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, 14C-labeled specifically at different C-atoms, was analysed by measuring the release of 14CO2 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, 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 Tu494 (Strauch et al. 1988; Wohleben 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 (Wohleben 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 (Wohleben 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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CH₃
HO-P=O
4CH₂
acetyl-CoA
HSCoA
3CH₂
Pat
H-C-NH₂
1COOH
L-PT

CH₃
HO-P=O
4CH₂
acetyl-CoA
HSCoA
3CH₂
L-ac-Pt
H-C-NH₂
1COOH
L-Pt

L-Pt L-ac-Pt

Fig. 1. Enzymatic reaction of phosphinothricin-N-acetyl-transferase (Pat). The positions of the radiolabeled ¹⁴C-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·1⁻¹) or ampicillin (100 mg·1⁻¹), 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-methylphosphinate-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).

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

The L-[1-¹⁴C]ac-Pt was produced by Pat-enzyme reaction using a crude bacterial extract (Strauch et al. 1988) and DL-[1-¹⁴C]Pt as substrate. Phosphinothricyl-alanyl-alanine (Pt-tripeptide; Ptt), ¹⁴C-labeled at the Pt CH₃-group, was prepared in vivo by S. viridochromogenes incubated with [¹⁴CH₃]methionine (Amer- sham, Braunschweig, FRG). The resulting [¹⁴C]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 Wohleben et al. (1988).

Table 1. Bacterial strains and plasmid vectors

<table>
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<tr>
<th>Strain</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
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<tr>
<td>S. viridochromogenes Tü494</td>
<td>Pat⁺, Ptt producer</td>
<td>H. Zähner, Tübingen FRG</td>
</tr>
<tr>
<td>E. coli S17.1</td>
<td>E. coli 294 derivative, chromosomal integrated RP4 derivative, Sm⁺, Km⁺</td>
<td>Simon et al. 1983</td>
</tr>
<tr>
<td>A. tumefaciens LBA4404</td>
<td>pAL4404 (T-DNA⁻, Vir⁺), Sm⁺, Km⁺</td>
<td>Hoekema et al. 1983</td>
</tr>
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<tr>
<th>Plasmids</th>
<th>Structure</th>
<th>Source or reference</th>
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<tr>
<td>pROK1</td>
<td>Plant vector derived from pBIN19 (Bevan 1984)</td>
<td>Baulcombe et al. 1986</td>
</tr>
<tr>
<td>pIB16.41</td>
<td>pROK1 derivative carrying the modified pat41 gene</td>
<td>This work, Fig. 2a</td>
</tr>
<tr>
<td>pSVB28</td>
<td>E. coli multicopy vector derived from pUC 8 with unique cloning sites for EcoRI and HindIII</td>
<td>Arnold and Pühler 1988</td>
</tr>
<tr>
<td>pWD26.41</td>
<td>pSVB28 derivative carrying the modified pat41 gene</td>
<td>This work, Fig. 2b</td>
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* Km, kanamycin; Ptt, Pt-alanyl-analine; Sm, streptomycin; †, resistant; ‡, sensitive