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Modified nicotine metabolism in transgenic tobacco plants expressing the human cytochrome P450 2A6 cDNA

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Abstract In this study, the human cytochrome P450 (CYP) 2A6 was used in order to modify the alkaloid production of tobacco plants. The cDNA for human CYP2A6 was placed under the control of the constitutive 35S promoter and transferred into *Nicotiana tabacum* via *Agrobacterium*-mediated transformation. Transgenic plants showed formation of the recombinant CYP2A6 enzyme but no obvious phenotypic changes. Unlike wild-type tobacco, the transgenic plants accumulated cotinine, a metabolite which is usually formed from nicotine in humans. This result substantiates that metabolic engineering of the plant secondary metabolism via mammalian P450 enzymes is possible in vivo.

Keywords: Cytochrome P450 2A6; Nicotine metabolism; Cotinine; Secondary metabolism; *Nicotiana tabacum*

1. Introduction

The cytochrome P450 monooxygenases (CYPs), heme-containing mixed-function oxidases of highly divergent function, constitute the largest family of plant enzymes. For *Arabidopsis*, 273 named genes have been listed [1] (http://www.p450.kvl.dk/ and http://arabidopsis-p450.biotec.uiuc.edu) and it is assumed that a similar or even higher number could be found in other plants [2,3]. Plant P450s have been reported to be involved in different physiological processes [4] with a limited number of functional similar P450s involved in the formation of essential components needed by virtually all plants. Examples are oxygenated fatty acids for cutin and suberin biosynthesis [5], phytohormones like gibberellin [6], or signaling molecules like oxylipins [7]. Another fraction of the plant P450s is involved in the detoxification of exogenous compounds like herbicides and pollutants and this particular set of P450s might account for the resistance to certain herbicides among plant species [2,8]. A third fraction of the P450s is responsible for essential steps in the biosynthesis of the vast amount of plant secondary metabolites [4]. Some of these metabolites could function as UV-protectants which are present in all plant species but others serve as defense compounds against bacterial, fungal, insect, or animal predators [9].

With the increasing knowledge about the biosynthetic capacity of P450s and availability of the encoding cDNA sequences, attempts have been made to manipulate plant metabolism by expressing foreign P450 genes. In several studies the tolerance of plants to herbicides was modified by the introduction of P450 genes from different donor species. For example, CYP71A10 from soybean enhanced the resistance against phenylurea herbicides when it was introduced into the tobacco genome [10]. CYP1A1 from rat mediated an enhanced chlortoluron resistance when introduced into the tobacco genome [11,12], proving that recombinant mammalian P450s have the potential to influence the metabolism of xenobiotics in plants.

Also the modification of biosynthetic pathways in plants is an enormous challenge since an improved secondary metabolism would increase the value of a crop or medicinal plant [13–15]. There are numerous attempts to influence the formation of secondary compounds in plants by genetic engineering with some employing the shuffling of P450s between different plant species. In this way, the phenylpropanoid pathway of plants has been successfully changed by the expression of foreign P450 genes [16]. For example, the transformation of the soybean 2-hydroxyisoflavanone synthase gene into *Arabidopsis* resulted in the formation of genistein which is not a constituent of the host plant [17]. However, with the narrow substrate specificity of most plant P450s it is unlikely that novel compounds could be formed by the heterologous enzymes.

In contrast to most plant P450 enzymes, some mammalian P450s are known for their broad substrate acceptance.

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Abbreviations: P450, cytochrome P450 monooxygenase; CaMV, cauliflower mosaic virus; LC/MS, liquid chromatography/mass spectrometry

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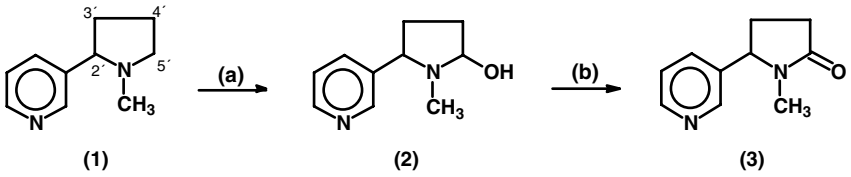

Fig. 1. Metabolism of nicotine (**1**) to cotinine (**3**). Compound (**1**) is hydroxylated by CYP2A6 (a) to 5′-hydroxynicotine. The conversion of the latter to (**3**) is catalyzed by a putative aldehyde oxidase (b).

Especially prominent in this group are P450s in human CYP families 1, 2, 3 that metabolize a huge variety of xenobiotics including pharmaceuticals and plant metabolites ingested with food [18]. Hence, it could be hypothesized that the expression of human P450 genes could enable transgenic plants to accomplish novel biochemical reactions and to accumulate new secondary compounds [19]. A limited number of publications report the expression of mammalian P450 genes in plants for purposes other than enhanced herbicide metabolism. Doty et al. showed that expression of drug metabolizing mammalian CYP2E1 resulted in an enhanced metabolism of halogenated hydrocarbons in tobacco [20] but changes in the alkaloid pattern have not been reported so far. Saito et al. [21] transferred rabbit CYP2C14 into tobacco and obtained plants which accumulated 2-propenylpyrrolidine, a degradative metabolite of nicotine. However, the occurrence of this compound was not due to the transgene but rather an effect of the enhanced senescence of the transgenic plants caused by other, so far not elucidated, effects.

To determine if a human drug metabolizing P450 enzyme could influence the pattern of secondary metabolites in plants, we expressed the cDNA encoding human CYP2A6 in tobacco plants which naturally accumulate the CYP2A6-specific substrate nicotine. In contrast to other human drug metabolizing P450s, the substrate specificity of CYP2A6 is limited to a relatively small number of chemicals. The CYP2A6-mediated hydroxylation of coumarin to 7-hydroxycoumarin (umbelliferone) and the C-oxidation of nicotine to *trans*-5′-hydroxynicotine are the most prominent examples [22]. In humans, 5′-hydroxynicotine is oxidized to the primary nicotine metabolite cotinine by aldehyde oxidase [23] (Fig. 1). Because *trans*-5′-hydroxynicotine and cotinine are not constituents of tobacco plants, their formation in transgenic tobacco plants would indicate the presence of active human CYP2A6 and prove that the enzyme has the ability to modify secondary metabolism in vivo.

2. Materials and methods

2.1. Plasmid construction

The cDNA encoding human cytochrome P450 2A6 was amplified by PCR to introduce an *Nco*I restriction site at the 5′-end for subsequent cloning into the plant expression vector pPS1 as a *Nco*I/*Xba*I fragment. In the resulting plasmid pPS12A6, the full-length CYP2A6 cDNA was placed between the tobacco etch virus (TEV) 5′-nontranslated leader which functions as a translational enhancer [24] and the 3′-untranslated sequences from the soybean *vspB* gene. The construct uses the cauliflower mosaic virus (CaMV) 35S promoter to drive translation and the *nptII* gene for kanamycin selection. The plasmid was cloned and transformed into *Escherichia coli* TOP 10 (Invitrogen) by methods described in [25] and was mobilized into *Agrobacterium tumefaciens* LBA4404 by the freeze–thaw method [26].

2.2. Plant material and transformation

Tobacco plants (*Nicotiana tabacum* cv. Petit Havana) were grown in the greenhouse with cycles of 16 h light and 8 h darkness. Growth temperature range was between 16 and 22 °C with a relative humidity of 60–70%. Transformation was achieved by cocultivating leaf discs from sterile grown plantlets with *Agrobacterium* strain LBA4404 harboring the expression plasmid [27]. Shoots were regenerated from transformed calli selected on medium containing 0.1 mg/ml kanamycin, subsequently rooted on MS medium and transplanted to soil.

2.3. Nucleic acid isolation and detection

DNA purification was performed with the CTAB-method described by Rogers et al. [28]. Detection of transgenic plants was achieved by PCR using a primer pair flanking the N-terminal 787 bp of the CYP2A6 cDNA and plant genomic DNA as a template.

Total RNA from plant tissue was isolated with TRItidy reagent (Applichem, Darmstadt, Germany) according to the manufacturer's protocol. RNA, 5 μg per lane, was loaded on a 1% denaturing gel. Electrophoretic separation of RNA and transfer to a nylon membrane was performed as described in standard protocols [25]. RNA bound to the membrane was transiently stained with methylene blue solution (0.25% methylene blue, 0.3% sodium acetate, pH 5.2). Specific detection of the target RNA was achieved with the non-radioactive nucleic acid labelling and detection system (DIG, Roche Molecular Biochemicals, Mannheim, Germany). For PCR amplification with the PCR DIG probe synthesis kit (Roche) the two oligonucleotides described above were used to generate a 787 bp digoxigenin-11-dUTP-labeled DNA fragment.

2.4. Protein analysis

Total leaf protein was extracted directly in 2× SDS sample buffer (100 mM Tris–HCl, pH 8; 200 mM dithiothreitol; 4% sodium dodecyl sulfate (w/v); 0.2% bromophenol blue (w/v); and 20% glycerol (w/w)). Frozen ground leaf (100 μg) was mixed with 200 μl of sample buffer and boiled for 10 min. After centrifugation (14 000 × *g*) samples were separated on a 10% polyacrylamide gel and subsequently transferred to a PVDF membrane, and the membrane was blocked overnight with 5% skim milk powder in phosphate-buffered saline with 0.05% Tween 20 (PBST). Blots were incubated with rabbit anti-human CYP2A6 (BioTrend, Köln, Germany) at a concentration of 1:2000 in PBST for 2 h. After washing, 1 h incubation with goat anti rabbit IgG HRP conjugated (1:20 000 in PBST), and detection with Super Signal chemiluminescent substrate (Pierce, Bonn, Germany) membranes were exposed to X-ray films.

2.5. Sample preparation and LC/MS analysis

Leaves of greenhouse-grown plants were harvested and immediately frozen in liquid nitrogen. After freeze-drying, 0.15 g of the powdered leaf material was extracted with 25% MeOH (v/v) for 30 min in an ultrasonic bath at 40 °C. After centrifugation (9000 × *g* for 10 min) 25 μl of the clear supernatant was injected onto a 250 × 4.6 mm, 5 μm analytical HPLC column (MetaChem Polaris C18-A; Varian, Darmstadt, Germany). The mobile phase consisted of 10 mM ammonium acetate/0.1% acetic acid (A) and MeOH (B). The flow rate was set to 0.8 ml/min and the following gradient (% B) was applied: 0 min: 20, 12.0 min: 65. After 12 min the column was flushed with 100% (B) for 5 min and re-equilibrated with 20% (A) for 5 min. The temperature for the analytical column was set to 25 °C. An ion trap mass spectrometer (Agilent, Waldbronn, Germany) consisting of the following series 1100 HPLC components: G1379A vacuum degasser, G1312A binary pump, G1313A autosampler, G1316A column

thermostat and G2445D mass selective detector was applied for the detection of cotinine using electrospray ionisation (ESI) in the positive ion mode. Nitrogen was used for the nebuliser (50 psi) and the drying gas (300 °C, 12 l/min). The capillary voltage was −3500 V and the skimmer, octopole 1, octopole 2 and capillary exit voltage were set to 40, 12, 1.7 and 104 V, respectively.

The quantification of cotinine in the tobacco plants was performed using external calibration. For this purpose, we prepared five calibration solutions with cotinine concentrations of 500, 250, 100, 25, and 10 ng/ml in MeOH 25 % (v/v). Every calibration sample was injected three times and the cotinine concentration was calculated after linear regression analysis ($y = 25332x + 133922$; $R^2 = 0.9999$). The limit of detection and quantification was 3 and 10 ng/ml, respectively.

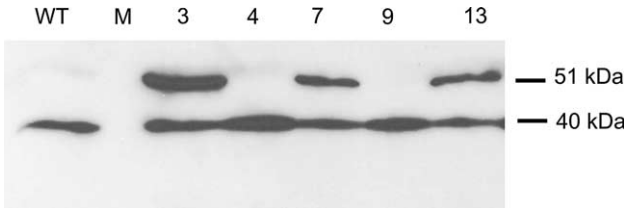

Fig. 3. Western blot analysis of CYP2A6 protein in plant extracts of wild-type and transgenic tobacco plants. WT: untransformed tobacco; M: marker lane; 3, 4, 7, 9, 13: transgenic lines.

3. Results

We cloned the full-length cDNA of human CYP2A6 in the plant transformation vector pPS1 under the control of the CaMV 35S promoter. This construct should provide constitutive expression in all parts of the tobacco plant and a high level of the CYP2A6 transcript due to the presence of 5′- and 3′-regulatory elements (TEV leader and *vsp*-terminator, respectively). After *Agrobacterium*-mediated transformation, independent kanamycin-resistant plantlets were obtained. RNA-blot analysis revealed that seven out of eleven plants exhibited CYP2A6 transcripts at different levels, plants number 3, 7, and 13 having the highest mRNA content (Fig. 2). Tobacco plants were transferred into the greenhouse and grown under standard conditions. No obvious changes could be observed in their phenotype. In contrast, Saito et al. [21] reported early senescence in tobacco plants expressing a different mammalian P450 gene (rabbit CYP2C14).

In order to determine the expression level of the P450-transgene, we tested plant protein extracts by immunoblot analysis using a commercially available antiserum against human CYP2A6. We were able to detect CYP2A6 protein in three independent plant lines (lines 3, 7, and 13 (Fig. 3)) indicating a clear correlation existed between the transcript abundance and the detectable amount of P450 enzyme.

No protein crossreacting with the CYP2A6 antibody could be detected in untransformed tobacco but all protein extracts tested exhibited an additional signal derived from a protein with a higher electrophoretic mobility and a molecular weight of approximately 40 kDa. This observation suggested the non-specific binding of the antiserum to an endogenous plant protein rather than to degradation products of the recombinant CYP2A6 protein.

For the determination of cotinine in plant material, leaves were harvested from greenhouse-grown plants, extracted, and subjected to liquid chromatography/mass spectrometry (LC/MS) analysis. In Fig. 4, the extracted ion chromatograms of *m/z* 177 ($[MH]^+$ of cotinine) are shown for extracts from the wild-type plants and lines 4 and 7 (lines 3 and 13 were identical to line 7 and are not shown in Fig. 4). Only the extracts from lines 3, 7, and 13 showed a distinct signal at the retention time of authentic cotinine suggesting that nicotine was oxidized to 5′-hydroxynicotine by the recombinant CYP2A6 protein and subsequently dehydrogenated to cotinine by an endogenous dehydrogenase (Fig. 4C with structures in Fig. 1). Quantification of the cotinine content in lines 3, 7, and 13 with LC/MS revealed cotinine concentrations between 2 and 7 ppm. Whereas these cotinine concentrations are rather low, we could not detect cotinine in lines 4, 9, and wild-type tobacco plants. The correlation between mRNA abundance, CYP2A6 protein accumulation and formation of the enzymatic product cotinine is shown in Table 1. As can be clearly seen for the plant lines 3, 7, and 13, only high levels of specific mRNA led to the detectable accumulation of the CYP2A6 protein and the production of cotinine (Table 1). No further changes in the metabolite pattern of the transgenic tobacco plants were observed after additional LC/UV and LC/MS fingerprint analysis (data not shown). Since the cotinine content in the plant lines 3, 7, and 13 was comparably low and an additional dehydrogenation is required for the formation of cotinine from nicotine (Fig. 1), we extracted additional signals of the total ion current in order to identify further oxidized metabolites of nicotine or cotinine. However, we obtained no additional signals in the

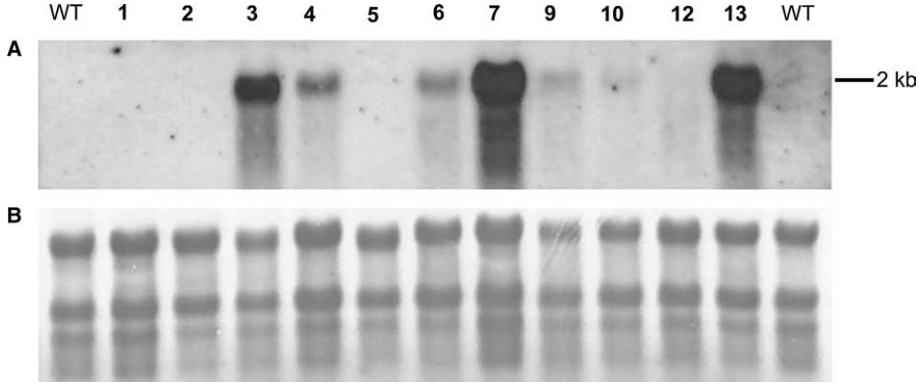

Fig. 2. Analysis of the CYP2A6 transcript abundance in transgenic tobacco plants. (A) Northern blot of total RNA from different plant lines hybridized with a CYP2A6-specific probe. (B) Methylene blue-stained total RNA. WT: wild-type tobacco plants. 1–13: Independent transformants.

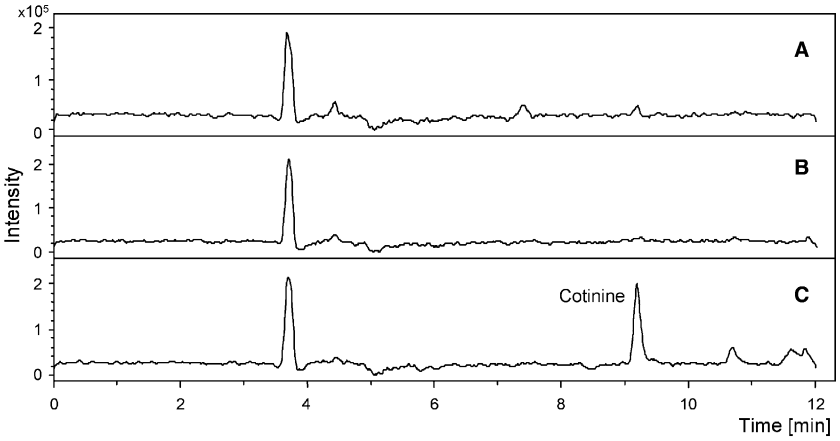

Fig. 4. LC/MS analysis of cotinine in wild-type and transgenic tobacco plants. The extracted ion electropherograms at *m/z* 177 correspond to the protonated molecule (MH^+ ion) of cotinine (M_r 176). (A) Wild-type tobacco. (B) Transgenic line 4 with low CYP2A6 transcript abundancy. (C) Transgenic line 7 with high CYP2A6 transcript accumulation.

Table 1
Dependence of cotinine production on CYP2A6 transcript levels and CYP2A6 protein accumulation in transgenic tobacco plants

Plant line	WT 1	WT 2	3	4	9	7	13
RNA	–	–	+++	+	+	+++	+++
Protein	–	–	+	–	–	+	+
Cotinine	–	–	+	–	–	+	+

–: not detectable.

extracted ion electropherograms indicating that nicotine was oxygenated to 5′-hydroxynicotine and subsequently dehydrogenated to cotinine without accumulation of oxygenated metabolites of nicotine or cotinine.

Because CYP2A6 was not present in the wild-type plants and no CYP2A6 protein could be detected in line 4 we conclude that in plant lines 3, 7, and 13 catalytically active CYP2A6 hydroxylated nicotine to 5′-hydroxynicotine, which was further metabolized to cotinine by an endogenous plant enzyme.

4. Discussion

In the present study, we demonstrated the heterologous expression of the human CYP2A6 cDNA in tobacco plants. Only modified plants with high expression levels of the transgene produced catalytically active CYP2A6 which resulted in the formation of cotinine, a metabolite usually not found in tobacco.

The amount of the new metabolite cotinine is relatively low and several reasons may account for this observation. P450 enzymes usually cooperate with a NADPH-P450 reductase as an electron donor and it is remarkable that the mammalian CYP2A6 is able to use a NADPH-P450 reductase from the plant host as redox partner although there is a moderate sequence identity of oxidoreductases from mammals and plants (e.g., 35% identity between human P450 reductase and *Arabidopsis* P450 reductase (ATR1)). However, inefficient coupling of this plant P450 reductases with the mammalian CYP2A6 could account for the reduced activity of the recombinant CYP2A6 in this system [29].

In humans, CYP2A6 is responsible for the 5′-hydroxylation of nicotine and a second enzymatic step catalyzed by an aldehyde oxidase is necessary to form cotinine (Fig. 1) [23]. Thus, the low cotinine content in transgenic tobacco leaves could also result from low levels of aldehyde oxidase, the second enzyme needed in cotinine formation. Since cotinine is found in plants we speculate that an enzymatic activity comparable to the human aldehyde oxidase must also be present in tobacco. The absence of 5′-hydroxynicotine leads to the conclusion that it is quantitatively converted to cotinine. Therefore, the limiting step in cotinine formation should be the 5′-hydroxylation of nicotine rather than the dehydrogenation of 5′-hydroxynicotine.

When analyzing the fingerprint spectra of both transgenic and wild-type tobacco extracts we noticed almost no changes or differences in the metabolite pattern. This is not an unexpected finding since the P450 used in this study is an enzyme which accepts and converts only a limited number of substrates and would not affect the majority of secondary metabolites found in tobacco. However, with the heterologous expression of the human CYP2A6 gene we have proven that the secondary metabolism of a plant can be selectively modified to produce structures usually not found in plants. By choosing genes whose encoded enzymes accept substrates which are naturally present in the host plants, novel compounds can be generated. Future experiments in which the transferred P450 gene (e.g., CYP3A4 which exhibits a very broad substrate acceptance) and the host plant are varied will show whether appreciable amounts of various novel compounds can be generated by the introduction of human P450 genes into plants.

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