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A selectable marker using cytochrome P450 monooxygenases for *Arabidopsis* transformation

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Abstract We have developed a selection system for transformation of *Arabidopsis thaliana* via *Agrobacterium tumefaciens*. This selection system uses human cytochrome P450 monooxygenases that metabolize herbicides. Herbicide-tolerant seedlings transformed with *CYP1A1*, *CYP2B6*, *CYP2C9*, or *CYP2C19* were selected with the herbicides acetochlor, amiprophos-methyl, chlorpropham, chlorsulfuron, norflurazon, and pendimethalin. The herbicide-tolerant plants transformed with *CYP1A1*, *CYP2B6*, and *CYP2C19* expressed the corresponding P450 cDNAs. Inheritance and segregation of the P450 genes were analyzed in T_2 progeny of herbicide-tolerant T_1 seedlings. Metabolism of [^{14}C]norflurazon by transgenic T_2 seedlings expressing *CYP1A1* produced non-toxic *N*-demethylated norflurazon. This result suggests that the P450 species expressed in transgenic *Arabidopsis* plants coordinately functioned as selectable markers because of active metabolism of the herbicides.

Key words: Cytochrome P450 monooxygenase, herbicide-tolerance, selectable marker, transgenic plant.

Plant transformation technology offers an array of opportunities for basic scientific research and for genetic modification of crops. Transgenic plants are typically produced from cultured cells or tissues in which foreign DNA is integrated in the nuclear or chloroplast genome by numerous methods. These methods can be biological (e.g. *Agrobacterium tumefaciens*), physical (e.g. electroporation, particle gun), or chemical (e.g. using polyethyleneglycol). Methods using *A. tumefaciens* are easy and inexpensive, and thus have been used extensively for transformation of several plant species (Lloyd et al. 1986; Binns and Thomashow 1988; Zambryski 1988).

Generally, successful genetic transformation requires not only efficient gene delivery, but also an efficient selection system. During transformation of plant tissues, only a limited number of cells are transformed. Co-introduction of a selectable marker gene conferring resistance to a selective chemical agent allows transformed plant cells to be selected. The selectable marker gene enables the transformed cells to survive on medium containing a selective agent, while non-transformed cells and tissues die or show retarded development. Commonly used selectable marker genes confer resistance to antibiotics and herbicides. The most widely used gene is *nptII*, which confers resistance to the aminoglycoside antibiotics, such as kanamycin. In addition, *hpt*, encoding hygromycin phosphotransferase, and *bar*, encoding phosphinothricin acetyl transferase (pat), are frequently used (Miki and McHugh 2004). Other selection schemes are based on positive selection with 2-deoxyglucose-6-phosphate phosphatase (Kunze et al. 2001) or phosphomannose isomerase (Joersbo et al. 1998). Isopentenyl transferase, encoded by the *ipt* gene from the Ti plasmid of *A. tumefaciens*, is also favored because no selective agents are required (Endo et al. 2001). Conversely, a negative selectable marker system has been developed using bacterial cytochrome (P450 or CYP) monooxygenase genes with pro-herbicides (O'Keefe et al. 1994; Koprek et al. 1999).

P450 monooxygenases are heme proteins that use electrons from NADPH to catalyze the activation of molecular oxygen. The catalyzed reaction is usually a mono-oxygenation, with the formation of a molecule of water and an oxygenated product. Mammalian P450 species show overlapping and broad substrate specificity and confer the ability to metabolize a number of chemicals, including herbicides. Most classes of herbicides are aryl- or alkyl-hydroxylated or *N*-, *S*-, or *O*-dealkylated by P450 species. The phenylurea herbicide

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Abbreviations: CYP: cytochrome P450, P450: cytochrome P450.

chlortoluron is detoxified either via hydroxylation of the ring-methyl or via di-*N*-demethylation (Gonneau et al. 1988). The introduction into plants of highly active mammalian P450 species that metabolize herbicides provides not only herbicide tolerance, but also selectable markers. Human P450 species have been used to generate herbicide-tolerant tobacco, potato, and rice plants (Shiota et al. 1994; Inui et al. 1999; Inui et al. 2001a). As these transgenic plants carrying human P450 species tolerate many herbicides, this selection system can be used to construct a system for selection of transgenic plants by using herbicides. Interestingly, the *CYP2B6* gene could also be used as a negative selectable marker gene, since it metabolizes the herbicides benfuresate and ethofumesate to more toxic compounds (Kawahigashi et al. 2002).

Human P450 species are much more effective at herbicide metabolism than plant P450 species (Ohkawa et al. 1998). In this study, we developed a selection method with human P450 genes and herbicides for transformation of *A. thaliana*. We tried to determine the optimum pair of P450 species and herbicide for the selection of transformants. Development of selectable markers from P450 species will be applied for multiple transformation with a pair of P450 species and herbicides.

Materials and methods

Bacterial growth and Agrobacterium strain

The expression plasmids pUHA1 for CYP1A1, pUHB6 for CYP2B6, pHU2C9 for CYP2C9 and pUHC19 for CYP2C19 were constructed as described previously (Inui et al. 2000). Each plasmid was introduced into *Agrobacterium tumefaciens* strain LBA4404 (pUHB6) or EHA105 (pUHA1, pHU2C9, and pUHC19) by electroporation (Walkerpeach and Velten 1994).

Plant materials and transformation

Arabidopsis thaliana (L.) Heynh. ecotype Columbia was used for all the experiments in this study. *Arabidopsis* plants were transformed by the method of Clough and Bent (Clough and Bent 1998). For tolerance and selection tests with herbicides and kanamycin, *Arabidopsis* seeds were germinated on a plate with Murashige and Skoog basal medium with Gamborg's vitamins (Sigma Chemical Co., St. Louis, MO, USA) containing 0.05% 2-morpholinoethanesulfonic acid and 0.8% agar at 23°C (Murashige and Skoog 1962).

Chemicals

Acetochlor (2-chloro-*N*-ethoxymethyl-6′-ethylaceto-*o*-toluidide), amiprophos-methyl (*O*-methyl *O*-2-nitro-*p*-tolyl isopropyl-phosphoramidothioate), chlorpropham (isopropyl *m*-chlorophenylcarbamate), chlorsulfuron (1-(2-chlorophenylsulfonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl) urea), norflurazon (4-chloro-5-methylamino-2-(3-trifluoro-*m*-tolyl) pyridazin-3(*2H*)-one), pendimethalin (*N*-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine) and trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl *p*-toluidine) purchased from Hayashi Pure Chemicals Ind., Ltd. (Tokyo, Japan) were used for selection of herbicide-tolerant seedlings with the concentrations that untransformed seedlings withered. These herbicides are metabolized by human P450 species (Inui et al. 2001b). Seedlings were also selected with kanamycin at 50 μg mL^{-1}. [^{14}C]-Labeled norflurazon (sp. act. 7.2 MBq mg^{-1}, radiochemical purity 99.3%) was purchased from Amersham Biosciences Corp. (Piscataway, NJ, USA).

Selection of seedlings by herbicides

Harvested T_1 seeds were spread on selection media containing herbicide or kanamycin. Seeds were incubated on the medium at 4 °C for 4 days, and then moved to a chamber at 23°C under 24-h light. After approx. 14 days, herbicide-tolerant plants were transferred to soil, and seeds were harvested after 4 to 5 weeks. T_2 seeds were similarly selected with these herbicides or kanamycin.

PCR

CYP1A1 cDNA in the genomic DNA of transformants was partly amplified with the primers 5′-ATGTCGGCCACGGAGTTTCTTCT-3′ and 5′-TGGTGAAGGGGACGAAGGAAGAGT-3′. The reaction conditions were 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. *CYP2B6* cDNA was amplified with the primers 5′-AACTCAGCGTCCTCCTCTTCCT-3′ and 5′-ATGGGGAGAAGGTCGGAAAATC-3′. The reaction conditions were 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min. *CYP2C9* cDNA was amplified with the primers 5′-TATTGTGTCCCTTGTGC-3′ and 5′-GTTGTGCTTGTCGTCTCTGT-3′. The reaction conditions were 35 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 2 min. *CYP2C19* cDNA was partly amplified with the primers 5′-TCCTTGTGCTCTGTCTCTCA-3′ and 5′-CCATCGATTCTTGGTGTTCT-3′. The reaction conditions were 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min.

Metabolism of [^{14}C]norflurazon

Transgenic *Arabidopsis* T_2 seeds expressing *CYP1A1* cDNA and wild-type seeds were incubated on 1/2 MS medium for 10 days. Thirty (approx. 0.4 g fresh weight for each treatment) *Arabidopsis* seedlings were incubated in 15 mL of distilled water containing 15 μL of Hyponex (Hyponex Corp., Marysville, OH, USA) and 5 μM [^{14}C]norflurazon (2.0×10^5 dpm). After 1 or 3 days' incubation, plants were ground with a mortar and a

Table 1. Selection of T_1 transgenic *Arabidopsis* plants by herbicides.

P450 species transformed	Selection reagent	Reagent concentration (μM)	Number of seedlings		Transformation efficiency (%)	PCR positive (%)*1
			resistant	total		
CYP1A1	amiprophos-methyl	0.2	2	1299	0.154	100
	chlorpropham	3	4	1544	0.259	100
	norflurazon	0.04	6	6055	0.0991	100
	pendimethalin	0.3	6	3245	0.185	100
	kanamycin	50*2	78	31333	0.249	100
CYP2B6	acetochlor	1	1	423	0.236	100
	pendimethalin	0.2	2	402	0.498	100
	kanamycin	50*2	4	456	0.877	100
CYP2C9	chlorsulfuron	0.005	12	2343	0.512	0
	kanamycin	50*2	5	6525	0.0766	100
CYP2C19	amiprophos-methyl	0.2	11	7512	0.146	100
	chlorpropham	3	4	4412	0.0907	100
	norflurazon	0.1	4	6863	0.0583	100
	pendimethalin	0.5	4	3961	0.101	100
	kanamycin	50*2	35	24728	0.142	100

*1 Herbicide-resistant seedlings were conducted to PCR analysis.
*2 $\mu g\,mL^{-1}$

pestle in ethanol and water (9 : 1, v/v). The extracts were centrifuged for 10 min at 2000×*g* and the supernatants were filtered through a 0.45-μm Minisart RC15 filter (Sartorius AG, Göttingen, Germany). The filtrates were dried and dissolved in a mixture of ethanol and water (9 : 1, v/v). The extracts (4.0×10^3 dpm) were separated by thin-layer chromatography on a silica gel $60F_{254}$ chromatoplate (20 cm×20 cm, 0.25 mm thickness; Merck, Darmstadt, Germany). The solvent system was a mixture of dichloromethane and methanol (98 : 2, v/v). Radioactivity of the separated bands was quantified with FLA-2000 Bio Imaging Analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Results

Use of herbicides to select transgenic Arabidopsis plants expressing P450 genes

We chose cell-division-inhibiting herbicides as selection reagents because they distinguished in the early stage between transformed and non-transformed tobacco seeds expressing α- and β-tubulin cDNAs (Anthony et al. 1999). Amiprophos-methyl, chlorpropham, chlorsulfuron, pendimethalin, and trifluralin inhibit cell division or root growth (Tomlin 1997); norflurazon inhibits carotenoid biosynthesis, resulting in bleaching of seedlings (Misawa et al. 1993). The concentrations of herbicides were determined from observations of the herbicide tolerance of wild-type seedlings on herbicide-containing media. Growth of wild-type seedlings was inhibited by 1 μM acetochlor, 0.2 μM amiprophos-methyl, 3 μM chlorpropham, 0.005 μM chlorsulfuron, 0.04 μM norflurazon, 0.2 μM pendimethalin, and 2 μM trifluralin (data not shown). The combinations of herbicides and P450 species are listed in Table 1. Each combination gave herbicide-tolerant plants (Table 1, Figure 1).

Herbicide-tolerant T_1/1A1 seedlings were revealed by the use of 0.2 μM amiprophos-methyl, 3 μM chlorpropham, 0.04 μM norflurazon, or 0.3 μM pendimethalin. Herbicide-tolerant T_1/2B6 seedlings were revealed by the use of 1 μM acetochlor or 0.2 μM pendimethalin. Herbicide-tolerant T_1/2C9 seedlings were revealed by the use of 0.005 μM chlorsulfuron. Herbicide-tolerant T_1/2C19 seedlings were revealed by the use of 0.2 μM amiprophos-methyl, 3 μM chlorpropham, 0.1 μM norflurazon, or 0.5 μM pendimethalin. PCR analyses showed that all herbicide-tolerant T_1/1A1, T_1/2B6, and T_1/2C19 plants contained the corresponding P450 cDNAs (Figure 2A, B, D, Table 1). The chlorsulfuron-tolerant T_1/2C9 seedlings did not contain *CYP2C9* cDNA (Figure 2C). Treatment with chlorpropham and amiprophos-methyl revealed the highest transformation efficiency in the transgenic plants expressing *CYP1A1* and *CYP2C19* cDNAs, respectively. These selection efficiencies were equal to that of kanamycin selection. Kanamycin-resistant plants were obtained in all transformations with a similar transformation efficiency. On the other hand, trifluralin gave almost no inhibition of growth.

Selection of T_2 plants

T_2 progeny of herbicide-tolerant T_1 seedlings (T_2/1A1, T_2/2B6, and T_2/2C19) were examined for resistance to different concentrations of herbicides and inheritance of the P450 cDNAs. The result for one concentration of each herbicide is shown in Table 2. The segregation ratios of tolerance to sensitivity toward herbicides or

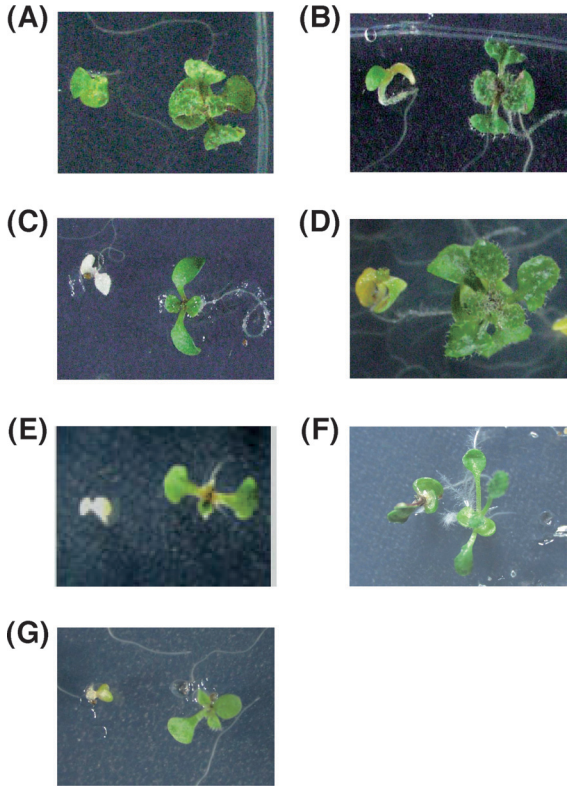

Figure 1. Selection of T_1 transgenic *Arabidopsis* plants expressing P450 genes on media containing herbicides or kanamycin. (A) T_1/1A1 seedlings were incubated on 0.2 μM amiprophos-methyl for 10 days. (B) T_1/1A1 seedlings were incubated on 3 μM chlorpropham for 10 days. (C) T_1/1A1 seedlings were incubated on 0.06 μM norflurazon for 7 days. (D) T_1/1A1 seedlings were incubated on 0.3 μM pendimethalin for 15 days. (E) T_1/1A1 seedlings were incubated on 50 μg mL^{-1} kanamycin for 8 days. (F) T_1/2B6 seedlings were incubated on 2 μM acetochlor for 14 days. (G) T_1/2C9 seedlings were incubated on 0.005 μM chlorsulfuron for 10 days. Left and right plants are sensitive and tolerant, respectively.

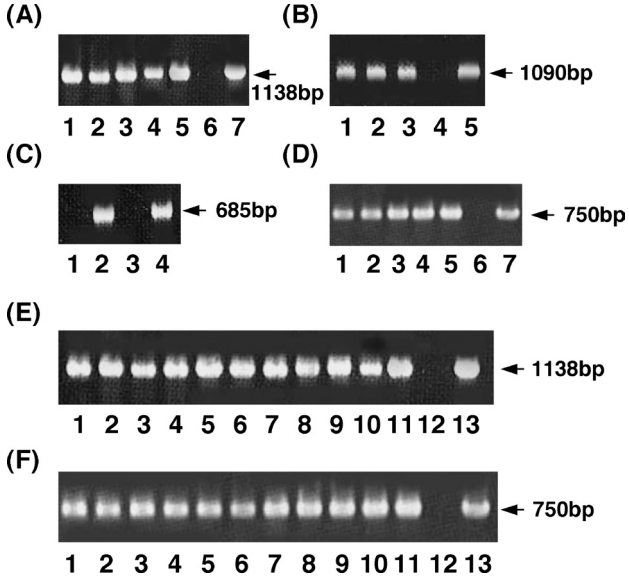

Figure 2. Introduction and inheritance of P450 genes in transgenic *Arabidopsis* plants. PCR was conducted with genomic DNA and each P450-gene-specific primer. (A) Lanes 1–5: T_1/1A1 seedlings selected by 0.2 μM amiprophos-methyl, 3 μM chlorpropham, 0.04 μM norflurazon, 0.3 μM pendimethalin, or 50 μg mL^{-1} kanamycin. Lane 6: non-transgenic plants. Lane 7: pUHA1 plasmid. (B) Lanes 1–3: T_1/2B6 seedlings selected by 1 μM acetochlor, 0.2 μM pendimethalin, or 50 μg mL^{-1} kanamycin. Lane 4: non-transgenic plants. Lane 5: pUHB6 plasmid. (C) Lanes 1 and 2: T_1/2C9 seedlings selected by 0.005 μM chlorsulfuron or 50 μg mL^{-1} kanamycin. Lane 3: non-transgenic plants. Lane 4: pHU2C9 plasmid. (D) Lanes 1–5: T_1/2C19 seedlings selected by 0.2 μM amiprophos-methyl, 3 μM chlorpropham, 0.1 μM norflurazon, 0.5 μM pendimethalin, or 50 μg mL^{-1} kanamycin. Lane 6: non-transgenic plants. Lane 7: pUHC19 plasmid. (E) Lanes 1–10: Independent T_2/1A1 seedlings selected by 0.06 μM norflurazon. Lane 11: T_1/1A1 seedlings selected by 0.06 μM norflurazon. Lane 12: non-transgenic plants. Lane 13: pUHA1 plasmid. (F) Lanes 1–10: Independent T_2/2C19 seedlings selected by 0.5 μM pendimethalin. Lane 11: T_1/2C19 seedlings selected by 0.5 μM pendimethalin. Lane 12: non-transgenic plants. Lane 13: pUHC19 plasmid.

Table 2. Selection of T_2 transgenic *Arabidopsis* plants by herbicides.

P450 species transformed	T_1 selection	Selection reagent	Reagent concentration (μM)	Number of seedlings		r/s	Segregation ratio (r:s)	X^2	p
				r*1	s*2				
CYP1A1	norflurazon	norflurazon	0.06	442	30	14.73	15:1	0.01	1
		kanamycin	50*3	263	13	20.23	15:1	1.12	0.99
CYP2B6	pendimethalin	pendimethalin	0.3	183	63	2.90	3:1	0.02	0.88
		kanamycin	50*3	210	73	2.88	3:1	0.42	0.81
CYP2C19	amiprophos-methyl	amiprophos-methyl	0.2	250	89	2.81	3:1	0.22	0.64
		kanamycin	50*3	168	44	3.82	3:1	1.82	0.18
	norflurazon	norflurazon	0.06	296	102	2.90	3:1	0.05	0.82
		kanamycin	50*3	180	62	2.90	3:1	0.02	0.88
	pendimethalin	pendimethalin	0.5	265	85	3.12	3:1	0.06	0.80
		kanamycin	50*3	221	74	2.99	3:1	0.001	0.97
	kanamycin	amiprophos-methyl	0.2	183	62	2.95	3:1	0.001	0.97
		norflurazon	0.06	212	70	3.03	3:1	0	1
		pendimethalin	0.2	189	64	2.95	3:1	0.001	0.97
		kanamycin	50*3	211	74	2.85	3:1	0.094	0.76

*1 resistant plants
*2 sensitive plants
*3 μg mL^{-1}

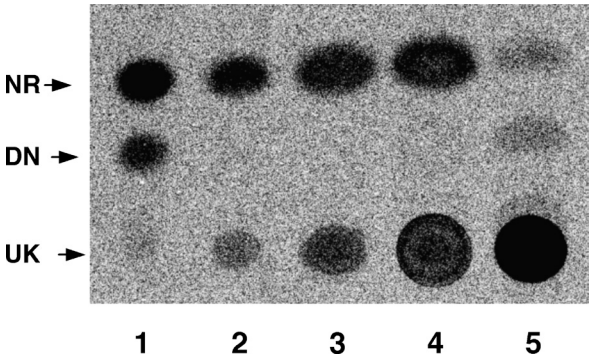

Figure 3. [^{14}C]Norflurazon metabolism activity of CYP1A1 in T_2/1A1 seedlings. Lanes 1–5: a recombinant yeast microsome expressing human *CYP1A1* cDNA, non-transgenic plants incubated for 1 and 3 days, T_2/1A1 seedlings incubated for 1 and 3 days. NR: norflurazon. DN: *N*-demethylated norflurazon. UK: unknown metabolites.

Table 3. Norflurazon *N*-demethylase activity of transgenic *Arabidopsis* plants expressing *CYP1A1* cDNA.

Plant	*N*-demethylase activity (pmol h^{-1} g $plant^{-1}$)
Non-transgenic plant	not detected
T_2/1A1 plant	397±50.6

kanamycin were the expected Mendelian ratios of 3 : 1 for insertion in a single locus or 15 : 1 for insertion in two independent loci, as determined by chi-squared analysis (χ^2). PCR analysis showed that T_2 progeny derived from T_1/1A1 (tolerant to norflurazon) and T_1/2C19 (tolerant to pendimethalin) had the corresponding P450 cDNAs (Figure 2E, F).

Norflurazon metabolism in transgenic plants expressing CYP1A1 cDNA

Analysis of [^{14}C]norflurazon metabolism revealed two metabolites, DN and UK. The Rf values of norflurazon, DN, and UK were 0.24, 0.15, and 0, respectively (Figure 3). DN had the same R_f value as the *N*-demethylated metabolite of norflurazon produced in the microsomal fraction of a recombinant yeast expressing human *CYP1A1* cDNA (Inui et al. 2001b). Norflurazon *N*-demethylase activity was detected in T_2/1A1, but not in a non-transgenic plant (Table 3).

Discussion

Most selectable marker genes in genetically modified crops belong to one of two types: those conferring antibiotic resistance and those conferring herbicide tolerance. In many cases, herbicide resistance genes have provided a more effective system for plant transformation than antibiotic resistance genes, for example, in legume species such as peas (Schroeder et al. 1993). Herbicide resistance marker genes may provide considerable advantages over antibiotic resistance genes in cases where high levels of the antibiotic may interfere with plant regeneration, resulting in the production of few transgenic plants, or plant tissues may exhibit a high level of intrinsic antibiotic resistance.

Transgenic *Arabidopsis* plants containing human P450 cDNAs as selectable markers were selected with several herbicides. Herbicide-tolerant seedlings were revealed by the combinations of *CYP1A1* and amiprophos-methyl, chlorpropham, norflurazon, or pendimethalin; *CYP2B6* and acetochlor or pendimethalin; *CYP2C9* and chlorsulfuron; and *CYP2C19* and amiprophos-methyl, chlorpropham, norflurazon, or pendimethalin. These results show that the combination of human P450 cDNAs and herbicides is useful for the selection of transgenic *Arabidopsis* seedlings. In particular, the combinations of *CYP1A1*-chlorpropham, *CYP2B6*-pendimethalin, and *CYP2C19*-amiprophos-methyl showed the highest transformation efficiency of each P450 gene, suggesting that inhibition of cell division by these three herbicides is critical for the survival of seedlings. On the other hand, no chlorsulfuron-resistant seedlings transformed with *CYP2C9* were positive by PCR. Inhibition of the pathway for branched-chain amino acid biosynthesis seems not to have given complete control of untransformed seedlings. This incomplete control would also have been caused by the low chlorsulfuron-metabolizing activity of the expressed *CYP2C9* (Inui et al. 2001b). *CYP1A1* and *CYP2C19* showed similar substrate specificity, but *CYP2B6* was different. This result is similar to that in transgenic potato plants expressing these P450 cDNAs (Inui et al. 1999). Selection with norflurazon was an easy system to distinguish transformants from non-transformants because of bleaching of leaves, but most selection methods gave appreciable differences in growth. Norflurazon tolerance in T_2/1A1 was derived from norflurazon metabolism by the expressed *CYP1A1*. This result strongly suggests that herbicides used as selection agents are metabolized by the P450 species expressed.

Bacterial $P450_{SU1}$ (*CYP105A1*) was used as a negative selectable marker in tobacco (O'Keefe et al. 1994), because it can metabolize the sulfonylurea herbicide R7402 to a more toxic metabolite. Thus, $P450_{SU1}$ expression and R7402 treatment can be used as a negative selection system in plants. The herbicides benfuresate and ethofumesate are also metabolized by several human P450 species to more toxic metabolites (Kawahigashi et al. 2002). Thus, human P450 genes can also be used as negative selectable markers. On the other hand, the *dao1* gene, encoding D-amino acid oxidase, was developed as a positive and negative selectable marker (Erikson et al. 2004).

We transformed *Arabidopsis* plants with human P450

genes as markers selectable by several herbicides. It is possible to excise these selectable marker genes from the genome before field testing (Hare and Chua 2002). The results of this study will allow the use of plant herbicide-metabolizing P450 species as acceptable selectable markers (Morant et al. 2003).

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