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Transgenic plants containing the phosphinothricin-*N*-acetyltransferase gene metabolize the herbicide L-phosphinothricin (glufosinate) differently from untransformed plants

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Abstract. L-Phosphinothricin (L-Pt)-resistant plants were constructed by introducing a modified phosphinothricin-*N*-acetyl-transferase gene (*pat*) via *Agrobacterium*-mediated gene transfer into tobacco (*Nicotiana tabacum* L), and via direct gene transfer into carrot (*Daucus carota* L). The metabolism of L-Pt was studied in these transgenic, Pt-resistant plants, as well as in the untransformed species. The degradation of L-Pt, ^{14}C-labeled specifically at different C-atoms, was analysed by measuring the release of $^{14}CO_2$ and by separating the labeled degradation products on thin-layer-chromatography plates. In untransformed tobacco and carrot plants, L-Pt was deaminated to form its corresponding oxo acid 4-methylphosphinico-2-oxo-butanoic acid (PPO), which subsequently was decarboxylated to form 3-methylphosphinico-propanoic acid (MPP). This compound was stable in plants. A third metabolite remained unidentified. The L-Pt was rapidly *N*-acetylated in herbicide-resistant tobacco and carrot plants, indicating that the degradation pathway of L-Pt into PPO and MPP was blocked. The *N*-acetylated product, L-*N*-acetyl-Pt remained stable with regard to degradation, but was found to exist in a second modified form. In addition, there was a pH-dependent, reversible change in the mobility of L-*N*-acetyl-Pt thin-layer during chromatography.

Key words: *Daucus* – Herbicide – *Nicotina* – Phosphinothricin-*N*-acetyl-transferase – Phosphinothricin metabolism – Transgenic plant

Introduction

Phosphinothricin [Pt; DL-homoalanine-4-yl-(methyl)-phosphinic acid], also known as glufosinate, is the active ingredient of the commercial herbicide BASTA® (Hoechst AG, Frankfurt/M, FRG). Phosphinothricin is a broad-spectrum, contact herbicide (Schwerdtle et al. 1981) in which the L-enantiomer, L-phosphinothricin (L-Pt), acts as a potent competitive inhibitor of glutamine synthetase (Bayer et al. 1972; Lea et al. 1984) which is a central enzyme of nitrogen metabolism in plants (Miflin et al. 1980). The resulting ammonia accumulation and the deficiency in glutamine lead to the rapid death of plant cells (Tachibana et al. 1986a, b; Sauer et al. 1987; Wild et al. 1987).

The uptake of Pt is restricted to leaves and roots of plants; woody parts are not affected. Therefore, the use of this non-selective herbicide is limited to specific applications, e.g. in vineyards and fruit orchards (Langelüdeke et al. 1981). Genetically engineered, herbicide-resistant plants promise to extend the agricultural application of BASTA®. Examples of transgenic plants that are resistant to the non-selective herbicides glyphosate (Comai et al. 1985) and some sulfonylureas (Chaleff et al. 1988) have already been described.

In recent papers, we reported the cloning and sequence analysis of a Pt-resistance gene (*pat*) from *Streptomyces viridochromogenes* Tü494 (Strauch et al. 1988; Wohlleben et al. 1988). The *pat* gene encodes a phosphinothricin-*N*-acetyl-transferase (Pat) which inactivates L-Pt by acetylation, resulting in the formation of L-*N*-acetyl-Pt (L-ac-Pt) (Fig. 1). The bacterial *pat* gene was modified to enable its expression in plants, and Pt-resistant tobacco was obtained by *Agrobacterium*-mediated gene transfer (Wohlleben et al. 1988; Broer et al. 1989).

A similar gene with the same function termed *bar* (bialaphos-resistance gene) which showed significant sequence homology to the *pat* gene (Wohlleben et al. 1988), was isolated from *S. hygroscopicus* (Thompson et al. 1987). The *bar* gene was also used to construct trans-

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Abbreviations: ac-Pt = *N*-acetyl-phosphinothricin; MPE = 2-methylphosphinico-ethanoic acid; MPP = 3-methylphosphinico-propanoic acid; *pat* = phosphinothricin-*N*-acetyltransferase gene; Pat = phosphinothricin-*N*-acetyltransferase; *pat*41 = *pat* gene modified for expression in plants; PPO = 4-methylphosphinico-2-oxo-butanoic acid; Pt = phosphinothricin

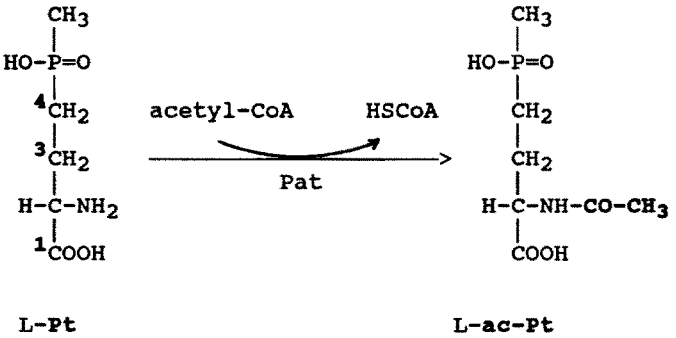

Fig. 1. Enzymatic reaction of phosphinothricin-*N*-acetyl-transferase (*Pat*). The positions of the radiolabeled ^{14}C-atoms are marked by numbers. *L-Pt*, L-phosphinothricin; *L-ac-Pt*, L-*N*-acetyl-phosphinothricin

genic plants resistant to the herbicide Pt (De Block et al. 1987).

Before Pt-resistant crops can be used in agriculture, Pt metabolism should be studied in detail. The degradation of Pt by soil microorganisms has been analysed by Tebbe (1988) and by Smith (1988). It was found that L-Pt was rapidly degraded to form the corresponding oxo acid 4-methylphosphinico-2-oxo-butanoic acid (PPO). This intermediate compound was subsequently decarboxylated to 3-methylphosphinico-propanoic acid (MPP) which formed the major catabolite in the soil (Götz et al. 1983; Tebbe 1988; Tebbe and Reber 1988; Bartsch and Tebbe 1989). To elucidate Pt metabolism in plants, Haas (1986) performed experiments using weeds grown under non-sterile conditions; both MPP and low levels of three unidentified metabolites were detected.

In this paper, we report on the metabolism of L-Pt in Pt-sensitive and Pt-resistant tobacco and carrot plants. We found that L-Pt was metabolized differently in untransformed and in transgenic plants carrying the *pat* gene.

Materials and methods

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this investigation, are listed in Table 1. The conditions of growth of *S. viridochromogenes* Tü494 (Bayer et al. 1972) have been published (Strauch et al. 1988). *Agrobacterium tumefaciens* was cultivated as recommended by Plant Collection, Leiden, The Netherlands. *Escherichia coli* S17.1 was grown at 37° C. Strains were selected on medium supplemented with kanamycin (25 mg · l^{-1}) or ampicillin (100 mg · l^{-1}), depending on the the plasmids used.

Chemicals. The following non-radioactive compounds were provided by Hoechst AG (Frankfurt/Main, FRG): DL-homoalanine-4-yl-(methyl)-phosphinic acid (DL-Pt), DL-2-acetamido-4-methyl-phosphinato-butanoic acid (DL-ac-Pt), 4-methyl-phosphinico-2-oxo-butanoic acid (PPO) and DL-3-amino-3-carboxypropyl-phosphinic acid. DL-2-amino-4-phosphono-butanoic acid and DL-2-amino-butanoic acid were purchased from Sigma (Deisenhofen, FRG).

^{14}C-radiolabeled Pt and its potential metabolites were synthesised by Hoechst AG: DL-[1-^{14}C]Pt (80.4 MBq · $mmol^{-1}$); L-[3,4-^{14}C]Pt (148 MBq · $mmol^{-1}$); L-[3,4-^{14}C]ac-Pt (379.7 MBq · $mmol^{-1}$); [3-^{14}C]methylphosphinico-propanoic acid (MPP) (134.3 MBq · $mmol^{-1}$); [2-^{14}C]methylphosphinico-ethanoic acid (MPE) (127.9 MBq · $mmol^{-1}$).

The L-[1-^{14}C]ac-Pt was produced by Pat-enzyme reaction using a crude bacterial extract (Strauch et al. 1988) and DL-[1-^{14}C]Pt as substrate. Phosphinothricyl-alanyl-alanine (Pt-tripeptide; Ptt), ^{14}C-labeled at the Pt CH_3-group, was prepared in vivo by *S. viridochromogenes* incubated with [$^{14}CH_3$]methionine (Amersham, Braunschweig, FRG). The resulting [^{14}C]Ptt, excreted from the Ptt-producing *Streptomyces* cells was isolated by preparative thin-layer chromatography (TLC).

Molecular cloning and bacterial matings. All DNA manipulations were performed using standard techniques as described by Maniatis et al. (1982). The oligonucleotides were synthesized with a DNA synthesizer (Model 380B; Applied Biosystems, Foster City, Cal., USA) by the phosphoramidite method (Beaucage and Caruthers 1981).

The plasmid pIB16.41 was transferred from *E. coli* S17.1 (Simon et al. 1983) to *A. tumefaciens* LBA4404 (Horsch et al. 1985) by biparental mating, modified as described by Wohlleben et al. (1988).

Table 1. Bacterial strains and plasmid vectors

Strain	Relevant characteristics[a]	Source or reference
S. viridochromogenes Tü494	Ptt^s, Ptt producer	H. Zähner, Tübingen FRG
E. coli S17.1	*E. coli* 294 derivative, chromosomal integrated RP4 derivative, Sm^s, Km^s	Simon et al. 1983
A. tumefaciens LBA4404	pAL4404 (T-DNA$^-$, Vir$^+$), Sm^r, Km^s	Hoekema et al. 1983

Plasmids	Structure	Source or reference
pES6.1	pSVB20 (Arnold and Pühler 1988) derivative carrying the *pat* gene from *Streptomyces viridochromogenes*	Strauch et al. 1988
pROK1	Plant vector derived from pBIN19 (Bevan 1984)	Baulcombe et al. 1986
pIB16.41	pROK1 derivative carrying the modified *pat*41 gene	This work, Fig. 2a
pSVB28	*E. coli* multicopy vector derived from pUC 8 with unique clonig sites for *Eco*RI and *Hin*dIII	Arnold and Pühler 1988
pWD26.41	pSVB28 derivative carrying the modified *pat*41 gene	This work, Fig. 2b

[a] Km, kanamycin; Ptt, Pt-alanyl-analine; Sm, streptomycin; ^{r}, resistant; ^{s}, sensitive

A successful plasmid transfer was proven by gel electrophoresis according to Eckhardt (1978).

Tobacco leaf-disk transformation. Leaf-disk transformation of *N. tabacum* Petit Havana SR1 (Maliga et al. 1973), was performed as described by Horsch et al. (1985) with slight modifications according to Wohlleben et al. (1988).

Direct gene transfer into carrot protoplasts. Carrot (*Daucus carota* cv. Rote Riesen) suspension cultures were grown in MS medium (Murashige and Skoog 1962) supplemented with 40 ml · l^{-1} coconut water (Gibco, Eggenstein, FRG) and 0.5 mg · l^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D), pH 5.8 (MSSC). Subculture was performed weekly. Protoplasts were isolated by a 15- to 17-h enzymatic treatment (2% Cellulase; 1% Macerozyme; 0.5% Driselase; 0.4 M mannitol; 0.025 M $CaCl_2 \cdot 2H_2O$; 0.5% 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 5.6) (Potrykus and Shillito 1986) of the suspension-culture cells. In order to remove cell debris, the protoplasts were filtered through a 60-µm mesh and washed three times in a wash buffer (0.3 M mannitol; 0.125 M $CaCl_2$; 0.5% Mes, pH 5.6). The protoplasts were subsequently collected by centrifugation (100 · *g*). Direct gene transfer was performed according to Hain et al. (1983) and Pröls et al. (1988); 10^6 protoplasts were treated with polyethyleneglycol (PEG) in the presence of 20–40 µg of pWD26.41 plasmid DNA. After DNA uptake, the protoplasts were cultured in a bead-type culture (Shillito et al. 1983) in MS protoplast culture medium (MSPC) which consisted of MS medium supplemented with 0.4 M mannitol, 1 mg · l^{-1} 2,4-D and 0.1 mg · l^{-1} kinetin (N^6-furfurylaminopurine), pH 5.7.

In order to select transgenic Pt-resistant calli, the growth medium was supplemented with 20 mg · l^{-1} Pt after 2 d of culture. The culture medium was changed every 10 d and the osmotic pressure of the medium was decreased by reducing the mannitol concentration. After eight weeks, Pt-resistant calli of approx. 4 mm in size were transferred to solid MS medium without hormones, so as to induce somatic embryogenesis and to regenerate transgenic carrot plants.

Plant culture conditions and treatment with radioactively labeled compounds. The transgenic and untransformed tobacco and carrot plants were grown on solid MS medium at 23° C and a 16-h daylight period, under sterile conditions.

Usually, 40–120 kBq of the radiolabeled Pt or its metabolites in aqueous solution was spread by means of a pipette on the surfaces of leaves of growing plants.

Experimental setup for measuring the release of $^{14}CO_2$. The [^{14}C]Pt-treated tobacco and carrot plants were cultured in a closed container under the continuous air flow generated by a peristaltic pump. The $^{14}CO_2$ formed by the Pt metabolism of the plant was transported by the air flow into 10 ml of 1 M NaOH solution. The radioactivity of the $^{14}CO_2$ trapped in the basic solution was determined by taking a scintillation count of a mixture of 4 ml 1 M NaOH solution, 4 ml of methanol and 12 ml of scintillation cocktail (Aquasol, Du Pont, Bad Nauheim, FRG) using a scintillation counter (TRI–CARB, Beckman, München, FRG). In order to study the kinetics $^{14}CO_2$ release, the amount of radioactivity measured was calculated as the percentage of the amount of [^{14}C]Pt applied (60 kBq) and plotted against the incubation time. Only the released $^{14}CO_2$ was detected; the fraction of $^{14}CO_2$ fixed by the plant could not be measured.

Preparation of plant extracts and TLC. Extracts were made of plant leaves by grinding the material, submerged in water or Tris buffer, in an Eppendorf tube. A metal rod made to fit the Eppendorf tube exactly was used as a pestle for the extraction procedure. The samples were placed at 90° C for 5 min to denature the proteins. Thereafter, the extract was cleared of debris by centrifugation.

The components of the supernatant (5–10 µl) were separated by TLC on high-performance (HP)TLC cellulose plates (Merck, Darmstadt, FRG). The plates were developed twice in the following solvent: pyridine, *n*-butanol, acetic acid and water (10:15:3:12, by vol.) (Strauch et al. 1988). The radiolabeled metabolites were visualized by autoradiography (X-ray film; Du Pont) and identified by the co-chromatography of reference compounds.

In order to isolate radiolabeled compounds from TLC plates, both HPTLC cellulose and preparative cellulose plates (0.5 mm; Merck) were used. The material corresponding to the radiolabeled spots was scraped from the plates and the substances were eluted in water.

Test for Pat activity. Plant leaf material (100 mg) was extracted in 50 µl of Pat extraction buffer (0.5 M Tris-HCl pH 7.5; 0.4 mM EDTA; 0.15 mg · ml^{-1} Leupeptin; 0.15 mg · ml^{-1} phenylmethylsulphonylfluoride (PMSF); 0.3 mg · ml^{-1} dithiothreitol (DTT); 0.3 mg · ml^{-1} bovine serum albumin (BSA) as described above. A 45-µl aliquot of extract was incubated with 2 µl of L-[3,4-^{14}C]Pt (4 kBq) and 3 µl of acetyl-CoA (100 mM) for 30 min at 30° C to enable the enzymatic reaction to occur. The reaction was stopped by placing the samples at 90° C for 5 min after which the extract was cleared by centrifugation. Finally, 5 µl of the supernatant was tested in the TLC system described above.

Specific staining of TLC plates. Organic acids and oxo acids were visualized on TLC plates by incubation with 2,6-dichlorophenol-indophenol-sodium salt (0.1% in ethanol). Red spots appeared after a short heat treatment (100° C) (Passera et al. 1964).

Results

Construction of transgenic, Pt-resistant tobacco and carrot plants. In order to study the metabolism of the herbicide Pt in transgenic Pt-resistant plants, a modified Pt-resistance gene from *S. viridochromogenes* encoding the *pat* gene, was introduced into tobacco and carrot. For this purpose, two plant transformation vectors were constructed (Fig. 2). The *pat*-gene coding sequence, isolated as an *Xho*II–*Bgl*II fragment from *S. viridochromogenes* (Strauch et al. 1988), was modified by the addition of two synthetic DNA fragments to enable its expression in plants. The first synthetic DNA fragment converted the original GTG start codon into an ATG codon and added a ribosome-binding site to enable expression in bacteria. The four bases upstream of the ATG codon were designed as proposed by Lütcke et al. (1987) to optimize translation in plants. The second DNA fragment was fused at the 3′ end of the coding region to provide a new translational stop codon, 3 base pairs (bp) upstream of the original one. This modification resulted in the addition of a phenylalanine to the Pat protein. This *Bam*HI-*Bgl*II fragment was inserted in the *Bam*HI site of the binary plant vector pROK1 (Baulcombe et al. 1986), leading to a gene fusion with the 35S promoter (p35S) of the cauliflower mosaic virus. Downstream of the *pat* coding sequence, the 3′ end of the nopaline-synthetase gene (3′ nos) provided the necessary polyadenylation signal. The resulting vector pIB16.41, carrying the *pat*41 fusion gene (Fig. 2a), was transferred to *A. tumefaciens* LBA4404 by conjugation and used to transform tobacco leaf disks. Subsequently, Pt-resistant tobacco plants were regenerated.

The Pt-resistant carrot plants were obtained by the following procedure: the *pat*41 gene was isolated as an *Eco*RI-*Hin*dIII fragment from pIB16.41 and inserted in

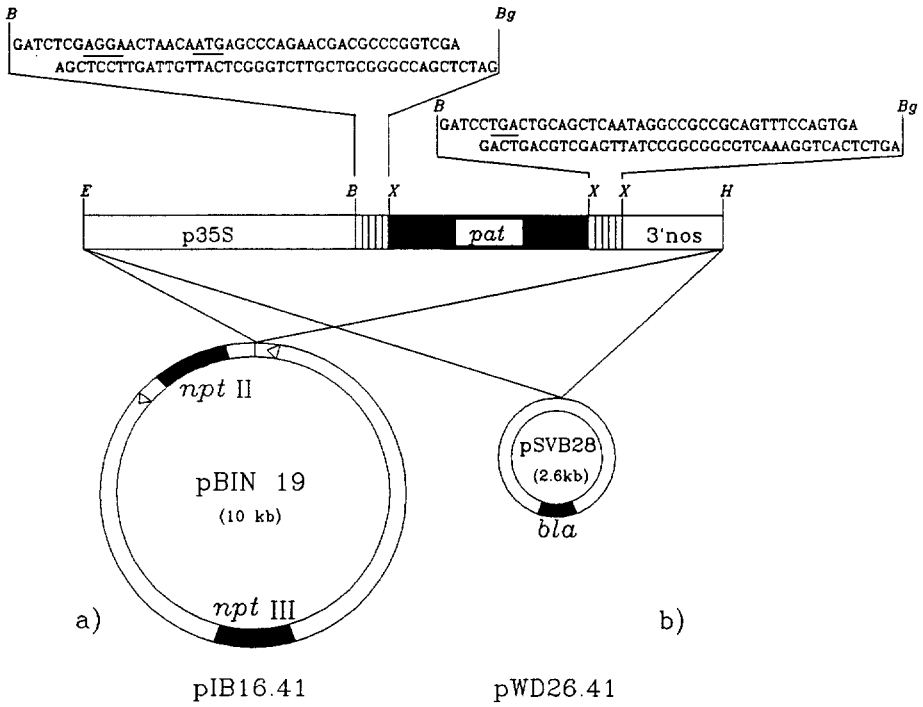

Fig. 2a, b. Plant transformation vectors carrying the phosphinothricin-*N*-acetyl transferase gene modified to enable its expression in plants (*pat*41): An *Xho*II-*Bgl*II fragment from *S. viridochromogenes* encoding the *pat* gene *(filled box)* was annealed to two synthetic oligonucleotides *(hatched boxes)* which changed the start and stop codons *(underlined)* and which included a bacterial ribosome-binding site *(underlined)*. The resulting fragment was fused to the 35S cauliflower-mosaic-virus promoter (p35S) and to the 3′ end of the nopaline-synthase gene (3′nos) *(open boxes)* (for details see text). **a** Restriction endonuclease map of the vector pIB16.41 which consists of the chimaeric *pat*41 gene cloned into the binary vector pBIN19 (Bevan 1984). **b** Restriction-endonuclease map of the vector pWD26.41 which consists of the *pat*41 gene cloned into the multicopy plasmid pSVB28 (Arnold and Pühler 1988). *npt*II, chimaeric gene encoding kanamycin resistance in plants; *npt*III, kanamycin resistance gene from *S. faecalis* used as a selection marker in bacteria; *bla*, ampicillin resistance gene; ◁ T–DNA border sequences; abbreviations of the restriction endonucleases: *H*, *Hin*dIII; *B*, *Bam*H1; *X*, *Xho*II; *Bg*, *Bgl*II; *E*, *Eco*RI

the high-copy-number vector pSVB28 (Arnold and Pühler 1988). The resulting plasmid was called pWD26.41 (Fig. 2b). The DNA of this plasmid was transferred into carrot protoplasts by direct gene-uptake experiments. Phosphinothricin-resistant carrot calli were regenerated on Pt-containing medium (20 mg·l^{-1}), while untransformed calli died. Regenerated plants showed Pt resistance and the expected Pat enzyme activity.

Degradation of L-*Pt in untransformed tobacco and carrot plants.* To analyse the metabolism of the herbicide Pt in planta, untransformed tobacco and carrot plants were incubated with radioactive [^{14}C]Pt in order to detect radioactively labeled degradation or modification products. The active form of the DL-Pt racemate is the L-enantiomer; hence, the metabolism of the L-Pt was investigated. However, since the commercial Pt formula BASTA® contains the DL racemate, the metabolism of D-Pt should also be analysed. Preliminary data obtained from transgenic, herbicide-resistant plants, which selectively acetylated the L-enantiomer of a DL-Pt racemate, indicated that D-Pt was not metabolized further by the plant (see below). Since D-Pt appears to be stable in plants, it can be regarded as a constant and, therefore, it is possible to use DL-Pt to investigate L-Pt metabolism.

The use of radioactive compounds, labeled at specific positions in the molecule, enabled the fate of Pt to be monitored and its metabolic pathway in plants to be elucidated. Radiolabeled L-[3,4-^{14}C]Pt and DL-[1-^{14}C]Pt were applied to examine differences between the degradation of the central C3,C4-atoms and the C1-atom of the COOH-residue (Fig. 1).

An aqueous solution of the radiolabeled herbicide was applied to a leaf surface, and its uptake and distribution was visualized by autoradiography of the whole plant. Plants grown under sterile conditions were used to exclude the additional metabolic activity of bacterial contaminants.

The COOH-group of L-*Pt is rapidly decarboxylated in untransformed plants.* Since the decarboxylation of the L-Pt COOH-group, a reaction likely to occur, was observed in microorganisms (Tebbe and Reber 1988), we investigated whether this reaction also occurs in plants by measuring the formation of $^{14}CO_2$ by plants incubated with DL-[1-^{14}C]Pt. The CO_2 gas released by the plant was trapped in NaOH solution, and the level of radioactivity determined by scintillation counting (Fig. 3).

The incubation of untransformed tobacco (Fig. 3A)

and carrot (Fig. 3C) plants with DL-[1-^{14}C]Pt led to the formation of $^{14}CO_2$. Approximately 6% of the radioactivity applied was trapped in the washing solution 600 h after application. This can be explained by a decarboxylation of the C1 COOH-group.

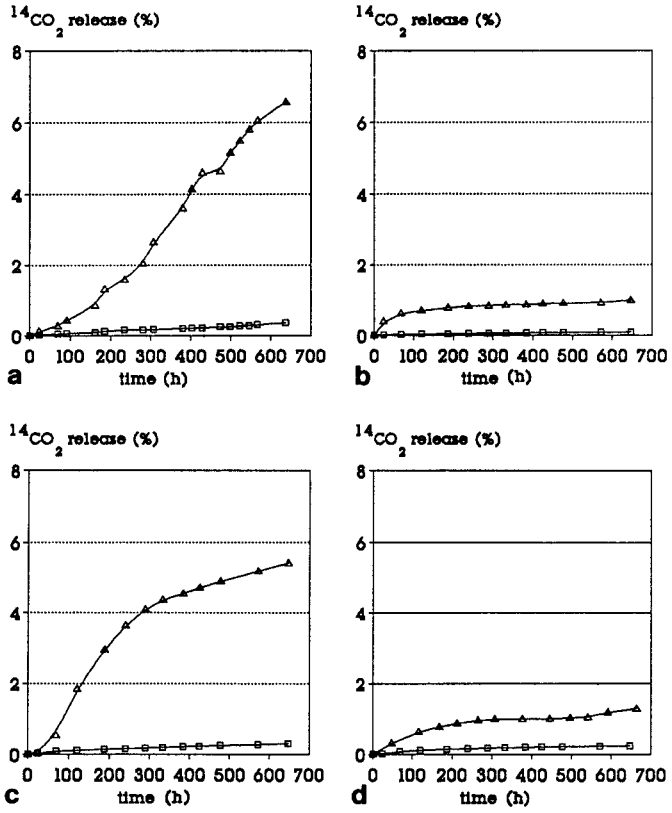

Fig. 3a–d. Cumulative $^{14}CO_2$ released from untransformed and transgenic tobacco (**a, b** respectively) and carrot (**c, d** respectively) plants treated with either L-[3,4-^{14}C]Pt (□–□) or DL-[1-^{14}C]Pt (△–△). The extent of Pt degradation to form CO_2 was determined by measuring the $^{14}CO_2$ released by the plant and trapped in NaOH. The amount of radioactivity is given as a percentage of the radioactivity applied

In contrast, there was no significant $^{14}CO_2$ release from untransformed tobacco and carrot plants incubated with L-[3,4-^{14}C]Pt (Fig. 3a, c). The amount of $^{14}CO_2$ released was comparable to that of the control experiment which did not contain plants. The C3,C4-backbone of the Pt molecule was evidently very stable with regard to oxidation. This finding was supported by the observation that there was no detectable $^{14}CO_2$ release when [3-^{14}C]MPP (see Fig. 6), a metabolite generated by successive deamination and decarboxylation of L-Pt in microorganisms (Tebbe et al. 1988), was applied to untransformed tobacco plants (data not shown).

Detection of three L-Pt metabolites in untransformed plants: PPO, MPP and an unknown metabolite. The leaves of tobacco and carrot plants were incubated with radioactive [^{14}C]Pt, and after defined periods of incubation the progressive degradation of L-Pt was monitored in leaf extracts by TLC. Radiolabeled degradation or modification products of the herbicide Pt could be detected by autoradiography, and were identified by comparing their migration with that of reference substances.

Incubation with L-[3,4 ^{14}C]Pt led to the formation of three different degradation products designated D2, D3, D4, while incubation with DL-[1-^{14}C]Pt resulted in the formation of only two compounds with the same mobilities as D2 and D3 (Fig. 4). An additional labeled compound migrating with the solvent front was found to be an impurity of the applied [^{14}C]Pt.

These experiments indicated that D2 and D3 still carried the C1-atom of the COOH-group, while D4 did not. D4 seemed to be the product of the decarboxylation reaction described above. Co-migration studies showed that D4 had the same mobility as MPP (Fig. 4). Since this compound does not carry the C1-atom of the Pt COOH-group, incubation with [1-^{14}C]Pt should not result in the formation of labeled MPP (Fig. 4a).

D2 showed the same mobility as the non-radioactive PPO reference (the colour staining of the thin-layer chromatogram is not shown). The D2 compound isolated by preparative TLC was not stable under sterile conditions. In water it decomposed spontaneously to form MPP.

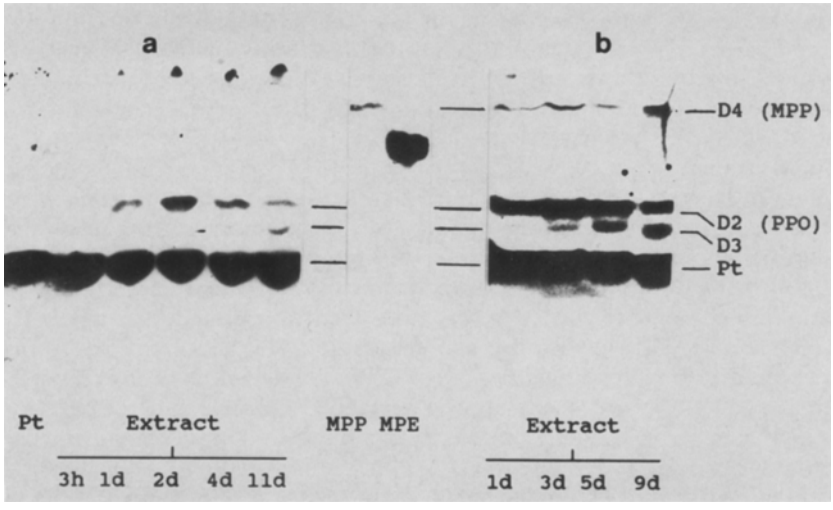

Fig. 4a, b. Cellulose TLC of extracts of untransformed tobacco plants incubated with DL-[1-^{14}C]Pt (**a**) for 3 h, 1 d, 2 d, 4 d, and 11 d or L-[3,4-^{14}C]Pt (**b**) for 1 d, 3 d, 5 d and 9 d. *D2, D3, D4,* degradation products; *MPP, MPE,* reference substances

These data indicate that a metabolic pathway leads from L-Pt via PPO to MPP (see Fig. 6), and correspond to the findings of the CO_2-release studies. The incubation of tobacco plants with radioactively labeled PPO isolated by preparative TLC from a tobacco plant extract, showed that decarboxylation occurred to form MPP. Concurrently PPO reverted to Pt (data not shown). Therefore, the first degradation step of L-Pt metabolism, the formation of PPO, is reversible in tobacco plants.

A further decarboxylation of MPP, resulting in the formation of MPE, seems likely and has been observed during the metabolism of Pt by soil microorganisms (K. Stumpf, Hoechst AG, Frankfurt, FRG, personal communication). Nevertheless, TLC studies of plant extracts did not reveal a radiolabeled compound with the mobility of MPE (Fig. 4). In order to study the possible degradation of MPP, tobacco plants were incubated with radioactive [3-^{14}C]MPP. Because MPP is not phytotoxic, the incubation could continue for a long period of time, and MPP was found to be a stable metabolite even after an incubation of 80 d.

After incubation of tobacco leaf extracts for a few days in the presence of L-Pt, D3 accumulated. Since D3 still contained the C1-atom, it is not an MPP metabolite. The increasing signal strength of D3 which was directly correlated with the decrease of PPO (D2), indicated that PPO was transformed to D3 (Fig. 4).

D3 was formed by either a modification of the L-Pt molecule, or by the cleavage of the P–C- or C–C-bonds. To test the possible cleavage of P–C or C–C bonds in the L-Pt molecule, tobacco plants were treated with the radiolabeled herbicide. The CH_3-group at the other end of the molecule was ^{14}C-labeled. Since this compound was not available commercially, [$^{14}CH_3$]Pt-alanyl-alanine (Ptt) was isolated from the Ptt-producing *S. viridochromogenes* which had been treated with [$^{14}CH_3$]methionine. In plant cells, Ptt is cleaved to form the active herbicide L-Pt (Wild and Ziegler 1989). In our experiments, a labeled compound was detected which showed the same mobility in TLC as the compound D3 after labeling at the C3, C4 or C1 position (data not shown). Hence, cleavage of a P–C bond in the Pt backbone does not occur.

To ascertain the chemical structure of D3, the following slightly modified Pt molecules were used as reference substances in co-migration studies: 2-amino-4-phosphono-butanoic acid and 3-amino-3-carboxypropyl-phosphinic acid. None of these compounds showed a mobility similar to that of D3 (data not shown).

Analogous experiments were performed with untransformed carrot plants. The radiolabeled metabolites detected in untransformed tobacco and carrot plants showed comparable mobilities in the TLC systems used (data not shown). Hence, the L-Pt metabolites seemed to be the same in untransformed tobacco and carrot plants.

Metabolism of Pt in transgenic, herbicide-resistant tobacco and carrot plants. Transgenic Pt-resistant tobacco and carrot plants were analysed in the same way as the untransformed plants. After the leaves were treated with a solution of radioactive [^{14}C]Pt, decarboxylation was determined by measuring the $^{14}CO_2$ released, and the radiolabeled Pt metabolites separated by TLC.

Decarboxylation of the COOH-group of L-Pt does not occur in transgenic, herbicide-resistant plants. The amount of $^{14}CO_2$ released was measured in sterile, transgenic tobacco and carrot plants which had been incubated with DL-[1-^{14}C]Pt. Only a low level of the radioactivity applied (approx. 1%) was detected as $^{14}CO_2$ (Fig. 3b, d). This is comparable to the rate of spontaneous decarboxylation of DL-[1-^{14}C]Pt in the absence of a plant. In contrast, untransformed plants released 6% of the applied radioactivity in the form of $^{14}CO_2$ during the same period of time.

Detoxification of L-Pt in transgenic plants occurs as a result of acetylation of the L-Pt amino residue. In untransformed plants, the metabolic pathway consists of a deamination step which precedes the decarboxylation. The different rates of C1 decarboxylation in transgenic and untransformed plants could be due to the *N*-acetylation of the L-Pt amino residue which prevents the deamination from occurring. To test this hypothesis, tobacco plants were incubated with L-[1-^{14}C]ac-Pt and no $^{14}CO_2$ was detected (data not shown). This indicates that L-ac-Pt is stable with regard to C1 decarboxylation, and supports the assertion that decarboxylation occurs only after deamination has taken place. After incubation with L-[3,4-^{14}C]ac-Pt no $^{14}CO_2$ was formed. Hence, the L-ac-Pt backbone showed the same stability with respect to oxidation as was observed for Pt.

In herbicide-resistant plants, L-Pt is rapidly converted to L-ac-Pt which exists in three different modified forms. Leaf extracts from transgenic plants were analysed for the presence of radiolabeled Pt metabolites. These metabolites were separated by TLC and visualized by autoradiography.

In transgenic tobacco and carrot plants incubated with DL-[1-^{14}C]Pt and L-[3,4-^{14}C]Pt, a compound with the same mobility as L-ac-Pt was found (Fig. 5). This rapid acetylation of L-Pt was detectable after only 2 h. Analogous to the CO_2-release experiments, the *N*-acetylation of the L-Pt amino-group prevents the normal metabolism of L-Pt. This result is supported further by the observation that only very low levels of PPO and MPP were detectable by TLC in transgenic plants.

After incubation with the racemic DL-[1-^{14}C]Pt, approx. 50% of the herbicide was *N*-acetylated (Fig. 5a) while the L-enantiomer was completely transformed to L-ac-Pt (Fig. 5b). Because of the stereospecifity of the Pat enzyme the D-enantiomer remained non-acetylated. The hypothesis that D-Pt is not transformed in plants was supported by the finding that no further D-Pt-specific metabolites were detected in the transgenic plants.

Figure 5 shows that in addition to L-ac-Pt a compound designated D1 appeared in transgenic plants. The incubation with DL-[1-^{14}C]Pt proved that both L-ac-Pt and D1 still carried the C1 COOH-group. The formation of D1 was independent of time, and no further degradation of the L-ac-Pt/D1 pair was observed over a period of 100 d. The incubation of transgenic plants with L-

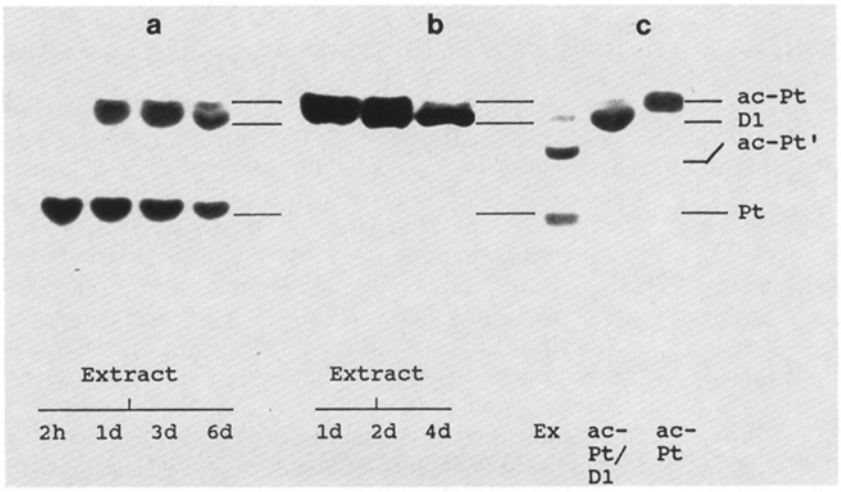

Fig. 5a–c. Cellulose TLC of extracts of transgenic tobacco incubated with DL-[1-^{14}C]Pt (**a**) for 2 h, 1 d, 3 d, and 6d or L-[3,4-^{14}C]Pt (**b**) for 1 d, 2 d, and 4 d. **c** Plant extracts showing a change of L-ac-Pt to ac-Pt' (*Ex*). Reference substances: *ac-Pt*, L-*N*-acetyl-Pt; *D1*, degradation product D1 isolated from plants

[3,4-^{14}C]-ac-Pt led to the formation of the same L-ac-Pt/D1 pair. No further degradation occurred and L-ac-Pt was not reversibly cleaved to form Pt. Therefore, both L-ac-Pt and D1 are stable in tobacco plants. Even a short treatment of L-ac-Pt with a plant extract led to the production of the L-ac-Pt/D1 signal, indicating that the conditions in the plant were responsible for the formation of D1.

Under different experimental conditions, another radiolabeled product, ac-Pt', appeared in tobacco plants and could be isolated by preparative TLC (Fig. 5c). An aqueous solution of ac-Pt' showed the same mobility as L-ac-Pt. It was demonstrated that the same shift in TLC mobility occurred after L-ac-Pt was treated with NaOH and that this conversion could be reversed by acid treatment. By contrast, Pt did not show this pH-dependant conversion. These data indicate that, depending on the pH, L-ac-Pt can reversibly convert into a second form (ac-Pt').

Discussion

In this work, tobacco and carrot were used as model systems to study the metabolism of the herbicide L-Pt in plants. It is well known that only the L-enantiomer of Pt has herbicidal activity. The Pt-degrading enzymes characterized in microorganisms selectively transform the L-enantiomer (Tebbe 1988; Bartsch and Tebbe 1989). Consequently, this work concentrated on the metabolism of L-Pt in higher plants. The commercially available herbicide BASTA®, contains the racemic DL-Pt. According to preliminary data on the metabolism of DL-Pt in transgenic, herbicide-resistant plants, D-Pt is stable in these plants which transform only half of the DL-Pt racemate by acetylation. This is due to the L-specific acetylation activity of the Pat enzyme; the remaining Pt can be regarded as the D-enantiomer. In these plants a specific metabolism of D-Pt can be distinguished from the acetylated L-enantiomer. Hence, we assume that D-Pt is stable in plants. Studies on the metabolization of D-Pt need to be conducted as soon as the radiolabeled compound is available. Furthermore, it was necessary to distinguish untransformed from transgenic plants.

Construction of transgenic tobacco and carrot plants carrying the Pt-resistance gene pat41. In order to obtain Pt-resistant plants, the detoxifying *pat* gene was modified to allow its expression in plants. In contrast to the modified *pat* gene described by Wohlleben et al. (1988) which encodes an extended fusion protein, the *pat*41 gene presented in this work results in a defined protein prolonged by one additional amino acid only. Besides this feature, the modified gene has the advantage of a 5′ ribosome-binding site which enables the gene to be expressed in bacteria. Two plant transformation vectors carrying this resistance gene were constructed. The binary vector pIB16.41, based on pBIN19, is a convenient vector for *Agrobacterium*-mediated gene transfer. Transgenic tobacco plants expressing the Pt-resistance phenotype, were obtained. A second plant vector pWD26.41, based on a multicopy replicon, enables the large amounts of DNA necessary for direct gene-uptake experiments to be isolated. Carrot protoplasts were transformed by the aid of this vector and Pt-resistant plants were regenerated.

In untransformed plants, L-Pt is metabolized by successive deamination and decarboxylation to finally yield MPP. In untransformed plants, a rapid decarboxylation of the L-Pt COOH-residue was detected by CO_2-release studies. The TLC analysis of the radioactively labeled metabolites revealed MPP and the 2-oxo acid (PPO) which is derived from Pt (Fig. 6). This indicates that the initial chemical change occurs at the amino terminus of the L-Pt molecule: L-Pt is deaminated to form PPO which is subsequently decarboxylated to yield MPP. This metabolic pathway (Fig. 6) also occurs in soil microorganisms. Bartsch and Tebbe (1989) described two different enzymatic activities in soil bacteria, an oxidase and a transaminase reaction which catalysed the degradation. Schulz et al. (1990) isolated from *E. coli* K12, a transaminase which was responsible for catalysing the reversible transamination reaction between Pt and PPO. The

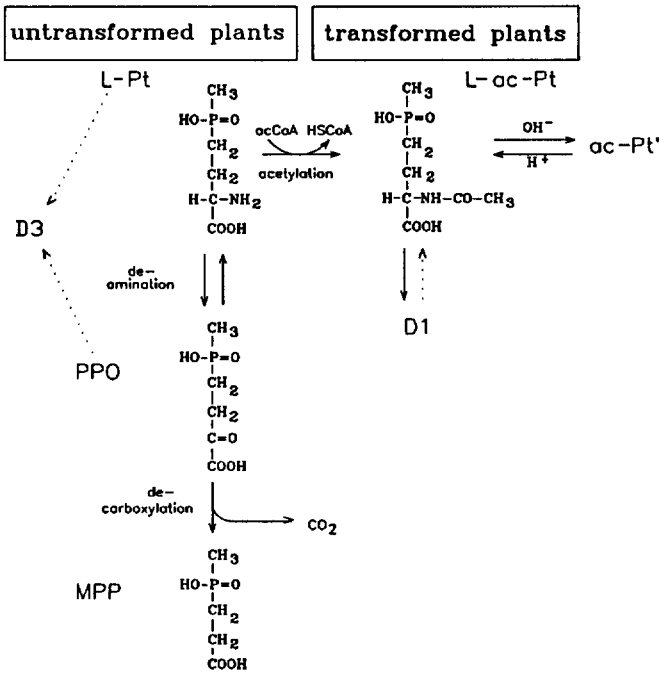

Fig. 6. Postulated metabolic pathway of L-Pt in plants. In untransformed plants, L-Pt is deaminated to form PPO; PPO is decarboxylated to yield MPP which is stable. D3 is an unidentified compound formed by PPO or L-Pt *(dotted lines)*. In transgenic plants, L-Pt is present in the form of *N*-acetylated L-ac-Pt which is partly converted into another substance (D1). Both are stable in plants. Depending on pH, an equilibrium reaction exists between L-ac-Pt and a modified form ac-Pt'

transaminase-encoding gene was cloned by Bartsch et al. (1990). In tobacco, the deamination reaction was reversible; after incubation with radioactive [3,4-^{14}C]PPO, Pt was detected. These findings indicate that enzyme activities comparable to those in bacteria are present in plants. A transamination reaction is likely to occur since an oxidative deamination is irreversible. The decarboxylation of PPO was found to occur spontaneously in aqueous solution under abiotic conditions. Therefore, an enzymatic activity in this second metabolic step is not necessary.

The rates at which Pt degradation occur in bacteria and plants are different. We found that Pt was rapidly C1-decarboxylated in unsterile soil (approx. 50% in 25 d, data not shown). Over the same time period, Tebbe (1988) found Pt-C1-decarboxylation rates of 75%, 74.5% and 68.5% in three different soils. In plants, only 6% of the Pt applied was decarboxylated in 25 d. Therefore, taking into consideration that D-Pt is stable, 12% of the applied L-Pt is decarboxylated. It is difficult to compare the decarboxylation rates of microorganisms and plants as the amount of $^{14}CO_2$ re-fixed by the plant cannot be determined; nevertheless, the difference is substantial.

The differences between bacteria and plants with respect to the CO_2 released from the C3, C4 positions, were even more pronounced. The degradation of DL-[3,4-^{14}C]Pt in unsterile soils was analysed by Tebbe (1988) who found that, depending on the type of soil, in 25 d 25.3%, 13.6% or 9.2% of the radioactivity applied was released by $^{14}CO_2$ formation. The CO_2-release studies performed by Smith (1988) resulted in comparable data. These findings support the hypothesis that the C3, C4 backbone of the Pt molecule is progressively degraded in soil. In agreement with these findings, the next possible decarboxylated compound, MPE, was detected during the metabolism of Pt by soil bacteria (K. Stumpf, personal communication). Studying the metabolism of Pt in rats, Dorn et al. (1986) found that MPP was modified further to form 3-methylphosphinico-3-oxo-propanoic acid. In contrast to these findings, MPP was not degraded further in tobacco plants, and was found to be stable even after 80 d of incubation. Hence, MPP seems to be the final metabolite in untransformed plants. It remains to be elucidated whether this compound is accumulated in plants or excreted into the soil.

Haas (1986) studied Pt metabolism in weeds grown under unsterile conditions and found MPP to be the major catabolite. Three further uncharacterised compounds were found at very low concentrations. In our study, the formation of PPO and a third compound, designated D3, exceeded that of MPP. These different findings may be a consequence of the experimental design or the plant species used.

Besides the metabolic pathway of Pt → PPO → MPP (see Fig. 6), a second pathway was investigated. The compound D3 was not generated from MPP, but was found to be a product of PPO or L-Pt. The precursor molecule could not be defined since the reaction between L-Pt and PPO was reversible. In order to identify the chemical structure of the compound D3, Pt radioactively labled at the C1, C3, C4 and C6 positions was used to study a possible cleavage of the P–C or C–C bond. All experiments revealed the same D3 labeled compound; hence, the cleavage of the Pt backbone could be excluded. These data correspond to those for Pt metabolism of bacteria where P–C cleavage was not detected (Tebbe 1988). Degradation of the structurally related herbicide glyphosate (*N*-(phosphonomethyl)-glycine) by P–C cleavage, has however, been observed (Talbot et al. 1984; Pipke et al. 1987).

In summary, D3 results from the modification of L-Pt or PPO and not from the degradation of L-Pt. Its precise chemical structure remains to be established.

In transgenic, Pt-resistant plants, N-acetylation prevents degradation of the herbicide. In transgenic tobacco and carrot plants, L-Pt was rapidly *N*-acetylated. The CO_2-release studies of transgenic plants incubated with DL-[1-^{14}C]Pt and L-[1-^{14}C]ac-Pt, demonstrated that decarboxylation of the COOH-group hardly occurred. This indicated that a deamination reaction, which was inhibited by an *N*-acetylation, was necessary for the subsequent decarboxylation observed in untransformed plants (see Fig. 6). Therefore, the progression of this pathway was prevented in transgenic plants. Hence, only low levels of PPO and MPP, the dominant metabolites in untransformed plants, were detected in transgenic plants after L-Pt treatment. These metabolites were probably the degradation products of the remaining, non-acety-

lated L-Pt. Degradation of L-ac-Pt was not observed; even the acetylation reaction was not found to be reversible. In summary, L-ac-Pt is a stable compound with respect to degradation in transformed plants.

Although L-ac-Pt was found to be stable, two other forms of the compound were identified as a result of different mobilities during TLC. Besides L-ac-Pt, we detected a second compound designated D1, which showed a very similar mobility. The formation of D1 was not time-dependent. Even a short incubation of L-ac-Pt with plant extracts led to the formation of D1, indicating that the formation of D1 depends on the biochemical conditions existing in the plant. A chemical equilibrium possibly exists between L-ac-Pt and D1. These two substances (L-ac-Pt and D1) were separable only in the cellulose TLC system described. Other TLC or high-performance liquid chromatography systems failed to reveal that there were two substances. Consequently, L-ac-Pt and D1 are probably chemically related compounds. D1 was isolated by preparative TLC in order to be chemically characterized. Both L-ac-Pt and D1 are stable in plants even after a long period (> 100 d), implying that these compounds accumulate in plants.

Under alkaline conditions, L-ac-Pt showed a considerable change in its mobility during TLC (ac-Pt'), enabling it to be separated from D1 which had a consistent mobility. This different chemical behaviour of L-ac-Pt proved that there were indeed two different compounds. The conversion of L-ac-Pt to ac-Pt' was reversible and depended on the pH conditions. Phosphinothricin did not show any pH-dependent mobility changes indicating that this phenomenon was not the result of a protonation/deprotonation reaction. The chemical structure of ac-Pt' is currently being analysed.

The same degradation products were observed in the tobacco and carrot species studied, indicating that L-Pt metabolism, as summarized in Fig. 6, is similar among dicotyledonous plants. Further characterization of single metabolites as well as toxicological studies are, however, necessary. It remains to be determined whether these substances are transported in plants, deposited in specific organs such as in fruits, or excreted.

Since the metabolism of the herbicide L-Pt is only one aspect of the evaluation of transgenic, Pt-resistant crops which are to be applied in agriculture, investigations addressing the release of transgenic plants, the stability of transformed genes under field conditions, as well as ecological and agricultural aspects, have to be undertaken.

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Note added in proof: Analysis of L-ac-Pt by NMR-spectroscopy did not reveal any differences dependent on pH. According to these data, L-ac-Pt' and L-ac-Pt cannot be regarded as two different chemical compounds.