1, COOH and UK2 indicate chlortoluron, demethylated chlortoluron, ring-methyl hydroxylated chlortoluron, didemethylated chlortoluron, demethylated ring-methyl hydroxylated chlortoluron, unknown metabolite 1, carboxylated chlortoluron and unknown metabolite 2, respectively.

[b] These values are the average of three independent experiments.

[c] -; not determined

The non-phytotoxic metabolites OH and DMOH were early accumulated and then decreased in T1977 and S1965, but not in the control. In S1972 and S1974, CT was more rapidly metabolized than in the control.

The amount of AT remained was 3.0, 0.7 and 1.0nmol/plant 8 days after incubation in the control, T1977 and S1965, respectively(TABLE 4-3). The non-phytotoxic metabolite deisopropylated deethylated AT(DIDE) was produced 6 times in T1977 and 5 times in S1965 higher than the control. The S1972 did not show any enhancement in AT metabolism.

[^{14}C]PC applied was extracted from both medium and plants(FIGURE 4-5). In medium, PC was metabolized to the level of 334.8pmol/8days/plant in the control, whereas it was hard to detect the parent compound in T1977(TABLE 4-4). The S1974 also metabolized much more rapidly PC than the control. On the other hand, UK3 was accumulated in the control as compared with other transgenic plants. In plants, T1977 did not accumulate PC, but did in the control. The metabolite *m-tert*-butylphenol(BP) was detected in T1977 and the other transformants. However, no BP was found in the control. In both medium and plants, it was observed that the largest amount of UK5 was in T1977.

Herbicide-tolerance tests

The strong tolerance toward AT, CT and MT was observed with T1977 but not with the control(FIGURE 4-6). The S1965 plant was also tolerant to CT and MT, and the S1974 plant was weakly to AT. The transgenic plants T1977, S1972 and S1974 clearly showed normal phenotype to each spraying of the herbicide AC and MC(FIGURE 4-7). However, it was observed in the

TABLE 4-3 Metabolism of [^{14}C]-atrazine in the transgenic potato plants

AT/ metabolite[a]	metabolite produced(nmol/plant)[b]																								
	control(day)					T1977(day)					S1965(day)					S1972(day)					S1974(day)				
	0	0.5	1	2	8	0	0.5	1	2	8	0	0.5	1	2	8	0	0.5	1	2	8	0	0.5	1	2	8
AT	<0.1	1.1	1.0	1.5	3.0	<0.1	1.2	1.1	1.2	0.7	<0.1	1.2	1.8	1.3	1.0	<0.1	1.9	2.3	2.1	2.1	<0.1	2.0	1.7	1.9	1.7
UK1	-[c]	-	-	-	-	<0.1	<0.1	<0.1	0.1	0.1	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	0.1	0.1
DI	<0.1	<0.1	0.1	0.1	0.8	<0.1	0.1	0.2	0.3	0.5	<0.1	0.1	0.2	0.3	0.5	<0.1	0.1	0.1	0.2	0.7	<0.1	0.1	0.1	0.3	0.8
UK2	<0.1	<0.1	<0.1	0.1	0.6	<0.1	0.2	0.3	0.6	0.5	<0.1	0.2	0.3	0.4	0.4	<0.1	0.1	0.1	0.1	0.4	<0.1	0.1	0.1	0.3	0.5
DIDE	<0.1	<0.1	<0.1	<0.1	0.2	<0.1	0.1	0.1	0.3	1.1	<0.1	<0.1	0.1	0.2	0.9	<0.1	<0.1	0.1	0.1	0.2	<0.1	0.1	0.1	0.1	0.3
UK3	<0.1	0.1	0.1	0.2	0.8	<0.1	0.2	0.3	0.5	1.7	<0.1	0.1	0.3	0.3	1.2	<0.1	0.1	0.2	0.3	0.7	<0.1	0.2	0.2	0.3	1.0

[a] AT, UK1, DI, UK2, DIDE and UK3 indicate atrazine, unknown metabolite 1, deisopropylated atrazine, unknown metabolite 2, deisopropylated deethylated atrazine and unknown metabolite 3, respectively.

[b] These values are the average of three independent experiments.

[c] -; not determined

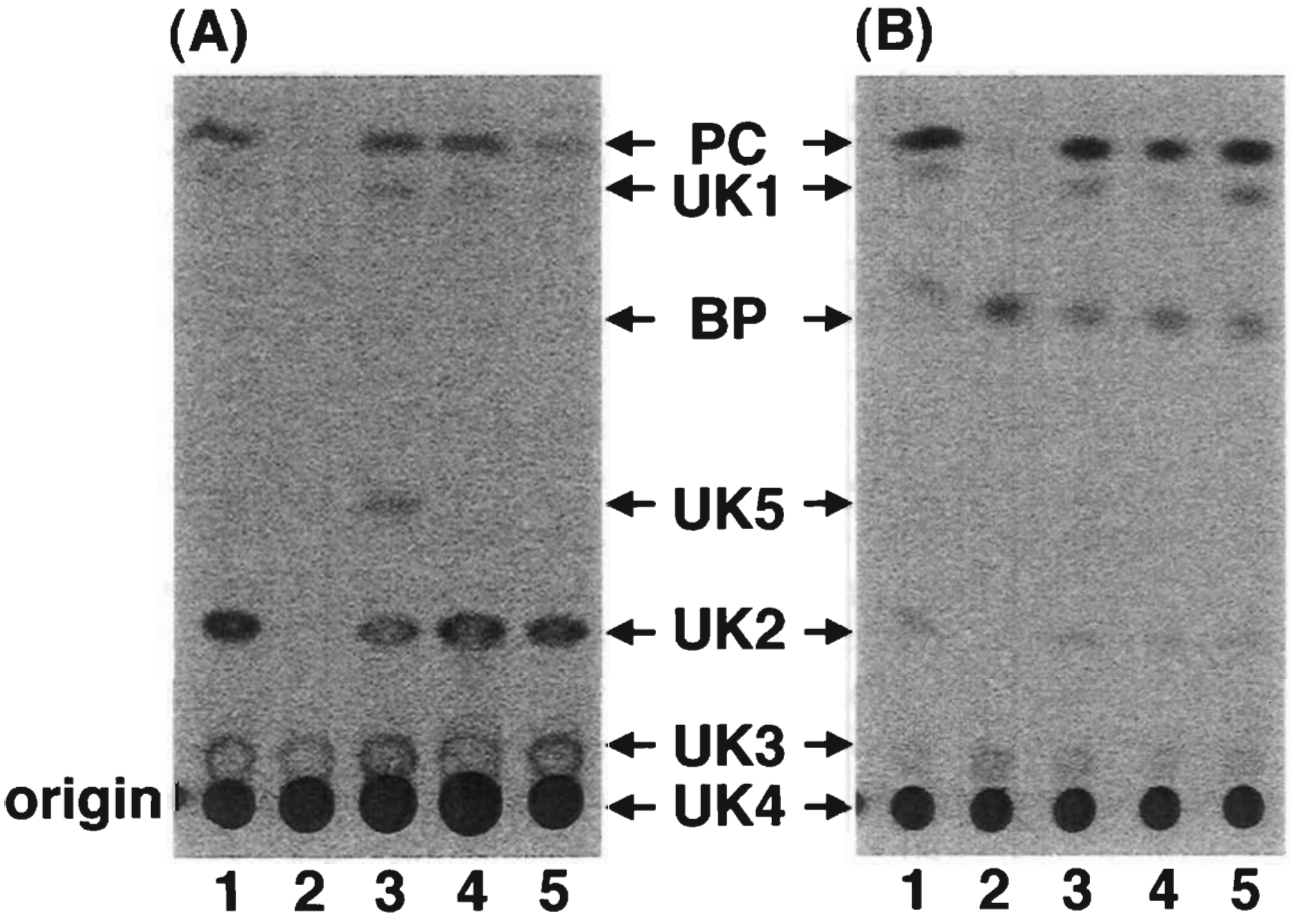

FIGURE 4-5 Metabolism of [^{14}C]pyributicarb in the transgenic potato plants

[^{14}C]Pyributicarb and its metabolites were extracted from medium(A) and plants(B) incubated for 8 days. PC, BP, UK1 to 5 represent for pyributicarb, *m-tert*-buthylphenol, unknown metabolites 1 to 5, respectively.

TABLE 4-4 Metabolism of [^{14}C]-pyributicarb in the transgenic potato plants

PC/ metabolite[a]	metabolite produced(pmol/8days/plant)[b]									
	medium					plant				
	control	T1977	S1965	S1972	S1974	control	T1977	S1965	S1972	S1974
PC	334.8	<0.1	96.8	91.3	57.5	2751.5	<0.1	1587.5	1439.1	1651.7
UK1	13.7	<0.1	7.6	<0.1	14.3	13.5	<0.1	82.3	<0.1	131.2
BP	<0.1	<0.1	2.7	0.2	1.2	24.7	384.7	75.2	229.6	253.9
UK2	<0.1	<0.1	58.7	<0.1	<0.1	-[c]	-	-	-	-
UK3	479.4	29.5	297.2	303.9	371.2	5.3	<0.1	<0.1	<0.1	<0.1
UK4	141.3	238.5	324.8	111.1	327.2	<0.1	51.1	7.9	<0.1	<0.1
UK5	1899.7	3038.7	3321.9	2408.6	2726.8	7623.4	11316.8	8111.9	12363.8	10450.8

[a] PC, UK1, BP, UK2, UK3, UK4 and UK5 indicate pyributicarb, unknown metabolite 1, *m-tert*-buthylphenol, unknown metabolite 2, unknown metabolite 3, unknown metabolite 4 and unknown metabolite 5, respectively.

[b] These values are the average of three independent experiments.

[c] -; not determined

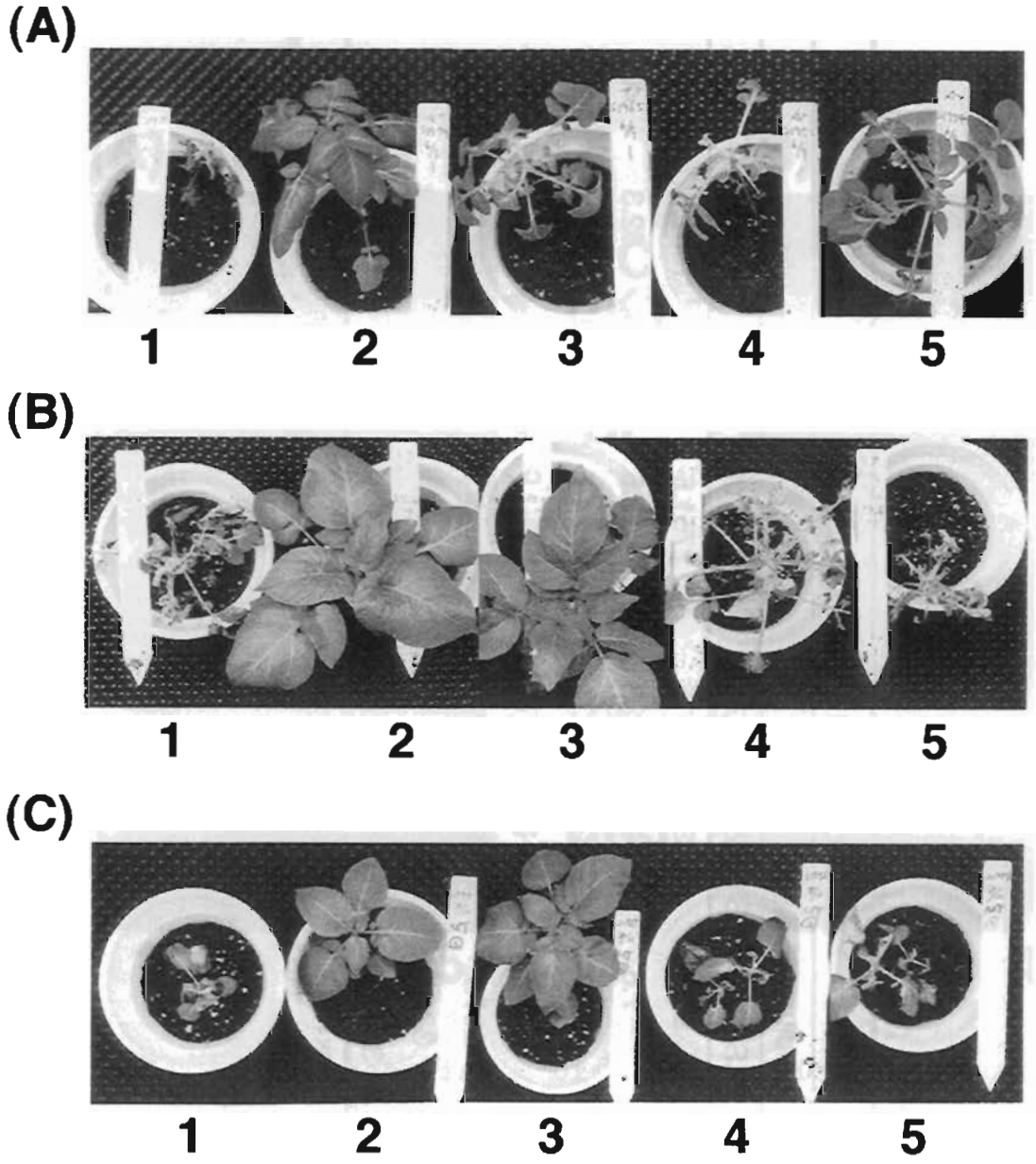

FIGURE 4-6 Herbicide-tolerance toward photosynthesis-inhibiting herbicides in the transgenic potato plants Lane 1 to 5 show the control, T1977, S1965, S1972, S1974, respectively. (A): The transgenic plants were sprayed with the herbicide atrazine at 1.2µmol/pot and observed after 11 days. (B): The transgenic plants were sprayed with the herbicide chlortoluron at 17.6µmol/pot and observed after 10 days. (C): The transgenic plants were sprayed with the herbicide methabenzthiazuron at 10µmol/pot and observed after 8 days.

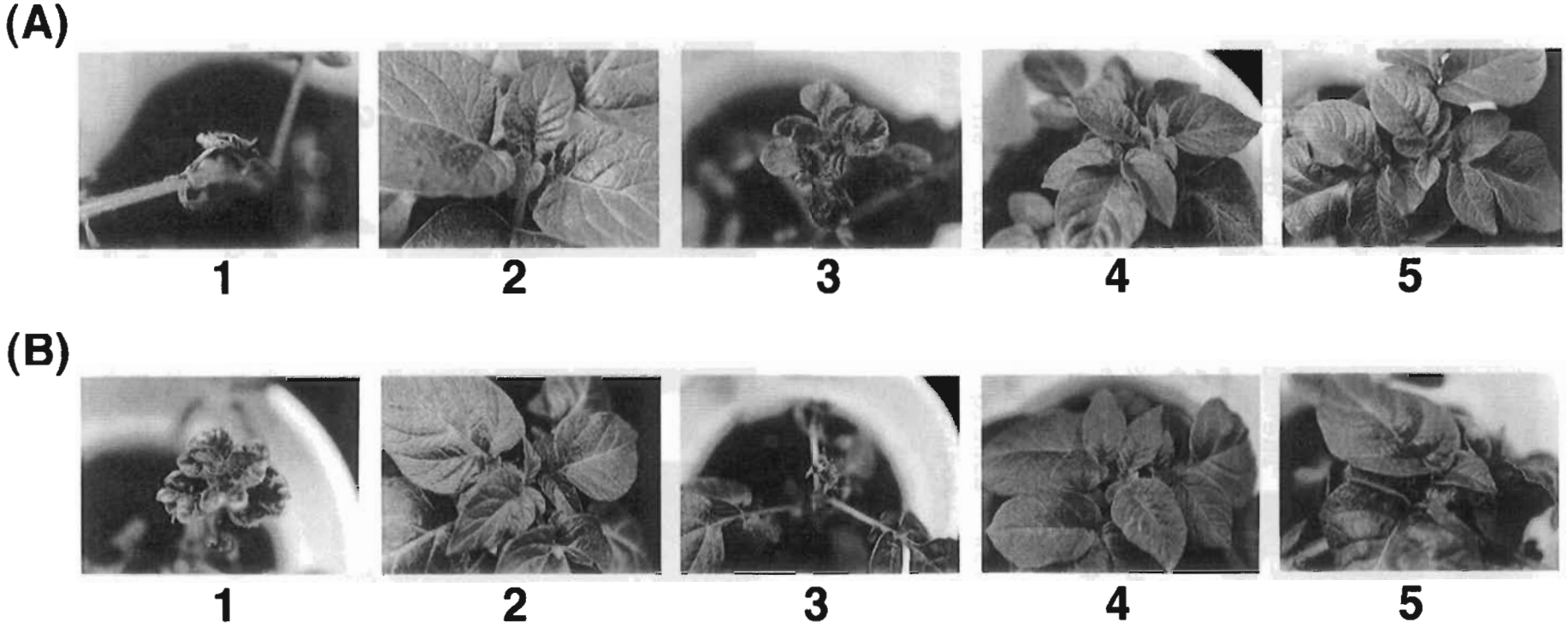

FIGURE 4-7 Herbicide-tolerance toward lipid and protein synthesis-inhibiting herbicides in the transgenic potato plants
Lane 1 to 5 show the control, T1977, S1965, S1972, S1974, respectively. (A): The transgenic plants were sprayed with the herbicide acetochlor at 20μmol/pot and observed after 15 days. (B): The transgenic plants were sprayed with the herbicide metolachlor at 30μmol/pot and observed after 15 days.

control and S1965 that apical buds were withered and showed abnormal phenotypes in AC and MC, respectively. Furthermore, T1977 and S1965 were greatly tolerant toward NR, although the apical parts of the control and other transgenic plants were completely bleached(FIGURE 4-8). The treatment of the herbicide PC showed that the root elongation and growth were observed only in the transgenic plant T1977, although the control, S1972 and S1974 did not show any tolerance(FIGURE 4-9).

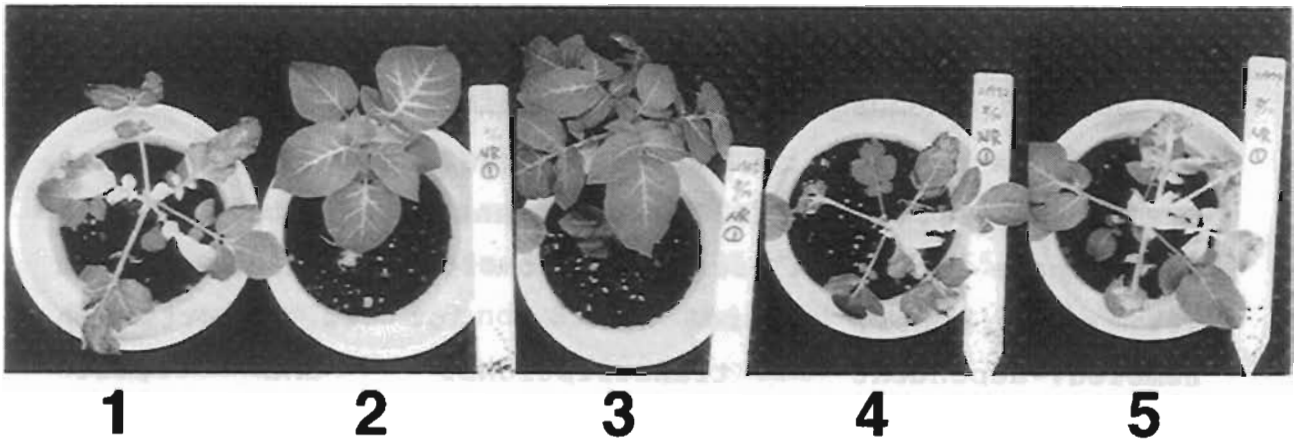

FIGURE 4-8 Herbicide-tolerance toward carotenoid biosynthesis-inhibiting herbicide norflurazon in the transgenic potato plants
Lane 1 to 5 show the control, T1977, S1965, S1972, S1974, respectively. The transgenic plants were sprayed with the herbicide norflurazon at 12μmol/pot and observed after 8 days.

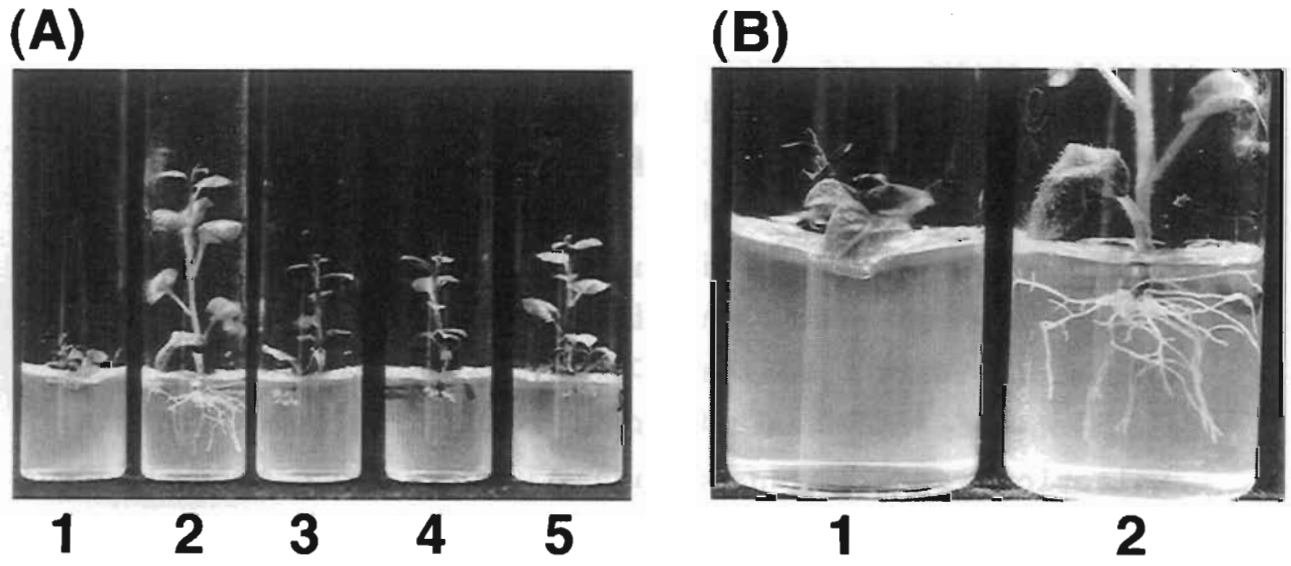

FIGURE 4-9 Herbicide-tolerance toward lipid biosynthesis-inhibiting herbicide pyributicarb in the transgenic potato plants
Lane 1 to 5 show the control, T1977, S1965, S1972, S1974, respectively. The transgenic plants were incubated with the herbicide pyributicarb at 10μM and observed after 23 days.

DISCUSSION

It was attempted to express three human P450 species, CYP1A1, CYP2B6 and CYP2C19 in transgenic potato plants. These have low DNA sequence homology with less than 40% each other. There were a lot of reports on homology-dependent transcriptional and post-transcriptional gene silencings(116). In addition, these three P450s showed high drug-metabolizing activity and relatively broad substrate-specificity. Thus, simultaneous expression of these P450 species seemed to metabolize a number of xenobiotics including herbicides.

It has been reported that a long intact T-DNA with LB-part was not always integrated into a genome(117). The plasmid pIKBAC with human CYP1A1, CYP2B6 and CYP2C19 cDNAs was 12kb-length in its T-DNA region. Thus, the T-DNA integrated into the plant genome might be truncated in LB-part including CYP2C19 cDNA. If so, it seemed to be rather difficult to select transformants having untruncated T-DNA. Consequently, in the present study the construction with the NPT II expression unit besides LB may be suitable for selection of transgenic plants containing intact T-DNA.

Selection of transformants based on the ECOD activity of leaf samples was simple and efficient to screen many regenerated shoots. Furthermore, it was confirmed that there was a correlation between ECOD activity of leaves and P450 protein level, since T1977 and T1979 showed higher accumulation of three P450 proteins than T1978 with a low ECOD activity.

The microsomal fractions from T1977 and S1965 showed a typical drug-metabolizing activity of CYP1A1 towards

7-ethoxyresorufin through *O*-deethylation. This activity was confirmed to be P450-dependent because of its inhibition with CO gas and in the absence of NADPH. These results revealed that human CYP1A1 was functionally expressed in T1977 and S1965. On the other hand, S1972 and S1974 had no activity of EROD, because CYP2B6 and CYP2C19 can not essentially metabolize 7-ethoxyresorufin.

The fact that T1977 expressing CYP1A1, CYP2B6 and CYP2C19 metabolized a more amount of AT and produced a large amount of DIDE 8 days after treated than S1965 expressing CYP1A1 suggested that CYP1A1 seemed to cooperatively work with CYP2C19 toward metabolism of AT. Because EROD activity of S1965 was 2 times higher than T1977. It strongly supported that a large amount of AT was metabolized by the combination of CYP1A1 and/or CYP2C19 in T1977. The CYP2C19 expressed in S1974 may not be fully functional for metabolism of AT, since CYP2C19 metabolized 2 times more than CYP1A1 expressed in the recombinant yeast microsomes(data not shown). Thus, it was suggested that both CYP1A1 and CYP2C19 cooperatively metabolized AT. Metabolism of [^{14}C]CT also seemed to be cooperatively carried out by both CYP1A1 and CYP2C19 in the transgenic plants.

[^{14}C]PC metabolism revealed that PC was completely metabolized by T1977, and some of PC were converted to BP which was an intermediate metabolite. Drastic decrease of PC in T1977 seemed to contribute to tolerance toward PC sprayed. PC and BP were found in the plants, whereas UK3 and UK4 were in medium, suggested that UK3 and UK4 were more water-soluble than PC and BP. The metabolite UK5 at the origin of

the TLC plate was found in both medium and plants. These highly hydrophilic metabolites may be presented in medium as well as aqueous fractions such as vacuoles in plants. The accumulation of UK3 in the control also seemed to be related to susceptibility to PC. Orally dose of PC to rat revealed that the major metabolite was BP in a liver(118). On the other hand, in the tolerant plant rice PC was mainly metabolized through conjugation of BP with glucose, glucose-xylose and glyoxalylglucose(119). Furthermore, human CYP2C19 sequentially metabolized PC to BP and BP to the other two unknown metabolites. These processes were confirmed by the addition of BP to the microsomal fractions expressing CYP2C19(data not shown). Thus, it was suggested that BP produced by CYP2C19 was further conjugated with sugar by endogenous conjugation enzymes and some of the unknown metabolites found in the present study may be conjugated with sugar(FIGURE 4-10).

The photosynthesis-inhibiting herbicides AT, CT and MT were sprayed to the transgenic plants grown in pots. The strongest tolerance toward AT and CT in T1977 were well met with the results of metabolism of [^{14}C]chemicals. No tolerance toward CT and MT was observed in S1974 expressing CYP2C19, which metabolized these two herbicides in the yeast microsomes. These results suggested that CYP2C19 metabolized a less amount of CT and MT than CYP1A1, and the dose level seemed to be too high for the ability of S1974 to metabolize them. In fact, AT was more rapidly metabolized by CYP2C19 than by CYP1A1 in the yeast microsomes. CT and MT were more rapidly metabolized by CYP1A1 than CYP2C19(data not shown). These

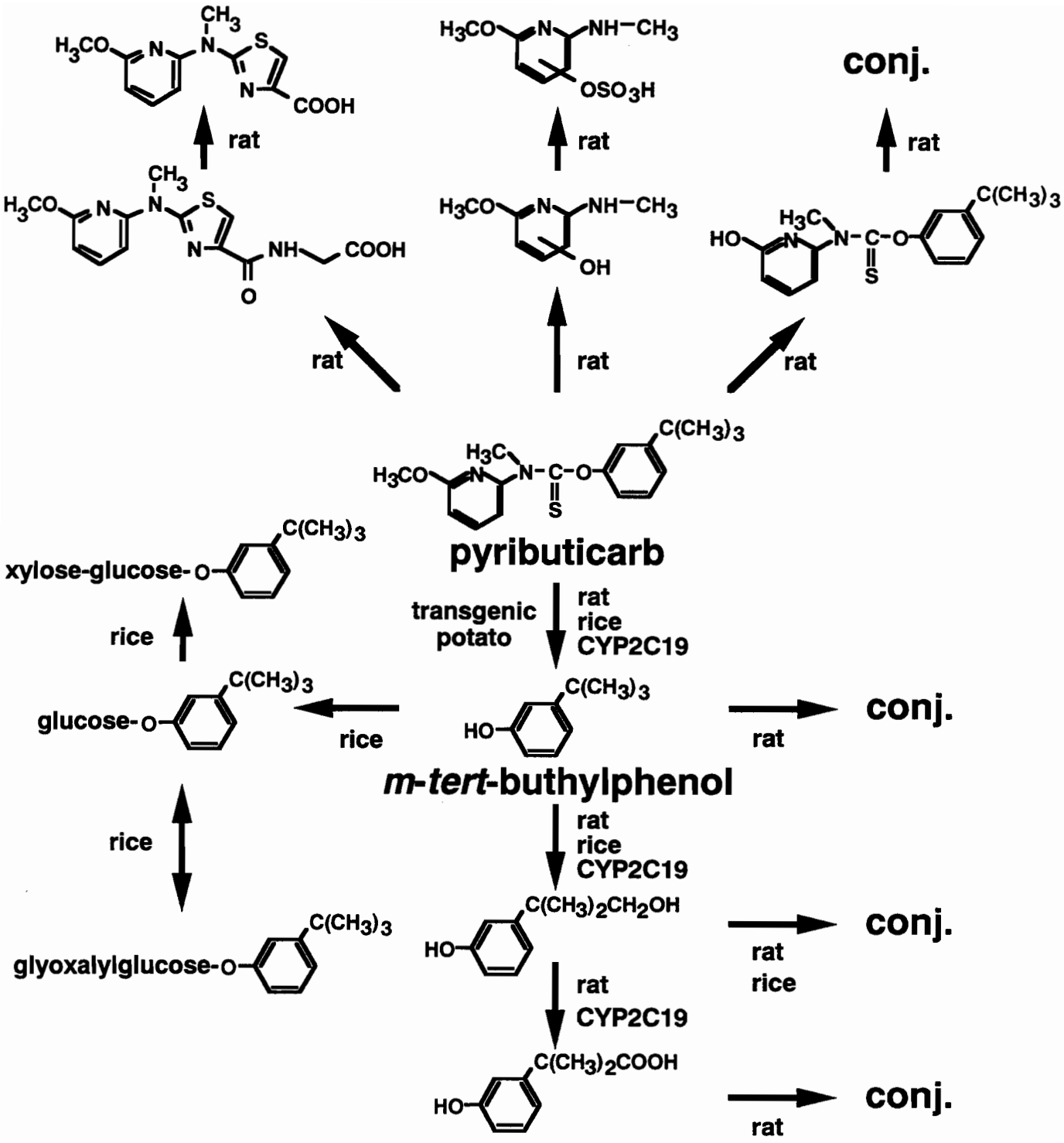

FIGURE 4-10 Proposed metabolic pathways for the herbicide pyributicarb in rat, rice, yeast microsomes expressing human CYP2C19 and transgenic potato plants
The rat and rice show references for Tsuzuki *et al.*(1991, 1993). The CYP2C19 and transgenic potato show references for a personal communication and the present study, respectively.

suggested that the metabolic capability of both CYP1A1 and CYP2C19 toward these herbicides well correlated with tolerance of S1965 and S1974 to the herbicides. The phenylurea herbicide CT was metabolized through ring-methyl hydroxylation and *N*-demethylation. However, MT was mainly metabolized in plants through side-chain methyl hydroxylation, its glucoside and *N*-demethylation, whereas in rat a combination of side-chain methyl and ring hydroxylation followed by sulfate ester conjugation was occurred(120).

It was reported that the microsomal membranes isolated from maize treated with ethanol metabolized about sixteen times larger amount of MC than the microsomes without treatment(121). Furthermore, eight ethanol-inducible CYP genes were cloned from ethanol treated maize. These were CYP71C5, CYP73A6, CYP73A7, CYP73A8, CYP81A1, CYP81A2, CYP81A3 and CYP81A4. These results suggested that MC metabolism in maize may be catalyzed by some of these P450 species. Moreland *et al.* also reported that the microsomal fraction from sorghum catalyzed P450-dependent *O*-demethylation of MC(63). However, in chloroacetanilide metabolisms, it was known that GSH conjugation by GST was a major pathway. Tolerant species maize and soybean metabolized AC through GSH and homo-GSH conjugations, respectively(122). In contrast, the susceptible species wheat metabolized AC to a lesser extent than the tolerant species, suggesting that MC selectivity was related to a degree of GSH conjugation in plant species(123). These results were supported by the report that safeners reduced injury in corn from the treatment of chloroacetanilide herbicides, because of the induction of GST and P450 in corn with the

safeners(124). On the other hand, cytosolic enzymes from rat liver converted MC to MC-GSH conjugation in the presence of GSH(125). Microsomal enzymes fortified with NADPH catalyzed *O*-demethylation, benzylic hydroxylation and *N*-dealkylation of MC. These findings suggested that the expression of human P450 species metabolizing chloroacetanilides, which inhibits lipid and protein synthesis, in the transgenic potato plants gives rise to tolerance toward these herbicides. *In vitro* metabolism of AC and MC with the recombinant yeast microsomes expressing CYP1A1, CYP2B6 and CYP2C19 exhibited that CYP2B6 and CYP2C19 metabolized these two herbicides. These results suggested that the transgenic potato plants expressing these P450 species show tolerance toward AC and MC. The present study described that T1977, S1972 and S1974 expressing CYP2B6 and/or CYP2C19 were highly tolerant to these herbicides. Moreover, a dose level of these herbicides to the plants was average in the field. On the other hand, AC and MC were known as contaminants for ground water(126). These transgenic potato plants may be useful for degradation of these contaminants, because these have a potential for absorption and metabolism of AC and MC. It seemed to be significant for the transgenic plants to metabolize MC, since MC is a suspicious environmental endocrine disruptor as with the triazine herbicide AT(127).

NR, which reduces carotenoid biosynthesis by inhibiting phytoene desaturase and fatty acid desaturation, has been known to metabolize through *N*-demethylation in both plants(128) and rat(129,130). The tolerance to NR might be added to the transgenic plants by the expression of CYP1A1 and/or CYP2C19,

since in the recombinant yeast microsomes NR was metabolized to *N*-demethylated as confirmed by LC/MS. However, we did not find any tolerance to NR in S1974, although the tolerance was observed in T1977 and S1965, suggesting that CYP2C19 may not be fully functional in S1974. Another approach to give a tolerance was the expression of phytoene desaturase gene *crtI* from a phytopathogenic bacterium *Erwinia uredovora* in the transgenic tobacco plants(16). These plants showed cross-tolerance toward several breaching herbicides including NR(131).

It was noteworthy that T1977 was a unique plant tolerant to PC, which interferes a squalene epoxidase resulting in the inhibition of elongation of roots in susceptible plants including corn, but not in tolerant plants including soybean(132,133). Both T1977 and S1974 expressing CYP2C19 were expected to show tolerance to PC, since PC was found to be metabolized by CYP2C19 expressed in the yeast microsomes. However, only T1977 exhibited strong tolerance toward PC. The reason why S1974 was not tolerant to PC seemed to be incomplete function of CYP2C19 in the transgenic plants. On the other hand, in T1977 CYP2C19 and the other P450s were cooperatively working for metabolism of PC. Namely the metabolites of PC derived from CYP2C19 may be sequentially metabolized by CYP1A1 and/or CYP2B6.

CHAPTER 5
CONCLUDING REMARKS

Cytochrome P450 monooxygenases metabolizing xenobiotics in mammalian livers were screened for P450 species CYP1A1, CYP2B6 and CYP2C19 metabolizing herbicides and then these selected P450 species were expressed in the transgenic potato plants. The transgenic plants expressing each of the selected P450 species and simultaneously expressing three P450 species clearly showed tolerance towards the herbicides with different structures and modes of herbicidal action which were metabolized by the corresponding P450 species expressed. This transgenic plant technology seems to be useful for engineering of low pesticide-residual crops, because the P450 species expressed metabolized not only the herbicides but also the other pesticides including insecticides and fungicides used on crops. The metabolites derived from the pesticides in these plants may be safer for human health, since the toxicity of the metabolites produced by mammalian P450 species were already tested with experimental animals. There is, however, a species difference in the metabolism of pesticides between human and experimental animals including the rat and mouse. It means that quality and quantity of the metabolites produced between human and experimental animals may be different. This problem may be also dissolved by the use of these transgenic plants expressing human P450 species. Namely, the transgenic plants expressing human P450 species seemed to be useful as a model system for human metabolism of pesticides. Expression of three P450 species in the transgenic potato plants is suitable for construction of a 'green liver' conceptualized by Sandermann as well as human metabolic system(134). Moreover, the

transgenic potato plants may be more reliable as a human metabolism system, when eleven human P450 species which involved in more than 90% of xenobiotic metabolism in human liver(76) were expressed as shown by the transgenic plants expressing human CYP1A1, CYP2B6 and CYP2C19 simultaneously.

The transgenic crops expressing useful traits such as herbicide-resistance, retardation of fruit softening and insect-resistance have been in the market since 1994. However, it is still under discussions that the transfer of transgenes into wild or weedy relatives may be occurred(135,136). There are many approaches to open a new avenue, which is the transformation of the chloroplast genome. This transgenic plant does not transfer their transgenes through pollen(137). For generating marker-free transgenic plants, isopentenyl transferase gene for MAT vector system(138), the bacteriophage P1 Cre/lox recombination system(139) and co-transformation with two Agrobacterium strains, each of them harboring two different plasmids(140) have been developed. On the other hand, human P450 species may be not only xenobiotic-metabolizing enzymes but also a selectable marker with herbicides metabolized by the corresponding P450 species. These trials should have public concerns over the field release of transgenic crops, because no transgenes from bacteria are contained in plant genomes.

Recently, it caused public discussion that some contaminants such as the organochlorines dibenzo-*p*-dioxins, dibenzofurans and coplanar polychlorinated biphenyls(PCBs) were distributed into the environment. It was also reported that the activities of P450 and GST in the hepatopancreases of freshwater crabs from

river were induced by organochlorine contaminants and there were correlation between the amount of contaminants and the enzyme activities induced(141). It means that environmental contaminants induced P450 and GST genes, suggesting that promoters and their regulatory regions of P450 and GST genes are useful for a biomarker, because these promoters are activated under the existence of such chemicals and consequently P450 enzyme induced can metabolize them for phytoremediation(113).

Contamination of environmental endocrine disruptors in the ground, water and atmosphere is a serious problem at the present time. There are many approaches to detoxify them. For example, *bphABC* genes from rhizobial strain was found to catabolize PCBs(142) and dioxin-like compounds were attacked efficiently by fungal and bacterial oxidases and dioxidases(143). Furthermore, plant P450 species CYP71B1 and P450 reductase were immobilized as a route to bioremediation and biocatalysis and this bioreacter catalyzed *N*-demethylation of CT(71). On the other hand, these transgenic potato plants were also able to metabolize environmental endocrine disruptors such as AT and MC. The transgenic plants may accumulate and metabolize them simultaneously.

Also, the expression of P450 species in transgenic plants seemed to reveal molecular mechanisms of herbicide selectivity and resistance(114), because P450 and GST are closely implicated. The enhancement of herbicide metabolism due to the expression of P450 or GST in plants may contribute to higher selectivity and resistance. On the other hand, the herbicides such as sulfonylureas which kill weeds at low rates

have been recently developed to provide herbicide selectivity and to consider human health as well as the environment.

Finally, it is said that these transgenic potato plants expressing mammalian P450 species have a great impact on phytoremediation of the environment as well as generation of herbicide-resistant crop and a model system for human metabolism of pesticides(FIGURE 5-1).

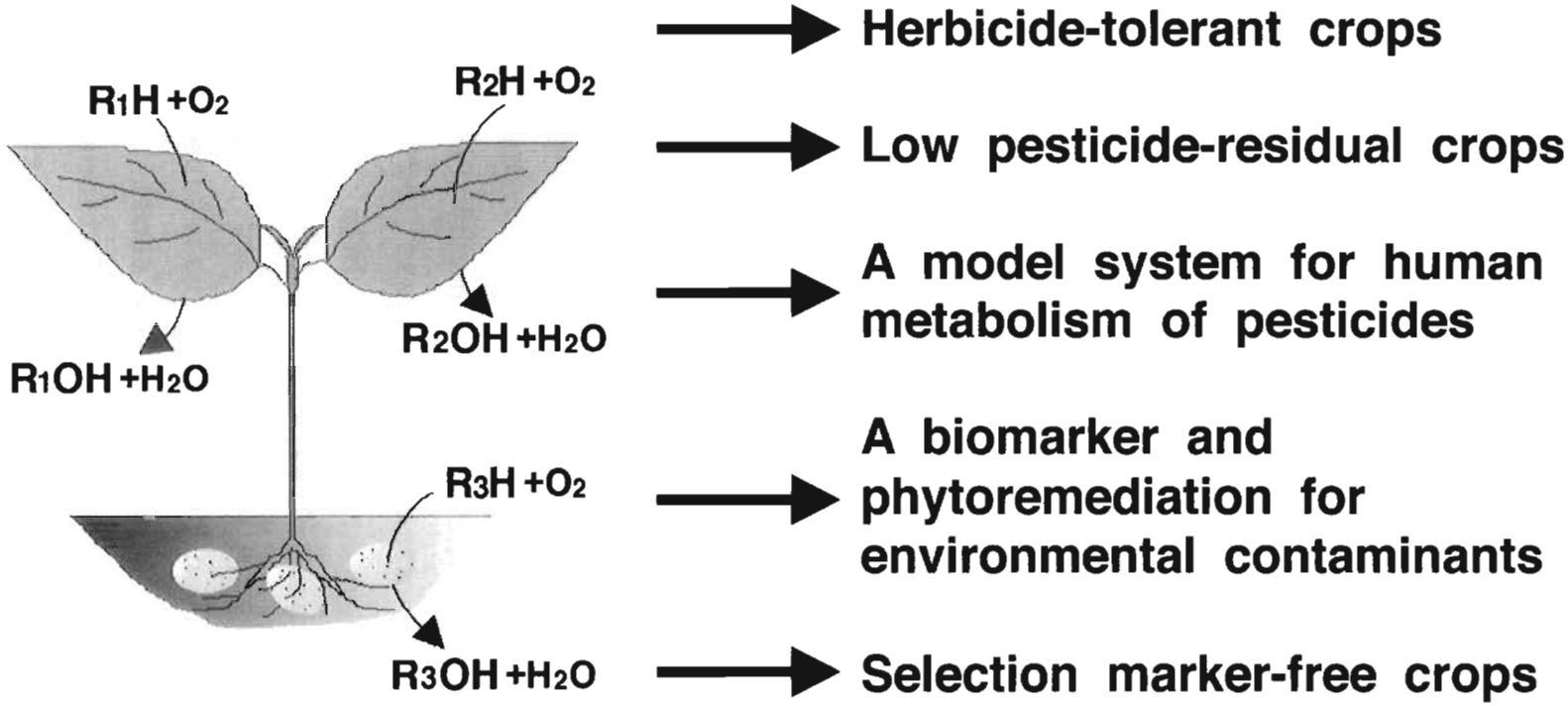

FIGURE 5-1 Prospect of the transgenic potato plants expressing cytochrome P450 monooxygenases metabolizing xenobiotics

SUMMARY

The transgenic potato plants expressing rat CYP1A1 and CYP1A1/YR fused enzyme were generated by Agrobacterium-transformation system. The S1160 and F1167 plants expressing rat CYP1A1 and the fused enzyme, respectively, selected showed two times higher 7-ethoxycoumarin *O*-deethylase(ECOD) activity with microsomes than the control. [^{14}C]CT metabolism revealed that S1160 metabolized CT via *N*-demethylation and ring methyl-hydroxylation more rapidly than the control. It was observed that S1160 was highly tolerant to the herbicides CT and DCMU, whereas F1160 did not show tolerance to CT. These results indicated that the transgenic potato plants expressing rat CYP1A1 metabolized the phenylurea herbicides and exhibited the herbicide-tolerance.

It was also attempted to express human CYP1A1 and CYP1A1/YR fused enzyme in transgenic potato plants. After transformation of potato microtubers with each of the constructed expression plasmids, S1384(expression of CYP1A1 with the vector pNG01), F1386(expression of CYP1A1/YR with the vector pNG01) and F1515(expression of CYP1A1/YR with the vector pUTR121H) were selected. The P450-dependent monooxygenase activity of the transgenic plants S1384, S1386 and F1515 was 3.5, 4.2 and 3.8 times higher in 7-ethoxycoumarin *O*-deethylation *in vitro*, and 6.4, 5.8 and 5.3 times higher in [^{14}C]CT metabolism *in vivo* than those of the control plants, respectively. In the metabolism of [^{14}C]AT, the deisopropylated deethylated metabolite DIDE, which is non-phytotoxic, was produced to a higher extent in S1384 and F1515 as compared with the control. The clear tolerance toward the herbicides CT, AT and PM was found in

S1384. These results suggested that a single expression of human CYP1A1 in the transgenic potato plants brought tolerance towards the herbicides with different structures and modes of herbicidal action.

The co-expression of human CYP1A1, CYP2B6 and CYP2C19 in transgenic potato plants was attempted. The transgenic plants T1977(CYP1A1, CYP2B6 and CYP2C19), S1965(CYP1A1), S1972(CYP2B6) and S1974(CYP2C19) were selected by the combination of kanamycin-resistance, PCR, ECOD activity and western blot analysis. It was found that both T1977 and S1965 expressing human CYP1A1 showed higher activities in the metabolism of [^{14}C]CT- and [^{14}C]AT than the control. In T1977, [^{14}C]PC was almost metabolized to *m-tert*-buthylphenol and unknown metabolites. The remarkable tolerance toward the photosynthesis-inhibiting herbicides AT, CT and MT, and the lipid biosynthesis-inhibiting herbicides AC and MC, the carotenoid biosynthesis-inhibiting herbicide NR was found in T1977, whereas the control did not show any tolerance. The S1965, S1972 and S1974 showed tolerance toward AT, CT, MT and NR, AC and MC, and AT, AC and MC, respectively. However, T1977 was a unique plant which showed high tolerance toward PC that inhibits root-elongation. These results indicated that T1977 expressing CYP1A1, CYP2B6 and CYP2C19 was tolerant towards all herbicides which were metabolized by these three P450 species.

The present technology opens a new avenue to approach to the novel field of breeding of herbicide-tolerant crops as well as phytoremediation for environmental contaminants.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Professor Dr. Hideo Ohkawa, Associate Professor Drs. Masanobu Nakata and Hiromasa Imaishi of Kobe University, and Dr. Noriaki Shiota for their guidance, precious advice and continuous encouragement during the course of this study.

I would like to express my gratitude to Dr. Teruo Ishige, Dr. Yasunobu Ohkawa and other researchers in National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries for many useful suggestions, valuable comments and technical guidance.

I greatly appreciate to Miss Akemi Konishi, Miss Yukiko Ueyama, Mr. Takuya Kodama and Miss Akiko Shinozawa for their technical supports and discussions.

I also greatly appriciate to Mr. Nikolai Kirilov Christov for his valuable discussions and encouragement through master and doctor courses. I thank all of the members of Agrochemicals and Biotechnology Laboratory, Department of Biological and Environmental Science, Faculty of Agriculture, Kobe University for their encouragement, precious discussions and relaxation with them.

Finally, I am deeply indebted to my family and my wife Kazumi. This work would not have been accomplished without their supports and encouragement.

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