Chapter 5

Agrobacterium mediated transformation of Anthriscus sylvestris with human cytochrome P450 3A4 followed by regeneration

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ABSTRACT

Metabolic engineering offers interesting perspectives to improve the productivity of the plant as a cell factory. The recently demonstrated conversion of deoxypodophyllotoxin into epipodophyllotoxin upon expression of human P450 3A4 in E. coli DH5α, has prompted us to investigate the activity of P450 3A4 in planta. Because of the endogenous production of deoxypodophyllotoxin in A. sylvestris, transformation of this plant may result in the production of (epi)podophyllotoxin. A. sylvestris callus tissue was successfully transformed with Agrobacterium tumefaciens carrying the human P450 3A4 gene and the cells were subsequently regenerated. The presence of the P450 3A4 gene in the regenerated transgenic A. sylvestris was confirmed by PCR. The transgenic plants were shown to form epipodophyllotoxin in low amounts whereas the wild type (control) plants do not. This opens the route for a novel plant source for the semi-synthesis of the important anticancer drugs etoposide and teniposide.

Keywords: Anthriscus sylvestris, epipodophyllotoxin, podophyllotoxin, deoxypodophyllotoxin, organogenesis, transformation, regeneration, human P450 3A4, CYP3A4
INTRODUCTION

Plants are a rich source of bioactive compounds. Compounds of plant origin can be used as drugs or as precursors of semisynthetic drugs, and may provide valuable leads for novel drug design. The amounts of secondary metabolites in plants are often low and the availability can often be a major bottleneck in furnishing the pharmaceutical needs. Chemists have in specific instances succeeded to synthesize typical plant compounds via organic synthesis, but often this is hampered by the high chemical complexity, specific stereochemistry and the economic feasibility. Metabolic engineering of plants offers interesting perspectives to improve the productivity of the plant as a cell factory. This approach may create new opportunities in agriculture, environmental applications, production of chemicals, and medicines.1, 2

Podophyllotoxin (PPT), obtained from Podophyllum species, is used for the semisynthesis of the anticancer drugs etoposide and teniposide. PPT and its derivatives are now intensively investigated for their use in anticancer therapy especially in combination treatment.3 Up to now, three different ways are known for the production of podophyllotoxin: direct isolation from Podophyllum species, via plant cell cultures, and by organic synthesis. Using organic synthesis or plant cell cultures is economically unfeasible. To date, PPT is only obtained by isolation from Podophyllum species. In the future, the availability of PPT from this source is likely to become a major bottleneck, as Podophyllum species have been listed on the endangered species list.4 An alternative and more sustainable source of PPT may be obtained by (biotechnological) hydroxylation of deoxypodophyllotoxin (DPT) at the C7 position (figure 1) yielding epipodophyllotoxin (epi-PPT), the diastereoisomer of PPT. This conversion has been achieved already on laboratory scale in E. coli DH5α transfected with the human cytochrome P450 3A4.5 Both epi-PPT and PPT can be used as a semisynthetic precursor for etoposide and teniposide.

In many plants, DPT serves as the precursor of PPT.6, 7 DPT is the main constituent of Anthriscus sylvestris (L.) Hoffm. (Apiaceae). A. sylvestris accumulates up to 1.5 µg/mg dried weight (d.w.) of DPT in the root part8 and trace amounts of PPT (<0.01 µg/mg d.w) in the aerial part.9 As a wild plant and generally occurring weed native to northwest Europe, the availability of A. sylvestris is hardly limited, in contrast to Podophyllum species.

The human P450 3A4 gene has been used to create different recombinant systems such as in the yeasts Schizosaccharomyces pombe10 and Saccharomyces cerevisiae,11 in the bacterium E. coli,5, 12 and in the insect cells Sf21 Spodoptera frugiferda.13 The transformation of human P450 3A4 has been achieved in plant cells, but so far only in Nicotiana tabacum.14, 15 For functional activity of the 3A4 P450 enzyme a P450 reductase has to be expressed from endogenous or exogenously added DNA.

The main objective of this study was to transform the human P450 3A4 gene in A. sylvestris plants and to determine the capacity of this system to in vivo transform deoxypodophyllotoxin into epipodophyllotoxin (see figure 1).
Agrobacterium transformation

Figure 1. Scheme of *Anthricus sylvestris* transformed with human P450 3A4. The expression of P450 3A4 is expected to result in the hydroxylation of deoxypodophyllotoxin (DPT) into epipodophyllotoxin (epi-PPT) based on the observed activity of *E. coli* produced 3A4 P450. The formation of its diastereoisomer, podophyllotoxin (PPT), is not expected to be catalysed by 3A4.

**RESULTS**

**Feeding of DPT to CYP3A4 expressing N. tabacum cells**

*N. tabacum* cells were transformed with the human full length CYP3A4 cDNA as described and the results of the feeding of DPT to transformed and to wild type cells are presented in table 1. The transformed cells (CYP3A4) as well as the wild type (control) cells were able to hydroxylate DPT into epi-PPT, the diastereoisomer of PPT.

**Table 1.** DPT and Epi-PPT concentrations, means are given ± SD (n=3), (µg/mg d.w. in cells and µg/ml in media) in tobacco plant cell suspension cultures of wild type (WT) and CYP3A4 (3A4) at different time points after feeding with DPT.

<table>
<thead>
<tr>
<th></th>
<th>WT DPT cells (µg/mg d.w.)</th>
<th>3A4-DPT in cells (µg/mg d.w.)</th>
<th>WT DPT media (µg/ml)</th>
<th>3A4-DPT in media (µg/ml)</th>
<th>WT epi-PPT cells (µg/mg d.w.)</th>
<th>3A4-Epi-PPT in cells (µg/mg d.w.)</th>
<th>WT epi-PPT media (µg/ml)</th>
<th>3A4-Epi-PPT in media (µg/ml)</th>
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<tr>
<td></td>
<td>Day 4</td>
<td>Day 7</td>
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<tr>
<td>WT DPT cells</td>
<td>0.102 ± 0.030</td>
<td>0.103* ± 0.026</td>
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<tr>
<td>3A4-DPT in cells</td>
<td>0.077 ± 0.002</td>
<td>0.035* ± 0.006</td>
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<tr>
<td>WT DPT media</td>
<td>1.316 ± 0.178</td>
<td>1.302 ± 0.138</td>
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<tr>
<td>3A4-DPT in media</td>
<td>0.721 ± 0.124</td>
<td>0.598 ± 0.144</td>
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<td>WT epi-PPT cells</td>
<td>0.029 ± 0.008</td>
<td>0.035 ± 0.034</td>
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<tr>
<td>3A4-Epi-PPT in cells</td>
<td>0.038 ± 0.020</td>
<td>0.102 ± 0.017</td>
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<tr>
<td>WT epi-PPT media</td>
<td>1.062 ± 0.109</td>
<td>2.940* ± 0.455</td>
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<tr>
<td>3A4-Epi-PPT in media</td>
<td>1.843 ± 0.244</td>
<td>4.748* ± 0.270</td>
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An asterisk (*) indicates a significant difference between wild type and 3A4 (p<0.05)
\emph{N. tabacum} does not contain the DPT. Therefore the epi-PPT formation was due to the addition of DPT. On day 7, the conversion of DPT into epi-PPT in the medium was significantly higher in transformed cells as compared to wild type cells.

In general, the concentration of epi-PPT was higher in the media than in the cells for both wild type and transgenic cells. Although the wild type was able to form epi-PPT, the conversion was 3 times lower than in the transformed cells and 1.5 fold lower in the media of the transformed cells on day 7 compared to the media of the wild type cells. In the transformed cells, the highest epi-PPT production was seen on day 7, in the cells (0.1 µg/mg d.w.) and in the media (4.8 µg/ml). The high concentration of the product in the medium as compared to the intracellular concentration suggests that there is an efficient secretion mechanism for the epi-PPT formed.

**Plant transformation and regeneration of \textit{A. sylvestris}**

We initiated callus cells from young sterile \textit{in vitro} seedlings (figure 2a-b). The calli were obtained by aseptically excising the hypocotyls of \textit{in vitro} seedlings (figure 2c). The calli were transformed by \textit{A. tumefaciens} harboring the binary plasmid pGreen029-3A4 (figure 3). YFP was fused to the P450 3A4 as an additional selection marker for visualization besides the kanamycin resistant gene. After the co-cultivation for 2 days, the calli were subcultured on shoot-inducing medium containing kanamycin for transformant selection and carbenicillin for elimination of overgrowth \textit{Agrobacterium}. The transformed calli will survive on antibiotics selection. Every 4 weeks, the calli were within 3 months while wild type would die. The non-transformed calli eventually died and the transformed calli grew well (figure 2d). About a hundred transformed calli clumps were subcultured to shoot-inducing medium (figure 2e) for up to 1 year and the regenerated shoots (figure 2f) were subcultured in the root-inducing medium (figure 2g) for 3 months. Eventually the transformed plants were transferred into the greenhouse (figure 2h) and grown under controlled conditions. After two independent \textit{Agrobacterium}-mediated transformations on callus cells, five kanamycin-resistant plantlets from two independent transformations were analyzed.

**Figure 2.** \textit{Anthriscus sylvestris} transformation and regeneration. (a) Seeds of \textit{A. sylvestris}. (b) Young sterile \textit{in vitro} seedling. (c) Callus induction by aseptically excised hypocotyls of the young sterile \textit{in vitro} seedlings. (d) Transformed callus selection on kanamycin and carbenicillin callus medium. (e) Shoot formation of transformed calli grown in shoot-inducing medium. (f) Elongation of shoot formation. (g) Root formation in shoot-inducing medium. (h) Young transformed plantlets grown in the green house. Bars equal to 1 cm (a-d, f, g), 0.5 cm (e) and 10 cm (h).
subcultured to fresh medium. The selection on kanamycin required 3 months and the elimination of *Agrobacterium* required up to 6 months. Kanamycin 100 mg/L was sufficient to kill the non-transformed cells within 3 months while wild type would die. The non-transformed calli eventually died and the transformed calli grew well (figure 2d). About a hundred transformed calli clumps were subcultured to shoot-inducing medium (figure 2e) for up to 1 year and the regenerated shoots (figure 2f) were subcultured in the root-inducing media (figure 2g) for 3 months. Eventually the transformed plants were transferred into the greenhouse (figure 2h) and grown under controlled conditions. After two independent *Agrobacterium*-mediated transformations on callus cells, five kanamycin-resistant plantlets from two independent transformations were analyzed.

**Figure 3.** Schematic map of plasmid construct used for *Anthriscus sylvestris* transformation; LB: left border, D 35 S: double CaMV 35S promoter, YFP: yellow fluorescence protein fused with human P450 3A4 gene, NOS: nos polyadenylation signal (black), NOS: nos promoter (orange), nptII: kanamycin resistance gene.

**Figure 4.** (a) PCR-amplified human P450 3A4 gene of 561 bp. (b) PCR-amplified kanamycin gene of 687 bp. (c) PCR-amplified YFP gene of 739 bp from two wild type plants (WT) and five transformed plants (Lane 1 – 5). M= molecular weight marker.

We tested that the transformed plants were free from *Agrobacterium* by growing the calli and the transformed plants in LB medium at 28°C for 3 days. No grow of *Agrobacterium* was observed. The presence of the P450 3A4, YFP and kanamycin gene in the transformed plants was confirmed by PCR (figure 4). The gene fragment could be amplified from the aerial part of the transformed *Anthriscus*, whereas no band appeared from the wild type aerial part of *Anthriscus* (negative control), confirming that the transformed plants could be considered as transgenic plants. The YFP expression of transformed plants was low and the differences compared to the wild type were not clear (not shown).
**Epi-PPT content in the transformed plants**

DPT, PPT or epi-PPT contents in the transformed and wild type plants are summarized in table 2. Figure 5 shows a typical HPLC chromatogram of *A. sylvestris* extracts. The enhanced formation of epi-PPT in transgenic *A. sylvestris* is clearly seen at a retention time of 7.65 min. The transformed plants were able to form epi-PPT and PPT. Wild type *Anthriscus* contained trace amounts of PPT in concert with earlier reports but no epi-PPT was formed. The PPT content in the transformed plants was slightly higher than in the wild type (areal part). No epi-PPT could be detected in the wild *Anthriscus* plant (figure 5). No obvious changes could so far be observed in the phenotype of the *in vitro* transformed regenerated plants.

**Table 2.** DPT, PPT, epi-PPT contents in regenerated wild type, regenerated transformed and wild type plants.

<table>
<thead>
<tr>
<th></th>
<th>Transformed plant</th>
<th>Wild type (control) plant</th>
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<tbody>
<tr>
<td></td>
<td>Aerial</td>
<td>Root</td>
<td>Aerial</td>
</tr>
<tr>
<td><strong>DPT</strong></td>
<td>0.105 – 0.230</td>
<td>*</td>
<td>0.3 – 0.5</td>
</tr>
<tr>
<td><strong>PPT</strong></td>
<td>0.006 – 0.133</td>
<td>*</td>
<td>0.006 – 0.011</td>
</tr>
<tr>
<td><strong>Epi-PPT</strong></td>
<td>0.0006 – 0.036</td>
<td>*</td>
<td>-</td>
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</table>

*not determined
^the concentration was calculated from highly concentrated extracts

**Figure 5.** HPLC chromatograms of transgenic and wild type *Anthriscus*. Epi-PPT (epipodophyllotoxin), PPT (podophyllotoxin), DPT (deoxypodophyllotoxin).
**DISCUSSION**

The production of bioactive compounds through *in vitro* transformed callus or cell cultures has been carried out in several plant species, especially in medicinal plants.\(^\text{16}\) The expression of human P450 genes in transgenic plants may lead to the formation of new secondary compounds.\(^\text{12}\)

It has been reported that P450 3A4 can be expressed in plant cells (*N. tabacum*) in an active form.\(^\text{15}\) Feeding experiments with loratadine to *N. tabacum* suspension culture cells expressing P450 3A4 led to the formation of desloratadine, an active metabolite of loratadine that is also used as a drug compound. Another feeding experiment with indole in *N. tabacum* suspension culture cells expressing human cytochrome P450 2A6 and 3A4 led to the production of indican, a metabolite that is usually exclusively present in indigoferous dye plants,\(^\text{14}\) but not in *N. tabacum*.

Based on the examples mentioned, it is illustrated that the human cytochrome P450 3A4 in *N. tabacum* may transform exogenously provided DPT into epi-PPT. In our studies, we showed that *N. tabacum* cell suspension cultures expressing the human P450 3A4 were able to hydroxylate DPT to epi-PPT with the highest concentration of epi-PPT in the cells and medium on day 7. Also the epi-PPT formation in medium was significantly higher on day 7 in transformed cells compared to wild type cells. Possibly, epi-PPT was excreted into the medium because it is toxic for the cells. Interestingly, the wild type tobacco cell suspension cultures were also capable of converting DPT into epi-PPT, although at a much lower level.

We used the *Agrobacterium* transformation technique to introduce the human P450 3A4 gene in the callus cells of *A. sylvestris*. Since callus of *A. sylvestris* lost the capacity of producing DPT because they are in undifferentiated form,\(^\text{9}\) it is crucial to induce differentiation in order to restore the capability of the cell to produce DPT. We have developed a regeneration protocol of *A. sylvestris*\(^\text{17}\) that indeed after regeneration does restore the ability of the cells to produce DPT. There was no obvious somaclonal variation with respect to DPT production.

The transformed *Anthriscus* plants were able to form clearly detectable amounts of epi-PPT and small amounts of PPT as well (figure 5). Wild type *Anthriscus* has been reported to contain trace amounts of PPT (<0.01 µg/mg d.w.).\(^\text{9}\) We found PPT (0.006-0.011 µg/mg d.w.) in wild type *Anthriscus* which is in line with this previous finding. The PPT content in the transformed plants was slightly higher than in wild type, however, the involvement of P450 3A4 in this conversion is unlikely. It was reported that DPT can be converted into PPT in *Linum album* cell cultures,\(^\text{18}\) but the enzyme responsible for the hydroxylation has never been identified.

The transformation efficiency of *A. sylvestris* was low (about 1%) compared to *N. tabacum* (about 30%). Five transgenic plants from two independent transformations were obtained. The shoot regeneration of *A. sylvestris* required up to a year in the transformed cells instead of 6 months in the wild type plant.\(^\text{17}\) This might due to carbenicillin which is extensively used for eliminating *Agrobacterium*. Carbenicillin has a broad spectrum of activity against bacteria and only low toxicity to eukaryotes.\(^\text{19}\)
Inhibition of callus growth and shoot regeneration has been observed in several plants such as *Daucus carota*, *Malus domestica*, *Populus* species, and *Lycopersicon esculentum*. Carbenicilin was removed from the medium once there was no *Agrobacterium* growth observed. Rooting took about 3 months which is similar to the wild type plant. Kanamycin showed no inhibition either in the transformed calli growth or the shoot and root formation.

We are the first to show the heterologous expression of the human cytochrome P450 3A4 in *A. sylvestris*. The construct under the control of a 35S promoter should provide constitutive expression in all parts of *A. sylvestris* plant. Fusion of a cytochrome P450 and a fluorescence protein has been reported in mammalian cells and plants. However, the YFP expression was low in our transformed plants (data not shown). This may explain why the amounts of epi-PPT formed in transformed plants are limited. Reverse transcriptase PCR will be carried out to confirm the transcription of the P450, YFP and kanamycin gene in the transformed plants and further work to screen high expression level of P450 3A4 is still in progress. In addition, the progeny will be screened on kanamycin and the segregation analysis will be done. For more effective biotransformation and high level of epi-PPT and / or PPT production, it is necessary to optimize the constructs used such as the promoter, the P450 3A4 gene, and the targeting signal peptide to the compartment where the DPT is produced. To the best of our knowledge, there is no report regarding where in the cell the DPT is produced. Recent papers suggest that the shikimate pathway occurs in the plastids because the shikimate enzymes are generally synthesized as precursors containing a plastid transit peptide that directs them to the plastids. Thus, DPT may be synthesized in the plastids. The 3A4 expression construct used in the *Anthriscus* study was devoid of any targeting signal as the original human signal sequenced was not copied in the PCR fragment. It should be noted that the construct used for expression in *N. tabacum* did contain an additional endoplasmic reticulum targeting signal.

**MATERIAL AND METHODS**

**Chemicals**

Epipodophyllotoxin (epi-PPT) was a gift from Dr. M. Angeles Castro (Salamanca University, Salamanca, Spain). The identity and purity of the compound was checked using HPLC. Acetonitrile and methanol were HPLC grade from Biosolve (Valkenswaard, the Netherlands). Dichloromethane was from Fisher Scientific (Landsmeer, the Netherlands). Formic acid, ammonium formate, podophyllotoxin, deuterated CDCl₃ (99.96 %) were from Sigma Aldrich (Zwijndrecht, the Netherlands). DPT was synthesized chemically from PPT as described below. PPT (98% purity by HPLC) was obtained from Sino Future Pharmaceutical Company (Xi’an, China).
Synthesis of deoxypodophyllotoxin from podophyllotoxin

Glacial acetic acid (100 mL) was added to PPT (5.0 g, 12 mmol) and Pd/C 10% (0.25 g, 2.4 mmol). The resulting suspension was reacted under hydrogen (H₂) atmosphere (3.0 bar) at 50°C for 16 h in a Parr apparatus. The suspension was diluted with ethyl acetate (200 mL) and filtered over Celite. The solvents were evaporated under reduced pressure and the residue was purified using column chromatography using ethyl acetate:hexane (1:1, v/v). The pure product was obtained as white powder in quantitative yield (85%). ¹H-NMR spectra are in agreement with the reported literature.²⁸,²⁹ ¹³C NMR spectra are in agreement with the reported literature.²⁹ MS ESI-MS m/z [M+H]+ C₂₂H₂₃O₇ calc 399.4, found 399.2, [M+NH₄]+ C₂₂H₂₆O₇N calc 416.4, found 416.1. [M+Na]+ C₂₂H₂₂O₇Na calc 422.4, found 422.1.

Plant material and callus induction

Fruits (ripe mericarps, hereafter called seeds) of Anthriscus sylvestris L. (Hoffm.) were collected from wild habitat in June 2007 in Groningen, the Netherlands (53° 13’ 34” N and 6° 32’ 43” E). Voucher specimen of the plants used have been deposited in our department, encoded Asylv2007-4. In vitro plants were grown and the calli were initiated as described recently.¹⁷

Plasmid construction

The cDNA encoding human P450 3A4 was a gift from F.P. Guengerich (Vanderbilt University School of Medicine, Nashville, USA). The plasmid construct used for Anthriscus transformation is shown in figure 3. Human cDNA devoid of the endoplasmic reticulum retention signal was amplified by PCR to create an overlapping sequence with YFP (pMON999, Monsanto, Missouri, USA), using a set of primers, a forward primer 5’-GCTTAGAGGTCCATGGCTCTGTTATTAGCAGTTTTTCT-3’ and a reverse primer 5’-CCTTGCTCACACCATTAGGCTCCACTTACGCTGCAATCCCTTGACT-3’. YFP gene was amplified by PCR to introduce an overlapping sequence with cDNA and an XmaI restriction site at the 3’-end, using a set of primers, a forward primer 5’-GGATGGCACCGTAAGTGGAGCGCTGAATGGTGAGCAAGGGCGAGGAGC-3’ and a reverse primer 5’-GGCCGCCGGGCGATCTAGTAACATAGATG-3’. The second round of PCR was performed to create cDNA::YFP::NOS with a set of primers, a forward primer 5’-GCTTAGAGGTCCATGGCTCTGTTATTAGCAGTTTTTCT-3’, and a reverse primer 5’-GGCCGCCGGGCGATCTAGTAACATAGATG-3’. All PCR was performed using phusion polymerase (Biolabs, Leiden, the Netherlands) and confirmed by sequencing. Thus, P450 3A4 and YFP were fused in frame. We cloned this sequence to plasmid pJIT145, which contained a double 35S promoter (a gift from Mark Smedley, John Innes Centre, UK). The plasmid was cut with KpnI and XmaI and cloned to pGreen029.³⁰ The binary pGreen029 contains the nptII gene for kanamycin selection of transformed plant cells. The resulting plasmid (pGreen029-3A4) was cloned and transformed into Escherichia coli DH5α and mobilized into Agrobacterium tumefaciens EHA 105 (a gift from P.J. Hooykaas, Leiden University) using the electroporation method (pulse cells at 1.8 KV in 1 cm cuvette, 600 Ω and 10 µF capacitance).
Callus transformation and regeneration

Agrobacterium strain EHA 105 harboring the pGreen029-3A4 was grown overnight in LB medium supplemented with rifampicin (50 mg l\(^{-1}\)) and kanamycin (100 mg l\(^{-1}\)) in a rotary shaker (225 rpm, 28°C). The Agrobacterium culture was centrifuged at 3000 g for 5 min and the pellet resuspended in Gamborg’s B5 medium supplemented with 40 g l\(^{-1}\) sucrose. Transformation was achieved by infecting A. sylvestris callus cells with Agrobacterium. Calli were dried on sterile filter paper, transferred to callus medium and incubated for 2 days at 24°C. Incubated calli were subcultured to callus medium supplemented with kanamycin (100 mg l\(^{-1}\)) and carbenicillin (250 mg l\(^{-1}\)). Shoots were regenerated on shoot-inducing medium as described,\(^\text{17}\) supplemented with kanamycin (100 mg l\(^{-1}\)) and carbenicillin (250 mg l\(^{-1}\)). Kanamycin was added to callus, shoot and root-inducing media. Carbenicillin was used until no Agrobacterium growth was observed (up to 6 months). Regenerated shoots measuring about 2 cm in length were transferred into root-inducing medium.\(^\text{17}\) The rooted plantlets were thoroughly washed with tap water to remove the agar and transferred to sterile soil in small pots and covered with plastic to maintain high humidity. They were placed in a climate chamber with 14-h light/10-h dark regimen, photosynthetic photon flux density 100 μmol m\(^{-2}\) s\(^{-1}\) at 22°C. After 2 weeks, the humidity was reduced to the climate room level by removing the plastic cover and 5 plants were transferred to bigger pots and kept in the greenhouse. The efficiency of the transformation was about 1%.

Nucleic acid isolation and detection

Plasmid isolation was performed using Nucleospin Plasmid Isolation kit (Macherey-Nagel). Total plant DNA was isolated using a DNA purification kit (NucleoSpin® Plant II, Macherey-Nagel). The presence of P450 3A4 in transformed plants was confirmed by PCR using a primer pair, a forward primer 5’-AACAGCCTGTGCTGGCTATC-3’ and a reverse primer 5’-GTGTATCTTTCGAGGCGACTT-3’ giving a product of 561 bp of an internal fragment of the P450 3A4 cDNA. The primer pair for YFP gene is 5’-GGAATTCATATGTGAGCAAGGGCGAGGCT-3’ and 5’-CGGGATCCTTACTTG- TACAGCTCGTCCATGC-3’ giving a product of 739 bp. The primer pair for kanamycin gene is 5’-GGAGCGGCGATACCGTAAAG-3’ and 5’-CGGCTATGACTGGGCACAAC-3’ giving a product of 687 bp. Plant genomic DNA was used as a template. The PCR condition was as follows: initial denaturation at 98°C for 5 min followed by 35 cycle of amplification at 98°C for 30 sec, annealing at 65°C for 30 sec, and extension at 72°C for 30 sec. The final extension step was at 72°C for 10 min.

Feeding experiments with Nicotiana tabacum suspension cultures

Nicotiana tabacum cv. Petit Havana suspension cultures were cultivated in a volume of 120 mL in 300 mL Erlenmeyer flasks, and subcultured on a weekly basis by transferring 60 mL of old culture to 60 mL of fresh LS medium.\(^\text{14}\) The P450 3A4 cDNA was cloned into the binary plasmid pPSI and cell suspension cultures of N. tabacum cv. Petit Havana were transformed as described.\(^\text{14}\) The cells transformed with human cytochrome P450 3A4 (CYP3A4) as well as the wild type (control) cells were used for the feeding
One day after subculture, one set of flasks of CYP3A4 and wild type were supplemented with 2.0 mg DPT in 100 µl DMSO and further cultivated until 7 days. A set of three flasks was used for each feeding experiment. The cells and the supernatant (media) were harvested on day 4, and 7 for analysis. Cells were separated with a nylon mesh in a Büchner-funnel and frozen in liquid nitrogen. Both media and cells were freeze-dried prior to extraction.

**Extraction, HPLC, LC-MS-MS and NMR analysis**

The extraction method of the plant material and the media was as described by Koulman et al.9 Shortly, 100 mg dried cells were weighed in a sovirel tube and all the supernatant were used for extraction. A 2.0 ml portion of 80% methanol was added and the mixture was sonicated during 1 hour. Subsequently 4.0 ml of dichloromethane and 4.0 ml H2O were added. The mixture was vortexed and centrifuged at 1,000 g for 5 minutes. The aqueous layer was discarded and 2.0 ml of organic layer were transferred into a 2 mL Eppendorf tube. The organic layer was left in the fume hood until dried. The residue was redissolved in 2.0 ml methanol and filtered over a 0.45 µm HPLC syringe filter (nylon). The samples were submitted to HPLC analysis. DPT, PPT and epi-PPT were analyzed by HPLC, LC-MS/MS and NMR analysis as described.31

**YFP visualization**

Calli of the transformed and wild type plants (control) were used to check the YFP expression. Visualization of the YFP expression was performed by using a Zeiss Axioskop fluorescence microscope (Zeiss Netherlands B.V. Weesp, The Netherlands).

**Statistical treatment of data**

All data presented in this study are the mean ± SD, of at least three independent experiments. Comparative statistical analyses of groups were performed using the student’s t-test. All statistical tests were considered significant at p values < 0.05.

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