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The Metabolites of the Herbicide L-Phosphinothricin (Glufosinate)[1]

Identification, Stability, and Mobility in Transgenic, Herbicide-Resistant, and Untransformed Plants

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The metabolism of the herbicide L-phosphinothricin (L-Pt) was analyzed in tobacco (*Nicotiana tabacum*), alfalfa (*Medicago sativa*), and carrot (*Daucus carota*). In transgenic, Pt-resistant plants expressing the Pt-*N*-acetyltransferase gene (*pat*), L-Pt was acetylated, resulting in two forms of *N*-acetyl-Pt (ac-Pt). In transgenic plants expressing only low *pat*-encoded acetylating activity as well as in genetically unmodified plants, three metabolic compounds 4-methylphosphinico-2-oxo-butanoic acid, 3-methylphosphinico-propanoic acid (MPP), and 4-methylphosphinico-2-hydroxy-butanoic acid (MHB) were identified. Hence, the transgene-encoded acetylation of L-Pt competes with a plant-specific degradation. The compounds MPP, MHB, and ac-Pt were found to be the final, stable products of the plant's metabolic pathways. The mobility of these stable compounds in the plant was investigated: L-Pt as well as the derived metabolites were found to be preferentially transported to the upper regions of the plant.

L-Pt [L-homoalanine-4-yl-(methyl)-phosphinic acid], also known as glufosinate, is the active ingredient of the broad spectrum herbicide Basta (Hoechst AG, Frankfurt/Main, Germany). L-Pt appears to be a phosphinic acid analog of L-glutamate and, therefore, acts as a potent competitive inhibitor of Gln synthetase (Bayer et al., 1972; Lea et al., 1984), a key enzyme of nitrogen metabolism in plants. After herbicide is applied a rapid accumulation of ammonia (Tachibana et al., 1986b), a deficiency in Gln (Tachibana et al., 1986a), and an inhibition of photosynthesis (Sauer et al., 1987) can be observed. Finally, the herbicide's action results in death of the plant cells (Bayer et al., 1972; Tachibana et al., 1986a, 1986b).

Being a nonselective herbicide, L-Pt is of limited use in agriculture. Hence, genetic engineering of Pt-resistant plants allows the enlargement of the application of the herbicide in transgenic crop cultures. The cloning of a Pt-resistance gene (*bar*) from *Streptomyces hygroscopicus* (Thompson et al., 1987) and the engineering of Pt-resistant plants has been described by De Block et al. (1987). Simultaneously, Strauch et al. (1988) isolated a similar gene (*pat*) from *Streptomyces viridochromogenes* Tü494 with the same function. We have used the *pat* gene to engineer herbicide-resistant tobacco (*Nicotiana tabacum*) (Wohlleben et al., 1988; Broer et al., 1989; Dröge et al., 1992) and carrot (*Daucus carota*) plants (Dröge et al., 1992). A *pat*-based synthetic gene with a codon optimized for plant systems has been used to transform alfalfa (*Medicago sativa*) (Eckes et al., 1989). The Pt-resistance gene directs the synthesis of the Pat, which specifically modifies L-Pt by *N*-acetylation and, therefore, detoxifies the herbicide (Wohlleben et al., 1988; Broer et al., 1989; Dröge et al., 1992).

Studies have been performed to evaluate the consequences of the application of the herbicide Pt and the resistant crops in agriculture. The toxicology of L-Pt has been intensively analyzed (Lejczak et al., 1981; Lacoste et al., 1985; Dorn et al., 1986; Logusch et al., 1989) and was summarized by Ebert et al. (1990). It is safe under the conditions of recommended use, but, according to European Economic Community directive 83/467/EEC, it has to be classified as harmful on the basis of testing for acute oral toxicity (Ebert et al., 1990).

The metabolism of Pt in soil has first been studied by Tebbe and Reber (1988, 1991) and also by Smith (1988, 1989). L-Pt is rapidly degraded to form the corresponding oxo acid PPO as an intermediate compound (Tebbe and Reber, 1988), which is subsequently decarboxylated to form MPP (Tebbe and Reber, 1988; Bartsch and Tebbe, 1989; Smith, 1989; Gallina and Stephenson, 1992). In soil, a second decarboxylation step results in MPE (Behrendt et al., 1990).

To consider Pt-resistant plants as a source of food production, it is absolutely necessary to analyze the metabolism in these plants. In genetically unmodified plants, compound

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Abbreviations: Ac_2O, acetic anhydride; ac-Pt, *N*-acetyl-L-phosphinothricin; AEP, 2-amino-ethyl-phosphoric acid; APB, 2-amino-4-phosphono-butanoic acid; D1, D2, D3, D4, L-Pt metabolites; DMP, 3-amino-3-carboxypropyl-phosphinic acid (desmethyl-phosphinothricin); HPTLC, high-performance thin-layer chromatography; MHB, 4-methylphosphinico-2-hydroxy-butanoic acid; MPE, 2-methyl-phosphinico-ethanoic acid; MPP, 3-methylphoshinico-propanoic acid; *pat*, phosphinothricin-*N*-acetyltransferase gene; Pat, phosphinothricin-*N*-acetyltransferase; PPO, 4-methyl-phosphinico-2-oxo-butanoic acid; Pt, phosphinothricin.

MPP was detected in addition to three other unidentified, low-level metabolites (Haas, 1986). Recently, we reported that the metabolism of L-Pt in transgenic, Pt-resistant plants differs from the pathways observed in genetically unmodified plants. In transgenic, Pt-resistant plants ac-Pt and an additional metabolite designated D1 have been detected. In genetically unmodified plants, a metabolic pathway starting from L-Pt to the intermediate PPO and finally resulting in MPP has been identified (Dröge et al., 1992) (see Fig. 6). The same metabolites occur in soil microorganisms (Tebbe and Reber, 1988, 1991; Bartsch and Tebbe, 1989). A third metabolite (D3) observed in genetically unmodified plants has remained unidentified (Dröge et al., 1992). Recently, Pt metabolism in genetically unmodified suspension cultures of soybean (*Glycine max*), wheat (*Triticum aestivum*), and maize (*Zea mays*) was reported, and four Pt metabolites were identified (Komossa and Sandermann, 1992).

In this paper the chemical structures of the two unidentified metabolites D1 and D3 are characterized by means of TLC, HPLC, and NMR. All detected metabolites of L-Pt are analyzed with regard to their stability and mobility in plants.

MATERIALS AND METHODS

Chemicals

The following nonradioactive compounds were provided by Hoechst AG (Frankfurt/Main, Germany): DL-homoalanine-4-yl-(methyl)-phosphinic acid (DL-Pt, Hoe 39866), L-homoalanine-4-yl-(methyl)-phosphinic acid (L-Pt, Hoe 35956), DL-2-acetamido-4-methyl-phosphinato-butanoic acid (DL-ac-Pt, Hoe 85355), PPO (Hoe 65594), and DL-DMP (Hoe 96203). DL-AEP and APB were purchased from Sigma.

^{14}C-radiolabeled Pt and its metabolites were synthesized by Hoechst AG: DL-[1-^{14}C]Pt (Hoe 35956, 80.4 MBq mmol^{-1}); L-[3,4-^{14}C]Pt (Hoe 35956, 148 MBq mmol^{-1}); [^{14}C]MPP (Hoe 61517, 134.3 MBq mmol^{-1}); [2-^{14}C]MPE (Hoe 64619, 127.9 MBq mmol^{-1}); [3,4-^{14}C]4-methylphosphinico-2-hydroxybutanoic acid (Hoe 42231, 123.4 MBq mmol^{-1}). L-[3,4-^{14}C]ac-Pt (Hoe 99730, 379.7 MBq mmol^{-1}) was obtained by synthetic derivitization of an aqueous L-[3,4-^{14}C]Pt solution with [1-^{14}C]Ac_2O after addition of NaOH (pH 7–8); [1-^{14}C]Ac_2O was purchased from Amersham.

Plant Material

Tobacco (*Nicotiana tabacum*) var Petit Havanna SR1 and carrot (*Daucus carota*) var Rote Riesen (Botana, Osnabrück, Germany) were transformed by leaf disc transformation and direct gene transfer, respectively (Wohlleben et al., 1988; Dröge et al., 1992). Transgenic alfalfa plants (*Medicago sativa*) var Ra3 were a gift from G. Donn (Hoechst AG).

Plant Culture and Treatment with the Radiolabeled Pt

Transgenic and genetically unmodified plants were grown on solidified Murashige-Skoog medium (Murashige and Skoog, 1962) at 23°C under aseptic conditions and a 16-h daylight period. Usually an aqueous solution of the ^{14}C-radiolabeled Pt or the corresponding metabolites was spread on the leaves of aseptically growing plants with a pipette. An aqueous volume of 100 μL containing 40 to 120 kBq of the radiolabeled compound was distributed on an area of approximately 10 cm^2. The Pt treatment of genetically unmodified plants led to the induction of necrotic spots after 5 to 10 d. Transgenic plants were found to be herbicide resistant (Wohlleben et al., 1988). Because of the plant's enzymic activity, PPO was aminated in vivo and, therefore, was found to be phytotoxic (Dröge et al., 1992). The other Pt metabolites tested did not lead to phytotoxic syndromes.

Preparation of Plant Extracts

Extracts were made from plant leaves by grinding the material submerged in water or Tris buffer in an Eppendorf tube. A metal rod 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 (Eppendorf centrifuge: 5 min, 13,000 rpm).

TLC

To separate the soluble L-Pt metabolites, approximately 5 μL of the cleared plant extract were analyzed in the following TLC systems: (a) cellulose HPTLC. The chromatographic plates (10 cm × 10 cm × 0.1 mm; Merck AG, Darmstadt, Germany) were developed twice in a solvent system consisting of pyridine:*n*-butanol:acetic acid:water (10:15:3:12) (Strauch et al., 1988). (b) silicagel HPTLC. The chromatographic plates (10 cm × 10 cm × 0.1 mm; Merck AG) were developed once in a solvent system containing 1-propanol:25% (w/v) ammoniacal solution (3:2). (c) SilG25 TLC. The SilG25-silicagel plates (10 cm × 20 cm × 0.2 mm; Macherey and Nagel, Düren Germany) were developed once in iso-propanol:acetic acid:water (2:1:1) (K. Stumpf, Hoechst AG, personal communication). Thereafter, the radiolabeled metabolites were visualized by autoradiography (x-ray film, Du Pont) and identified by co-chromatography of reference compounds.

HPLC

HPLC was conducted by using a Spherisorb column (250 mm × 4.6 mm; Bishoff) and a solvent system containing 0.001 M KH_2PO_4 and 10% methanol (pH 1.9) (K. Stumpf, Hoechst AG, personal communication). For radiodetection of the labeled metabolites, a Ramona D detector (Raytest, Pittsburgh, PA) was used.

Synthetic Acetylation of Pt and Its Derivatives

A 34% (w/v) solution of NaOH was used to adjust the pH of a 5-mM solution of Pt (or its derivative) to a value of 8. After [1-^{14}C]Ac_2O (Amersham) was added in excess, the mixture was shaken vigorously. To complete the derivitization, the procedure was repeated once.

NMR

^{13}C-NMR spectroscopy was conducted in D_2O (Sigma) using a Bruker (San Jose, CA) AM 300/CPX 100 NMR spectrograph.

Autoradiography of Plants Treated with the Radiolabeled Pt Metabolites

To analyze the mobility of the Pt metabolites in plants, an aqueous solution of the radiolabeled compound was spotted on the surface of a leaf in the middle of the plant. After 6 d of incubation, the plants were exposed to x-ray film (DuPont) for 5 d.

RESULTS

Identification of a Second Form of ac-Pt Resulting from the L-Pt Metabolism of Transgenic, Herbicide-Resistant Plants

The application of transgenic, herbicide-resistant plants in agriculture combined with the use of the corresponding herbicide depends on the formation of herbicide metabolites in these plants. To analyze the metabolism of the herbicide L-Pt, three different plant species, tobacco (*N. tabacum*), alfalfa (*M. sativa*), and carrot (*D. carota*), were compared to study possible species-specific differences. The aim of this work was to analyze the plant's metabolic activities separately from bacterial influences. Hence, leaves of aseptically grown plants were treated with an aqueous solution of the ^{14}C-radiolabeled L-Pt. To identify even low-level metabolites, unusually high doses of the radiolabeled herbicide were applied. After defined incubation times, plant extracts were examined by TLC or HPLC. To detect only the herbicide metabolites, radiodetection systems were applied. The same metabolites were identified in all three plant species tested.

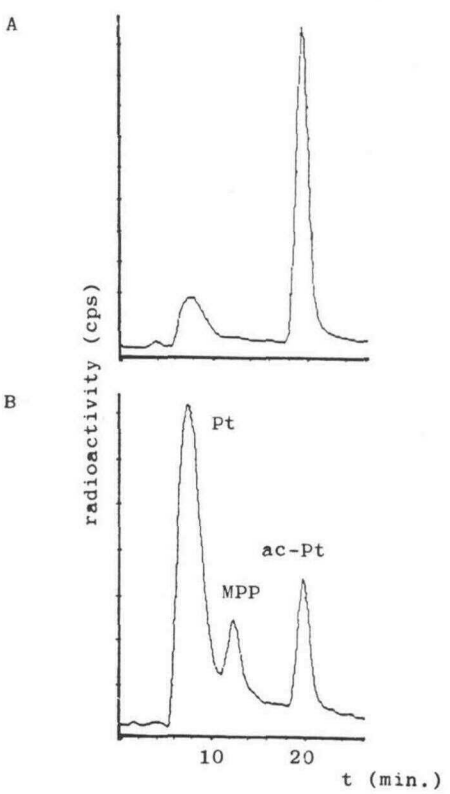

Figure 1. Analysis of the L-Pt metabolites formed in transgenic, herbicide-resistant plants. A, Plants were incubated with radiolabeled [^{14}C]Pt. Subsequently, radiolabeled L-Pt metabolites were extracted and separated by HPLC. Depending on elution time, the amount of eluted radioactivity (cps, counts per second) is given. B, HPLC separation of reference compounds Pt, MPP, and ac-Pt.

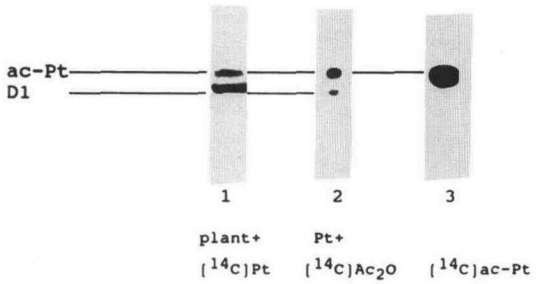

Figure 2. Analysis of the L-Pt metabolites formed in transgenic, herbicide-resistant plants. Lane 1, Tobacco plants were incubated with radiolabeled [^{14}C]Pt for 6 d. Extracts of these plants were analyzed by cellulose HPTLC. Two radiolabeled metabolites were separated and visualized by autoradiography of the TLC plate. One compound has already been identified as ac-Pt (Dröge et al., 1992). The second metabolite was designated D1. Lane 2, Synthetic derivatization of DL-Pt with [1-^{14}C]Ac_2O. The reaction products were separated by cellulose HPTLC, and the labeled, acetylated compounds were detected by autoradiography. Lane 3, Aqueous solution of the reference compound [^{14}C]ac-Pt (the occurrence of the two conformations depends on solution conditions such as buffer, pH, or presence of plant extract).

In transgenic, Pt-resistant plants analyzed by HPLC, one further metabolite was detected in addition to the remaining unacetylated Pt (Fig. 1A). By means of co-chromatography of reference compounds the metabolite was identified as ac-Pt, the product of the Pat enzymic reaction (Fig. 1B). By means of two different TLC systems (silicagel HPTLC and SilG25 TLC) ac-Pt was again the only metabolite found. Surprisingly, after a cellulose HPTLC separation system was applied, a second metabolite was found, which showed mobility similar to that of ac-Pt (Fig. 2). The nearly identical physical behavior indicates that this additional compound, designated D1, is chemically related to ac-Pt. Similar to ac-Pt, D1 occurred only in plants expressing the *pat* gene. Hence, it might be an acetylated derivative as well.

To identify D1, Pt was synthetically acetylated using [1-^{14}C]Ac_2O. Only the resulting acetylated products were ^{14}C-labeled and thus could be detected by TLC and autoradiography (Fig. 2). Two compounds showing the mobility of ac-Pt and D1 were labeled. Hence, D1 represents an acetylated form of Pt carrying a radiolabeled [^{14}C]acetyl residue.

To investigate whether the metabolite D1 is acetylated twice or at a different molecule position than the amino function, some Pt derivatives were used in the acetylation experiments. Again, the reaction products were monitored by TLC and autoradiography (Fig. 3). Only the compounds exposing the NH_2 residue resulted in radiolabeled signals (AEP, DMP, APB). Derivatives without the NH_2 group (PPO) or with a blocked amino function (ac-Pt) did not lead to radiolabeled acetylation products. Hence, as is expected

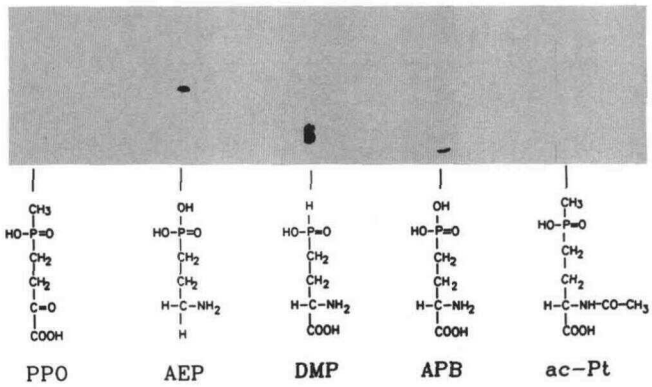

Figure 3. Analysis of possible acetylation sites in the Pt molecule. Synthetic derivatization of Pt-related compounds by acetylation with [1-^{14}C]Ac_2O. The radiolabeled acetylated products were separated by cellulose HPTLC and monitored by autoradiography. Compounds used in the acetylation reaction are ac-Pt, PPO, AEP, DMP, and APB.

chemically, acetylation did not occur at a functional group (-COOH or -OH) other than the amino residue. In conclusion, the radiolabeled acetylation product, showing the mobility of the plant metabolite D1, must be *N*-acetylated Pt as well. It had to be determined whether it is a new chemical structure. For instance, it could be postulated that new chemical structures could be formed by cyclization of ac-Pt. By NMR spectroscopy, these compounds should become detectable. The products of the acetylation reaction of L-Pt and D,L-Pt were examined with ^{13}C-NMR spectroscopy. Nevertheless, differences from an ac-Pt reference compound were not found (^{13}C-NMR: 300 MHz, D_2O; δ 181.393, 176.789, 59.355, 59.153, 31.830, 30.610, 28.958, 24.823, 19.098, 17.881; ^{31}P-NMR, 300 MHz, D_2O: δ 43.75). These data indicate that D1 was not a new, chemically distinct metabolite but appeared to be another conformation of ac-Pt. The acetylation experiments gave additional information about these conformations. Acetylation of the chemically related compound DMP also resulted in the formation of two products (Fig. 3), whereas APB led to only one compound. Pt, DMB, and APB differ only in their P substitution (Fig. 3). In view of the chemical structures of ac-Pt and DMP, two asymmetric centers (C-2 and the P atom) exist in these molecules. In contrast, APB exposes no second asymmetric center. Therefore, these data indicate that diastereomers, which may differ in their physical behavior, are the reason for the two ac-Pt signals observed in TLC.

In conclusion, D1 shows the chemical structure of an *N*-acetylated Pt. Therefore, it is not a new metabolite.

Identification of the L-Pt Metabolite MHB in Genetically Unmodified Plants

In genetically unmodified plants three further metabolites were detected by cellulose HPTLC (Fig. 4A). The same compounds were found in some transgenic plants (Dröge et al., 1992). These plants also still harbor substantial amounts of remaining L-Pt not used in the transgene-encoded acetylation reaction. Hence, these plants seem to express only a weak Pat activity. Therefore, the three additional metabolites can be characterized as products of the normal plant-specific L-Pt metabolism. Two of them have already been identified as products of successive deamination and decarboxylation reactions leading to the compounds PPO and MPP (Fig. 4A) (Dröge et al., 1992). By means of ^{14}C-labeling experiments using Pt labeled at the positions C-1, C-3, C-4, and C-6, it could be demonstrated that uncharacterized metabolite D3 still carries all C atoms of the Pt backbone (data not shown). To identify metabolite D3, possible Pt derivatives were tested in co-chromatographic studies using different TLC systems. An aqueous solution of plant metabolite D3 isolated from the cellulose TLC plate showed the same mobility as the reference substance MHB. Depending on the presence of plant extracts, the mobility of this compound in the cellulose HPTLC system changed (data not shown). Therefore, a SilG25 TLC and a silicagel HPTLC system were used to verify the result. Figure 4B shows the autoradiography of a silicagel HPTLC separation. Again, D3 showed mobility identical with that of the reference substance MHB, the hydroxy analog of L-Pt.

Stability of the L-Pt Metabolites in Plants: MPP, MHB, and ac-Pt Are Not Further Metabolized

When one considers a possible metabolite deposition in the plant, it is important to know whether the herbicide metabolites remain stable or are further degraded. In addition, the development of a metabolic scheme requires an analysis of possible reactions of the metabolites. Therefore, tobacco plants were incubated for 10 d with the different radiolabeled

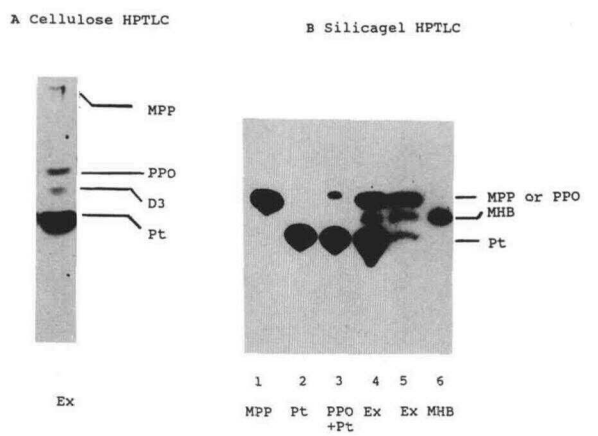

Figure 4. Identification of the plant-specific L-Pt metabolite D3. Extracts of genetically unmodified tobacco plants incubated with [^{14}C]Pt were analyzed by TLC. The radiolabeled metabolites were shown by autoradiography of the TLC plates. A, Cellulose HPTLC separation of the L-Pt metabolites PPO, MPP, and the unidentified compound D3 as described in Dröge et al. (1992). B, Silicagel HPTLC separation of the L-Pt metabolites. D3 was identified by co-chromatography to be MHB. Lane 1, MPP; lane 2, Pt; lane 3, Pt and PPO; lanes 4 and 5, extracts (Ex) of plants incubated with [^{14}C]Pt; lane 6, MHB.

metabolic compounds detected in this study. The plant's metabolism of these compounds was assayed by TLC.

Figure 5a shows that ac-Pt is not further metabolized after treatment of plants with the radiolabeled compound. Even deacetylation, which should result in L-Pt, could not be measured (Fig. 5a). Hence, ac-Pt appeared to be a stable metabolite in plants. PPO was found to be very unstable: it spontaneously decarboxylated in an aqueous solution, resulting in MPP (Fig. 5b, lane 2). In plants, it was decarboxylated to form MPP as well, and, additionally, it was reversibly aminated to its ancestor compound L-Pt (Fig. 5b, lane 1).

Figure 4c shows the metabolism of MPP. This compound, resulting from the decarboxylation of PPO, was not further degraded and remained stable in plants. In contrast to the metabolism observed in soil bacteria (Behrend et al., 1990), decarboxylation of MPP resulting in the formation of MPE was not detectable (Fig. 5c). In plants, the compound MHB was stable as well. In contrast to the oxo acid PPO, the hydroxy acid MHB was not decarboxylated (Fig. 5d).

The plant's L-Pt metabolism, based on these data, is summarized in Figure 6. In transgenic, Pt-resistant plants two stable forms of ac-Pt are generated. This acetylation reaction competes with a plant-specific degradation of L-Pt. L-Pt is deaminated to PPO, which subsequently forms MPP by decarboxylation. The metabolite MHB, the hydroxy acid of L-Pt, might be generated from PPO as well. MPP cannot be the ancestor of MHB, because this compound has already lost by decarboxylation the C-1 atom that is still contained in MHB (Dröge et al., 1992). In view of the chemical structure of MHB, a reduction of the oxo acid PPO resulting in MHB is likely to occur. This hypothesis cannot be proven by the incubation of plants with radiolabeled PPO, because PPO is reversibly aminated to L-Pt; therefore, L-Pt cannot be excluded as the ancestor of MHB (Fig. 5b). However, when MHB occurrence is observed throughout time, MHB always becomes detectable some days after PPO (Dröge et al., 1992), and therefore, these data suggest the hypothesis that MHB results from the reduction of PPO.

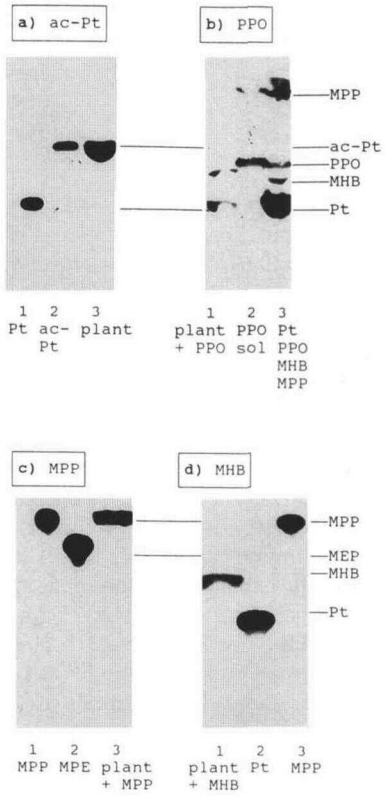

Figure 5. Stability of the L-Pt metabolites in plants. Tobacco plants were incubated with the ^{14}C-radiolabeled Pt metabolites ac-Pt, PPO, MPP, and MHB. After 10 d of treatment, plant extracts were analyzed by cellulose HPTLC and autoradiography. a, Stability of ac-Pt in transgenic plants: lane 1, L-Pt; lane 2, ac-Pt; lane 3, extract of a plant incubated with ac-Pt. b, Stability of PPO in genetically unmodified plants: lane 1, extract of a plant incubated with PPO; lane 2, aqueous solution (sol) of PPO; lane 3, reference compounds Pt, MHB, PPO, and MPP. c, Stability of MPP in genetically unmodified plants: lane 1, MPP; lane 2, MPE; lane 3, extract of a plant incubated with MPP. d, Stability of MHB in genetically unmodified plants: lane 1, extract of a plant incubated with MHB; lane 2, Pt; lane 3, MPP.

Mobility of the L-Pt Metabolites: ac-Pt, MPP, and MHB Are Preferentially Transported to the Upper Parts of the Plant

Since only defined parts of the plant, such as fruits, are used for food production, it is important to know whether the L-Pt metabolites are translocated in the plant or whether they accumulate at their place of origin. To investigate the mobility of the L-Pt metabolites in the whole plant, one defined leaf located in the middle of the plant was treated with ^{14}C-labeled L-Pt. After 6 d of incubation, the whole plant was exposed to x-ray film to study the translocation of the radiolabeled compound (Fig. 7, left). In plants treated with L-Pt, the radioactivity was distributed mainly in the leaf to which the compound was applied. Additionally, part of the label was found in the stem and was preferentially transported into the upper leaves. In small amounts, translocation into the roots was observed as well. In this experiment the transport of the herbicide itself or its metabolites cannot be distinguished. Therefore, experiments were conducted to study the translocation of the stable metabolites. The metabolites MPP (Fig. 7, middle), ac-Pt (Fig. 7, right), and MHB (data not shown) were distributed in the same way as it was for the herbicide itself.

DISCUSSION

As part of a study evaluating the consequences of the use of transgenic, herbicide-resistant crops in agriculture, the aim of this work was to analyze the metabolism of the herbicide Pt in these plants. The commercial formulation Basta consists of a racemate of the D and L enantiomers. As previously described (Dröge et al., 1992), D-Pt is stable in plants, and therefore, this study dealt with the metabolism of the L enantiomer. Additionally, this study was limited to soluble metabolites; bound residues of the herbicide or its metabolites were not studied. Studies of Pt metabolism in genetically unmodified suspension cultures have found only a very small amount of the applied radiolabeled Pt in bound residues

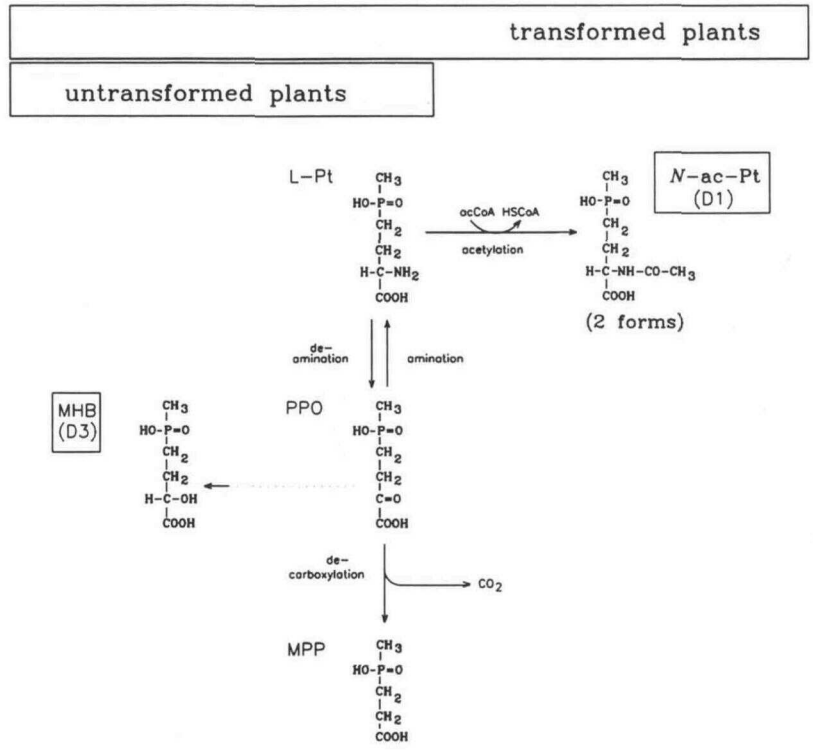

Figure 6. Metabolic reaction scheme of the herbicide L-Pt in plants. In transgenic plants expressing the *pat* gene, L-Pt is acetylated to result in two stable forms of *N*-acetyl-Pt. The transgene-encoded acetylation of L-Pt competes with a plant-specific degradation, occurring in untransformed plants as well. Two metabolic pathways were elucidated: a successive deamination and decarboxylation resulting in the formation of PPO, and the finally stable MPP. PPO is likely reduced to result in the stable metabolite MHB (dotted line).

(<0.1%) (Komossa and Sandermann, 1992). Hence, the soluble compounds seemed to be of main interest.

In most of the independently transformed plants analyzed, L-Pt was quantitatively modified by acetylation. Only in those plants that showed a weak *pat* gene expression were three further metabolites detected (PPO, MPP, MHB). These metabolites were found in genetically unmodified plants as well. Hence, they could be interpreted as typical compounds generated by the plant's metabolic activities. Therefore, a competition between a transgene-encoded acetylation of L-Pt and a plant-specific degradation occurs in transgenic, Pt-resistant plants (Fig. 6).

Two classes of metabolites have to be distinguished. First, the two forms of *N*-acetyl-Pt occurring at high concentrations and, second, the low-level metabolites PPO, MPP, and MHB.

In transgenic plants with high acetylation activity, L-Pt was quantitatively *N*-acetylated. Additionally, no further metabolism of the *N*-acetylated Pt occurred. Therefore, substantial amounts of the modified herbicide remained in the plant. It was demonstrated that two forms of a *N*-acetylated Pt are generated. In view of the evaluation of the metabolite contamination of these plants, it is important to know whether these compounds are distinct chemical structures. NMR spectroscopy and synthetic acetylation studies were used, and the formation of new derivatives of ac-Pt could be excluded. Both forms were characterized as having the same chemical structure. To explain the different physical behaviors of these two forms in TLC, we suggest that two conformers exist. Since two products were observed only after acetylation of potentially diastereomeric compounds (ac-Pt and DMP), the data obtained may be explained by the separation of diastereomers. In aqueous solution, Pt derivatives are deprotonated and, therefore, should not harbor a P asymmetric center. Consequently, these compounds should not be diastereomeric. Nevertheless, under the conditions of TLC, the compound was not free in an aqueous solution, and therefore,

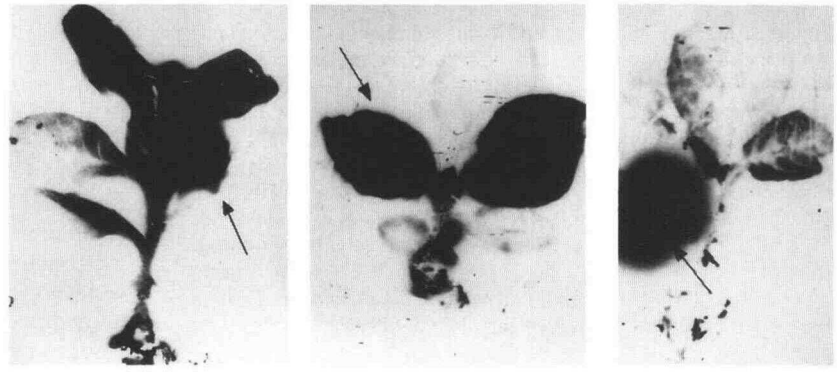

Figure 7. Analysis of the mobility of Pt and its metabolites in the whole plant. Genetically unmodified tobacco plants were treated with ^{14}C-radiolabeled L-Pt (left) and the stable metabolites MPP (middle) and ac-Pt (right). The compounds were applied in an aqueous solution on a middle standing leaf (arrow). After 6 d of incubation, the plants were exposed on x-ray film.

interaction with the thin-layer material may have stabilized diastereomeric forms. This may be the reason that a separation was detected only in one of the four applied chromatographic systems. Additionally, in the case in which ionic interactions have stabilized an intramolecular salt of ac-Pt, the existence of diastereomeric compounds can be postulated. Whatever the conformations look like, the most important result for the evaluation of the metabolite contamination of transgenic plants is that D1 is not an additional new metabolite.

The second class of the three low-level metabolites PPO, MPP, and MHB was formed by a plant-specific degradation of L-Pt. Similarly to the L-Pt metabolism in soil microorganisms (Smith, 1988, 1989; Tebbe and Reber, 1988, 1991; Bartsch and Tebbe, 1989), the metabolites PPO and MPP were identified as products of a successive deamination and decarboxylation (Dröge et al., 1992). The third, formerly unidentified metabolite was chemically characterized to be the 2-hydroxy analog of L-Pt, MHB. The same metabolites have recently been found in genetically unmodified suspension cultures incubated with L-Pt (Komossa and Sandermann, 1992). Additionally, in this study 4-methylphosphinico-butyric acid has been described as an L-Pt metabolite in suspension cultures of maize (*Z. mays*) and wheat (*T. aestivum*) but not in soybean (*G. max*). It can be suggested that the formation of this additional compound was due to the artifical suspension culture system. However, experiments conducted in carrot (*D. carota*) suspension cultures did not lead to the identification of additional metabolites like 4-methylphosphinico-butyric acid (W. Dröge-Laser, A. Pühler, I. Broer, unpublished data). Our studies were limited to plant species belonging to three different dicot families: Solanaceae, Apiaceae, and Fabaceae. Therefore, we suggest that 4-methylphosphinico-butyric acid may be a specific metabolite formed in some monocot plants only. In conclusion, it cannot be excluded that even more metabolites are generated in other plant species.

The compound PPO was found to be very unstable, and, therefore, it did not accumulate in high concentrations. In contrast to the metabolism observed in soil, the metabolite MPP was stable in plants. The decarboxylation of MPP to MPE described in soil microorganisms (Behrendt et al., 1990) did not occur in plants. The only metabolism of MPP found in a mammalian consumer, the β-oxidation to 3-methylphosphinico-3-oxo-propionic acid in rat liver (Dorn et al., 1986), was not detected. Additionally, MHB was found to be stable in plants as well. Therefore, MPP and MHB may be deposited in the plant.

It is important to know whether the stable herbicide metabolites remain at their place of origin and are stored in cell compartments such as the vacuole or whether they are transported through the plant to be finally deposited in defined tissues such as seeds or fruits. These parts of the plant normally have no direct contact with the herbicide, because the treatment takes place in advance of fruit ripening. Hence, deposition of stable herbicide metabolites in these parts of the plant depends on storage or translocation. Similarly to L-Pt, all L-Pt metabolites were found to be translocated preferentially into the upper regions of the plant. Hence, driven by transpiration, this transport likely took place in the xylem. At a low level, a translocation into the lower parts of the plant was observed, suggesting that transport takes place in the phloem. These data contrast with translocation studies conducted with the chemically related herbicide glyphosate, which is transported by xylem and phloem into the metabolic sinks (Sprankle et al., 1985). Substantial amounts of this herbicide were detected in roots and rhizomes. In conclusion, transport of the L-Pt metabolites into the fruits or seeds may occur. Quantitative measurements of the metabolite concentrations in these parts of the plant have to be conducted.

In this study, we have presented the chemical structures of the L-Pt metabolites formed in transgenic, herbicide-resistant as well as genetically unmodified plants. Using this knowledge, we can analyze the possible influence of these compounds on the consumer organism. Studies of toxicology will help to evaluate the consequences of the use of herbicide-resistant plants in agriculture.

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