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RESEARCH ARTICLE

PHYTOREMEDIATION OF THE HERBICIDE SIMAZINE BY P450 TRANSGENIC TOBACCO PLANTS

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ABSTRACT

The human Cytochrome P 450 CYPIA2 gene was introduced into tobacco plants (*Nicotina tabacum* cv. Virginia tobacco). CYP IA2 plants grown in soil clearly showed a healthy growth and tolerance to simazine, but non transgenic plants were completely damaged by the herbicides. The results proved that simazine was completely metabolized by the CYPIA2 transgenic plants to prevailingly non phytotoxic metabolites, the herbicide was biotransformed to a minor extent in non transgenic plants to metabolites with residual phytotoxicity. Thus, CYPIA2 tobacco plants can remove simazine herbicide from the soil, whereas non transgenic tobacco was damaged. The results revealed that in the NT tobacco plantlets and excised leaves, 32% and 50% respectively of the simazine absorbed by the tissues was metabolized into Des. The T-E plantlets metabolized 83% of the absorbed parent herbicide into different metabolites, while only 8.3% remained non-metabolized in the T-V plantlets. The main metabolite resulting from degradation of simazine by CYP1A2 transgenic plants was DiDes representing 41% (plantlets) and 37.3% (excised leaves) in case of T-E, and 35% (plantlets) and 60% (excised leaves) with T-V. Doses ranging between 4 and 40 mg simazine per plant were enough to inhibit the growth, finally causing the death for tobacco plants. When simazine was applied to the CYP1A2 transgenic tobacco plant (T-E and T-V) under similar conditions, the plants showed considerable resistance with all concentrations used.

INTRODUCTION

The proper use of herbicides is a labor saving means of improving crop yield and quality, it reduces weed infestation and increases market prices. In addition, because properly used herbicides consume less soil and moisture than mechanical weed control, they minimize loss of moisture and erosion. However, pollution arising from the agricultural use of herbicides is diffuse because the compounds are distributed over larger areas. For example most of the transport of atrazine in runoff occurs during the first rains or during irrigation events following its application. Therefore, non target effects in the environment should be considered when applying herbicides. Herbicide residues may simultaneously have positive and negative effects on parasite transmission. The net effect of exposure to environmentally realistic levels of pesticide may elevate amphibian trematode infections (Rohr *et al*., 2008). Simazine is herbicide that was discovered in the 1950 and inhibits electron transport during photosynthesis. It is mainly observed through roots but also through leaves and is translocated acroprtally in the xylem. In sensitive plants, unaltered simazine accumulate in apical meristems and leaves and cause chlorosis and death through inhibition of electron transport during photosynthesis (Shamer, 2003; Kawahigashi *et al*., 2005b). Simazine is metabolized by N-dealkylation in many plant species to from mono- and di dealkylated products (Fig. 1). N-Dealkylation of a single side chain results in partial loss of phytotoxicity and that both side chains cause complete loss of phytotoxicity (Lamoureux *et al*., 1998). Susceptible and tolerant plant species show important difference in the extent to which they can dealkylate these herbicides (Ahrens, 1994). The N-dealkylate seems to be catalyzed by cytochrome 450 monooxygenases (CYP). In particular, tulip (*Tulipa generianal* L.) cytochromeP450 causes in vitro metabolism of atrazine, resulting in the production of double dealkylated metabolites (Topal *et al*., 1996). The cytochromeP450 inhibitor 1-aminobenzotriazole inhibited N-dealkylation of simazine and reduced the plant weight of biotypes of *lolium rigidum* resistant to triazine herbicides with the combination of simazine (Burnet *et al*., 1993). P450 monoxygenases insert one atom of oxygen into hydrophobic molecules to make them more reactive and water soluble (Hatzois, 1997). Genome sequence analysis revealed that plant genomes can include hundreds of P450 genes (e.g. 273 in *Arabidopsis thaliana* and 356 in *Oryza sativa*) (Nelson *et al*., 2004). The varied substrate specificities of these P450 complicate prediction of their functions from sequence similarities only. However, a few P450 genes related to herbicide metabolism have been cloned and characterized from plants, including Jerusalem artichoke

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(*Helianthus tuberosus*) (Pierrel *et al*., 1994; Robineau *et al*., 1998), tobacco (*Nicotiana tobacum*) (Yamada *et al*., 2000) and soybean (*Glycine max*) (Siminszky *et al*., 1999; Siminszky, 2006).

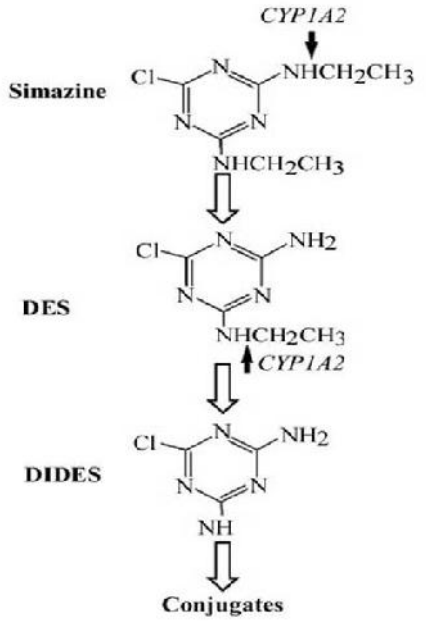

Figure 1. Metabolism of simazine in plant. DES, deethylated; DIDES, di-deethylated simazine

It is well known that microsomes of mammalian livers contain several P450 involved in xenobiotic metabolism and these enzymes have been reported to show broad overlapping substrate specificity toward Lipophitic xenobiotics, including herbicides (Nebert and Russell, 2002). In vitro metabolism studies using hepatic microsomes from several animals including rat, goat, pig and rabbit showed that atrazine and simazine were converted into dealkylated metabolities (Adams *et al*., 1990). Inui *et al*. (2001) used an in vitro yeast microsome system to demonstrate that atrazine and simazine were metabolized by human Cyp1 A1 and Cyp1 A2 through dealkylation. Phytoremediation is the process by which plants detoxify soils, sediments and aquatic sites contaminated with organic and inorganic pollutants (Gao and Zhu, 2004; Kamahigashi *et al*., 2005 a and b; Mills *et al*., 2006; Whitfield Aslund *et al*., 2007).

It is much less expensive and destructive than physical or chemical remediation. The process can be used to remove herbicides from water and soil because most herbicides are moderately hydrophobic. Phytoremediation has been shown to be useful in the removal of herbicides such as atrazine (Marcacci and Schwitzguebel, 2007) and metolachlor (Moore *et al*., 2001). Despite the rate of contaminant removal using conventional plants is inadequate (Goel *et al*., 1997). To produce plants that can more effectively metabolize chemical pollutants, transgenic plants have been developed by endowing them with genes from organisms that can degrade chemical pollutants (Linacre *et al*., 2003; Davis, 2006; Doty, 2008). Phytoremediation is also a possible method to remove pesticides from contaminated water. Uptake of pesticides by plants plays an important factor in phytoremediation. It was found that the partition coefficient (Log K_{ow}) of substances and their solubility in water give a good indication for their uptake. According to their Log K_{ow} substances may be subdivided to three categories: hydrophilic (Log K_{ow} < 0.5), moderately lipophilic (Log K_{ow} 0.5-3) and highly lipophilic substances (Log K_{ow} > 3). Compounds ranging between Log K_{ow} 0.5 and 3 are expected to be good targets for phytoremediation since these compounds are adsorbed only moderately to soil organic matter and exhibit favorable plant uptake, can be transported inside the plant and generally have the ability to penetrate the plasmalemma (Reichenauer and Germida, 2008). On the other hand, some compounds in the range of Log K_{ow} between 0.5 and 3 are expected to contaminate groundwater (Cunningham and Berti, 1993). Since simazine has moderate water solubility, a Log K_{ow} = 2.1 (Tomlin, 1994), a low volatility and a long soil half life, it may contaminate ground and surface waters (Ahrens, 1994; Knuteson *et al*., 2002) and thus is thought to be a good target for phytoremediation. The aim of the present study was to produce transgenic tobacco plants expressing human CYP1A2 that showed herbicide tolerance towards simazine by detoxifying it. Also to produce corresponding P450 transgenic tobacco plants suitable for phytoremediation.

MATERIALS AND METHODS

Chemicals

^{14}C-ring-labeled simazine [6-chloro-N, N-diethyl-1,3,5-triazine-2, 4-diamine] (specific activity, 1.93 MBq mg^{-1}; radiochemical purity, 97.9%) were provided by Novartis Crop Protection, Inc. (Basel, Switzerland). Reference compounds simazine, deethyl simazine (Des) and dideethyl simazine (DiDes) were purchased from Riedel de – Haen (Seelze, Germany). For the metabolism experiments the radio labeled compounds had a final specific activity of $2x10^6$ dpm per 40 µg simazine in 100 µl methanol, or $1x10^6$ dpm per 20 µg simazine in 100 µl methanol.

Plant materials

Nicotiana tobacum (cv. Virginia tobacco; Terra Pflanzenzucht GmbH. Rosdorf, Germany) plantlets with an age of 2 weeks or leaves derived from 6 weeks old mature plants. All plantlets and mature plants were derived from aseptically grown shoot cultures. The CYP1A2 transgenic shoots were produced from leaves of aseptically grown tobacco plantlets according to commonly utilized procedures essentially as described for the transgenic cell cultures before. After as election period using kanamycin, the plant materials were grown as shoot cultures and were regularly sub-cultivated.

Shoot culture

The cultured shoots (transgenic and non-transgenic) were maintained at 25 ºC in a phytochamber on MS medium (Murashige and Skoog, 1962) with illumination (16/8 h light/dark) using a light intensity of 150-200 $\mu molm^{-2}S^{-1}$ (Universal White Standard F 58 W/125 Lamps; Silvania, Osram, Germany). Shoots showing clearly differentiated leaf structures were transferred to round glasses (0.5 L) containing root induction medium (Murashige and Skoog, 1962), and

were then kept until root development. Well grown plantlets were subsequently transferred to soil. According to growth and expression of CYP1A2, transformants V- and E- were regarded as suitable candidates for the subsequent studies. These were designated as T-V and T-E, the non-transgenic tobacco as NT.

Soil culture

The plants were grown in soil (TYP ED 73, Einheiserde, Germany). The plantlets were transferred to the soil and were gradually exposed to fresh air during the first week of soil culture. The plants were watered every day. After two months, Wuxal Super Fertilizer (Aglukon Spezial dunger, Dusseldorf, Germany) N:P:K (8:8:6 w/w/w) was added once a week (2 mm^{-1}). For the entire cultivation period, the plants were grown in green house light chamber (16/8 light/dark; 22-25 ºC) and received irradiation (PFD) of 200-300 µmol cm^2 $S^{-1}$from Osram Superstar HQI-T 400 W/HD Lamps.

DNA extraction and Polymerase Chain Reaction (PCR)

The presence of human CYP1A2 gene in the transgenic tobacco plants was detected by PCR using primers specific to the CYP1A2 gene. Total DNA of the transgenic plants or non-transformed plants were extracted following the procedure described by Mettler (1987). PCR analysis was executed using primers P1A2-5 (5-CTGAGAGTAGCGATGAGA-3) and P1A2-3 (5-CTGCCACTGGTTTACGAA-3). The PCR program was 94 ºC for 5 min, 94 ºC for 30 sec, 56 ºC for 30 sec and finally 72 ºC for 80 sec using 35 cycles. Amplified PCR products were analyzed by electrophoresis in 1% agarose gel, and DNA fragment bands stained with ethidium bromide were visualized using UV illuminator.

Application of ^{14}C labelled simazine

The metabolism studies using non-transgenic and CYP1A2 transgenic tobacco plantlets were performed using plantlets from the transgenic and non-transgenic tobacco plant with an age ranging between 2 and 2.5 weeks. Excised leaves were derived from transgenic and non-transgenic plants with an age of 6 weeks. The plantlets were transferred to the application solution containing ^{14}C-simazine ($2.1x10^6$ dpm) 40 µg simazine per plantlet in/ml sterilized tap water. Leaves were cut from plants with scissors and were then rapidly transferred to the application solution containing 20 µg of ^{14}C-simazine per g (leaf fresh weight) in 1 ml of sterilized tap water. After 8 h most of the application solution was absorbed by the plant tissues. All plantlets and excised leaves were incubated at 28 ºC at 70 µmol m^{-2} Sec^{-1} light for 48 h. Prior to extraction, actual fresh weights of all samples were determined.

Extraction and analytical procedures

After incubation of plantlets and leaves, lower parts of tissues were washed to remove ^{14}C adhering to outer surfaces. Both plantlets and leaves were examined for translocation and distribution of ^{14}C by autoradiography using 20 x 40 cm BAS-MS 2040 imaging plates and a Fujifilm BAS-1000 Bio-Imaging Analyzer (Raytest, Straubenhardt, Germany). Plates were exposed for 24 h regarding plantlets and 4 h regarding leaves and were then analyzed. Portions of radioactivity present in the incubation solutions were determined by liquid scintillation counting (Lsc; Camberra Packard, TRI-CARB 2250 CA, Dreieich, Germany). Plant tissues were cut into pieces, freezed and crashed under liquid nitrogen. The plant materials were then extracted with water : methanol 1 : 1 (v/v) by homogenization using ultrasonication for 20 min. Extract and cell depris were separated by suction fitration resulting in extracted plant residues and filtrate. Insoluble plant depris were air dried and combusted using Biological Oxidizer OX 500 (Zinsser/Harvey Instrument Corporation, Frankfurt, Germany). To remove chlorophyll, the methanolic extract was extracted twice with 50 ml of cyclohexane. Methanolic phases were collected, while the upper phases were discarded. The lower phase consisted of the final plant extract, which was examined for ^{14}C as described. All analytical procedures (e.g. thin-layer chromatography, TLC and HPLC) were as described (Bode *et al*., 2004).

Phytotoxicity of simazine to transgenic and non-transgenic tobacco plants

Non-transgenic and transgenic Cyp 1A2 tobacco plants grow in pots 13x13x13 cm (W/L/H) containing 500 g soil are examined after dosing 0.5, 1, 2, 5, 10, 20, 40 mg of simazine per plant added in 10 ml of water. After application of simazine, water was added to the plants every day. Toxic effects of simazine were examined daily.

RESULTS

Expression of the CYP1A2 gene in transgenic tobacco plants

Polymerase Chain Reaction (PCR) analysis was performed to confirm the presence of human CYP 1A2 cDNA in the transgenic plants. As shown in Figure 2, clear PCR amplification products (590 bps) were detected with transgenic plants examined (Lanes 1-6).

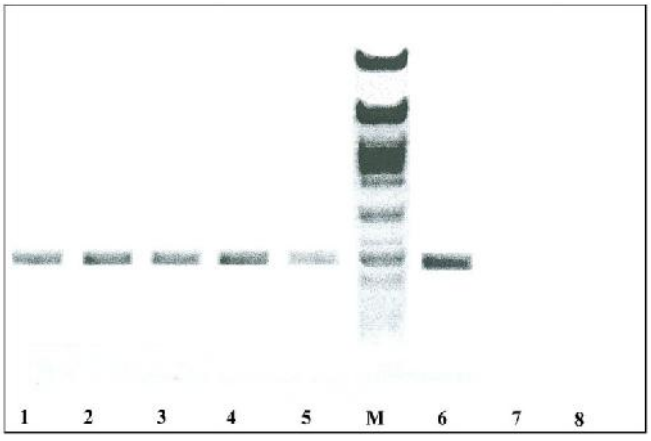

Figure 2. PCR analysis of transgenic tobacco plants by ethidium bromide mediates fluorescence of CYP1A2 PCR products after UV excitation

The PCR products were subjected to a 1% (w/v) agarose gel in 1 x TAE buffer for 120 min at 90 V. One clear band of the expected size range corresponding to CYP1A2 (590 bps), M = λ Pstl DNA marker. Lane 1-4 different CYP1A2-transgenic plants T-E; Lane 5, 6 different CYP1A2-transgenic plants T-V; Lane 7: NT and Lane 8: negative control.

No amplification products were detected in case of the non-transgenic plants (lane 7). Immediately after transformation and establishment of the CYP1A2, transgenic shoot cultures, a similar result was obtained using PCR analysis. One clear band of the expected size range corresponding to CYP1A2 (590 bps), M = λ pstl DNA marker. Lane 1-4 different CYP1A2 transgenic plants T-E; Lane 5, 6 different CYP1A2 – transgenic plants T-V; Lane 7: NT and Lane 8: negative control.

Distribution of ^{14}C in plantlets and excised leaves

The distribution of radioactivity applied as ^{14}C–simazine within the plantlets and excised leaves was first examined by autoradiography. Figure 3 show that simazine was rapidly obsorbed by the roots and translocated in the xylem to the entire shoot reaching the whole plantlets. The radioactivity especially accumulated in the leaves with little accumulation in the lower plant parts. Similar results were obtained with all samples.

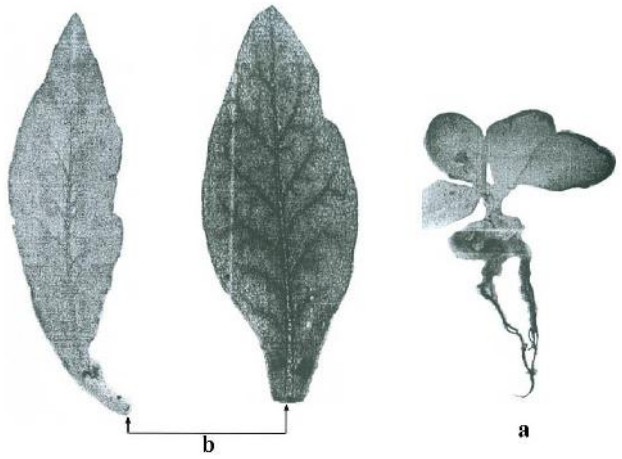

Figure 3. Autoradiography of a non-transgenic tobacco plantlets (a) and leaf (b) treated with ^{14}C – simazine via the roots (a) and the cut surface (b) for 4 h, and kept for a total incubation period of 48 h

Simazine was applied to both, the non-transgenic and CYP1A2 transgenic tobacco plantlets and leaves. The distribution of radioactivity observed after 48 h of incubation among the fractions examined, i.e. incubation solutions, plant extracts and non-extractable radioactivity are shown in Table 1. Since recoveries of ^{14}C were between 77.4% and 99.2%, it was assumed that only minor portions of the parent simazine were mineralized to $^{14}CO_2$ or transformed to other volatile products. In case of plantlets 31.0% of applied radioactivity was found in the extracts of the non-transformed plants, whereas 43.0% were detected in those of transformant T-E. While 50.2% of ^{14}C was detected in those of the excised leaves of transformant T-E, 52.6% was detected in those of the leaves detached from non-transgenic tobacco plants. Both transform T-V plantlets and leaves showed high rates of absorption (48.1% and 80.0% applied ^{14}C respectively). The non-extractable ^{14}C-residues detected in the T-V plantlets and leaves showed slightly higher portions (2.9 and 4.4% respectively) as compared to other plantlets and leaves examined in the study. TLC analysis of the cell extracts showed that in the non-transgenic tobacco plantlets and leaves 39.4% and 27.5% respectively of applied simazine was recovered non-metabolized, while the T-V plantlets and leaves metabolized 94.5% and 89.9% respectively of the applied herbicide. T-E plantlets (72.3%) and T-V leaves (83.9%) exhibited the highest percentages of soluble metabolites. Based on fresh weight of the tobacco tissues examined, transformant T-V showed the highest rate of metabolic activity.

Table 1. Distribution of radioactivity in transgenic and non-transgenic tobacco plantlets and leaves after application of ^{14}C simazine

Fraction of applied radioactivity (amount of simazine)	NT[a]	T-E[a]	T-V[a]	NT[a]	T-E[a]	T-V[a]
	40 µg plantlet^{-1}			20 µg g^{-1} leaf		
Incubation solution	48.8	45.3	36.3	31.4	25.4	14.8
Plant extract	31.0	43.0	48.1	52.6	50.2	80.0
Non-extractable ^{14}C	0.9	2.5	2.9	1.4	1.9	4.4
Recovered ^{14}C	80.7	90.8	87.3	85.3	77.4	99.2
Recovered simazine[b]	39.4	6.7	5.5	27.5	15.8	10.1
Soluble metabolites[b]	26.9	72.3	70.3	49.7	53.0	83.9

Assays: 48 h of incubation, three parallels; 20 µg simazine assays per g (leaf) and 40 µg (plantlets). Data are averages of parallels given as % of applied ^{14}C.

[a]NT = non-transgenic tobacco, T-E, T-V = tobacco transformants harbouring human P450 CYP1A2. Fresh weights at the beginning of the experiment were:plantlets: NT: 0.49 g, T-E: 1.24g, T-V: 1.35g; leaves: NT: 1.74g, T-E: 2.48g, T-V: 2.0g.
[b]According to TLC analysis of extracts.

Identification and isolation of metabolites from plantlets and excised leaves

The extracts from non-transgenic (NT) and CYP1A2 transgenic transformants (T-E and T-V) plantlets and plant leaves were first analyzed by TLC. Simazine and its expected metabolites 2-chloro-4-(ethylamino)-6-amino-s-triazine (Des) and di-dealkylated product, 2-chloro-4,6-diamino-s-triazine (DiDes) were generally identified by HPLC analysis with the corresponding reference compound in all extracts examined. With HPLC analysis, simazine, Des and DiDes were eluted as described before. Additionally, two unidentified metabolic products (UN1, UN2) appeared after 3.8 and 9.7 min (Fig. 4).

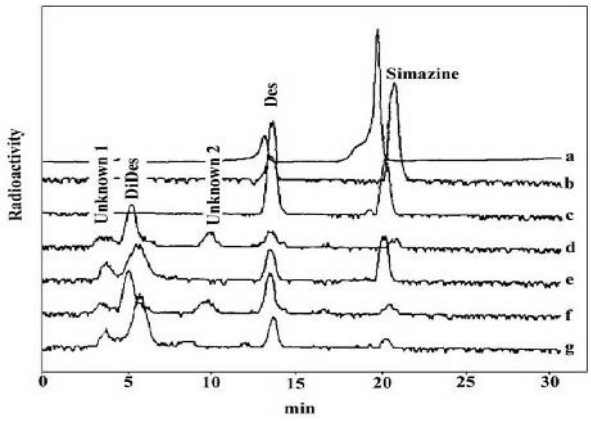

Figure 4. HPLC analysis of seedling extracts derived from studies using 40 µg simazine per assay and leaf extracts derived from studies using 20 µg simazine per assay.

a) Chromatogram (UV detection) of reference compounds, b) NT plantlet, c) NT leaf, d) transgenic T-E plantlet, e) transgenic T-E leaf, f) transgenic T-V plantlet and g) transgenic T-V leaf. Des = de-ethyl-simazine and DiDes = di-de-ethyl-simazine.

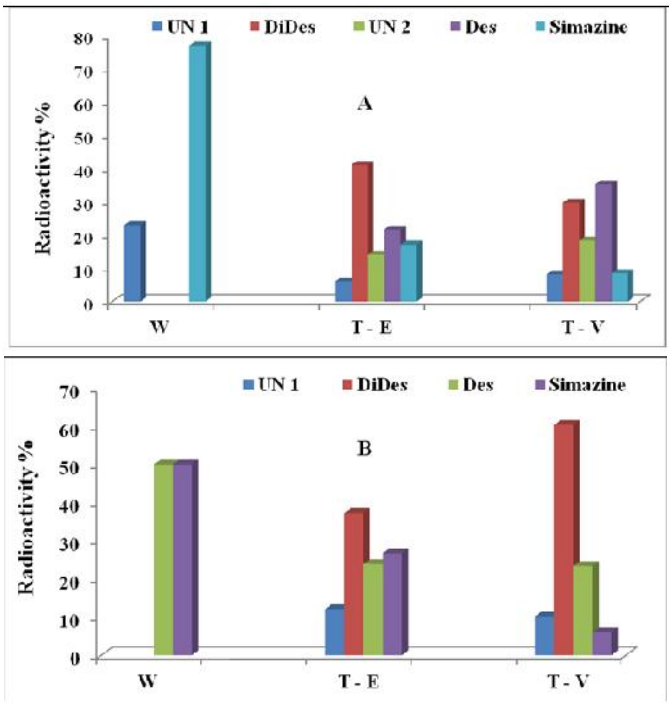

Figure 5. Metabolites distribution in (a) cell extract of plantlets and (b) cell extract of plant leaves according to HPLC

NU1,UN2 are unknown metabolites detected above the threefold background level. (W) non-transgenic tobacco, (T-E, T-V) transgenic tobacco integrated human P450 species CYP1A2.

In the NT plantlets and excised leaves, 23% and 50% respectively of the simazine absorbed by the tissues was metabolized into Des. The T-E plantlets metabolized 83% of the absorbed parent herbicide into different metabolites, while only 8.5% remained non-metabolized in the T-V plantlets. These results almost agree with those obtained with the excised leaves: 73.4% of the absorbed simazine was metabolized in T-E, while only 6% simazine was detected non-metabolized in T-V. The main metabolite resulting from degradation of simazine by the CYP1A2 transgenic plants was DiDes representing 41% (plantlets) and 37.3% (excised leaves) in case of T-E and 35% (plantlets) and 60% (excised leaves) with T-V. In addition, further metabolic products were found. Des represented 23.9%, 23.4% in the excised leaves of both transformants (T-E, T-V) and 21.6% and 29.7% of absorbed ^{14}C in the transgenic plantlets. Besides, unidentified metabolites were detected in both the T-E and T-V excised leaves and plantlets. These results suggested that the CYP1A2 transgenic T-V plants possessed the capability to biotransform almost completely the amounts of simazine applied in this experiment.

Table 2. GC-MS analysis of simazine and Des (silylation with MSTFA 70 °C, 30 min for Des)

Substance M_w[g mol^{-1}]	Retention time [min]	m/z (relative intensity)
Simazine M_w = 201	13.87	201 M^+ (100), 186 (63), 173 (37), 158 (18), 138 (20), 123 (13), 110 (6), 96 (30), 93 (23), 71 (34), 68 (65), 53 (16).
Des M_w = 317	13.86	317 M^+ (30), 302 (100), 282 (5), 274 (16), 244 (9), 232 (5), 207 (15), 194 (10), 171 (25), 143 (13), 140 (22), 116 (16), 95 (62), 73 (83), 59 (8).

Parent ^{14}C simazine and Des contained in the extract of all cultures were separated by preparative HPLC and were then analyzed by GC-ELMS. EL mass spectra obtained from isolated parent simazine and its metabolite Des were identical to those of the respective reference compounds. Prior to GC-EL MS, the fraction containing Des was reacted with MSTFA to obtain the trimethylsilyl derivative of this metabolite. The corresponding mass spectra given as m/z (relative abundance) was described in Table 2.

Toxic effects of simazine on CYP1A2 transgenic and non-transgenic tobacco plants

A comparison of the transgenic and non-transgenic tobacco plants in the green house (Fig. 6) showed that the Cyp1A2 tobacco plants exhibited normal morphology, including normal height and leaf color. It was obvious that the photosynthesis inhibiting herbicide simazine had negative effect on the growth of the non-transgenic tobacco plants grown in soil. Doses ranging between 5 and 40 mg simazine per plant were enough to inhibit the growth finally causing the death for tobacco plants. When simazine was applied to the Cyp1A2 transgenic tobacco plants (T-E and T-V) under similar conditions, the plants showed considerable resistance with all concentrations used. Thus, the adult CYP1A2 transgenic plants appeared to metabolize simazine to non-or less phytotoxic metabolites as shown before using the corresponding plantlets, excised leaves and cell cultures.

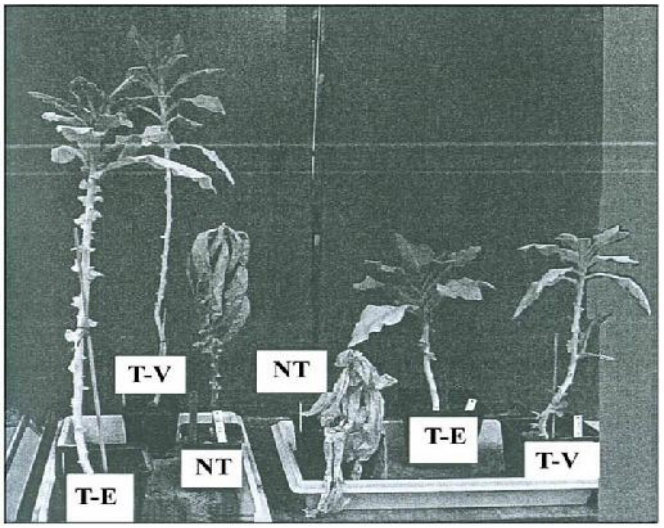

Figure 6. Phtotoxicity of 40 mg simazine herbicide per pot (500 g of soil) toward non-transgenic and transgenic tobacco plant expressing CYP1A2

CYP1A2 transgenic tobacco plants grow well, but non-transgenic tobacco plants were completely damaged by the photosynthesis-inhibiting herbicides.

DISCUSSION

Phytoremediation is a relatively new field of science and technology that uses plants to clean polluted soil, water or air (Salt *et al.*, 1998). Many plants including corn, sorghum, cotton, citrus, black walnut, yellow poplar, Canada thistle and parrot feather can take up and metabolize s-triazine herbicides (Lamoureux *et al.*, 1998; Knuteson *et al.*, 2002). It has been

proposed that genetic engineering can improve the extent to which plants extract, sequester and detoxify diverse environmental contaminants (Meagher, 2000; Linacre *et al*., 2003). Cyp1A1 rice plants showed broad herbicide resistance and also showed the ability to reduce the concentration of herbicide in the culture medium (Kawahigashi *et al*., 2005b). Similarly, cultured tobacco cells transformed with CYP1A1 or CYP1A2 show high metabolic activity towards atrazine to produce N-dealkylated metabolites. They metabolize atrazine faster than non-transformed tobacco cells (Bode *et al*., 2004). In the transgenic plants expressing human CYP1A2 higher, but different turnover rates were observed. This result is supported by a recent publication reporting that simazine was transformed more rapidly by human CYP1A2 than by CYP1A1 or CYP3A4 contained in microsomes isolated from corresponding transformed yeast cell (Inui and Ohkawa, 2005). Accordingly, the tobacco plants expressing human CYP1A2 showed high transformation rate of simazine in the present study.

The herbicide was metabolized into the major metabolite DiDes followed by an unidentified more polar metabolite and Des (Figs. 4 and 5). Des and DiDes are known to exhibit a lower phytotoxicity than the parent simazine. Thus, human CYP1A2 expressing tobacco plants appeared to be promising as model in order to study the application of P450 transgenic plants for phytoremediation. Immediately after production of the CYP1A2 transgenic tobacco plants by commonly utilized procedures, the presence of CYP1A2 cDNA and expression of the corresponding monooxygenase in two transformants (T-V and T-E) was proven by PCR. The subsequent metabolism studies clearly showed in the CYP1A2 transgenic plants examined as plantlets and excised leaves, simazine was metabolized to a high degree and almost completely as compared to the non-transformed plants, where transformation of the herbicide was significantly lower. In addition, simazine was metabolized differently. The main product of the CYP1A2 transgenic plants was the doubly dealkylated simazine (DiDes) besides lower portions of the non-dealkylated product, Des, whereas the main product in the non-transgenic tobacco plants was the mono-dealkylated product (Fig. 5). The transformants T-V metabolized simazine to a higher degree than the transformants T-E, possibly due to a higher expression of the CYP1A2 gene in the transformants T-V plants.

Winkelmann and Klaine (1991) demonstrated that the metabolite resulting from removal of one of the N-attached alkyl side-chains (Des) is almost as toxic as simazine, while N-dealkylation of both side-chains (DiDes) was shown to lead to a non-phytotoxic metabolite. However, N-dealkylation was identified as major detoxification pathway for triazine herbicides in non-tolerant plants (Kawahigashi *et al*., 2005b; Kawahigashi *et al*., 2008; Line *et al*., 2008). Data on the phytotoxic properties of the unidentified metabolite detected in the persent study are not available. It may be assumed that this compound is less phytotoxic than parent simazine. In addition, the non-extractable residues formed might be regarded as detoxification products. The present P450 transgenic tobacco plant is regarded a good candidate for phytoremediation purposes. Since it can be remove contaminants or at least triazines from soil by uptake and detoxification. In this regard, heterologous expression of human CYP1A2 in the transgenic plants proved to be especially useful for phytoremediation. The comparison of the phytotoxic effect of simazine on the CYP1A2 transgenic and non-transgenic tobacco plants using large concentrations of the herbicide. The transgenic tobacco plants exhibited a healthy growth over a period of one month using doses of up to 40 mg simazine per plant cultivated in pots containing 500 g of soil. Under the same conditions, the non-transgenic tobacco plants were severely damaged with doses above 2 mg per plant. The symptoms were consistent with the inhibition of photosynthetic electron transport (Hooker and Skeen, 1999; Doty *et al*., 2000; Dietz and schnoor, 2001; Linacre *et al*., 2003). Thus, the CYP1A2 transgenic tobacco plants showed a higher tolerance towards the herbicide simazine and appear to be useful for the remediation of contaminated sites.

Conclusion

CYP1A2 tobacco plants showed broad herbicide resistance towards various herbicides and well also prove useful in degrading, and thus decreasing the environmental load of herbicides in soil. CYP1A2 showed broad and overlapping substrate specificity towards foreign chemicals with different chemical structures, and thus it is necessary to study further the substrate specificity of the introduced CYP1A2 towards xenobiotics and secondary metabolites in plants (e.g. regarding metabolic activation to toxic or genotoxic products). Further experiments on environmental safety assessment are also needed before such transgenic plants can be put to practical use. In further, transgenic plants expressing $P450_s$ should be good not only for developing herbicide resistant tobacco but also for reducing the environmental impacts of agrochemicals.

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