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Accumulation of tyrosol glucoside in transgenic potato plants expressing a parsley tyrosine decarboxylase

Jörn Landtag[a], Alfred Baumert[b], Thomas Degenkolb[c], Jürgen Schmidt[c], Victor Wray[d], Dierk Scheel[a], Dieter Strack[b], Sabine Rosahl[a,*]

[a]*Department of Stress and Developmental Biology, Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle/Salle, Germany*
[b]*Department of Secondary Metabolism, Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle/Salle, Germany*
[c]*Department of Bioorganic Chemistry, Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle/Saale, Germany*
[d]*German Research Center for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany*

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Abstract

As part of the response to pathogen infection, potato plants accumulate soluble and cell wall-bound phenolics such as hydroxycinnamic acid tyramine amides. Since incorporation of these compounds into the cell wall leads to a fortified barrier against pathogens, raising the amounts of hydroxycinnamic acid tyramine amides might positively affect the resistance response. To this end, we set out to increase the amount of tyramine, one of the substrates of the hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)-transferase reaction, by placing a cDNA encoding a pathogen-induced tyrosine decarboxylase from parsley under the control of the 35S promoter and introducing the construct into potato plants *via Agrobacterium tumefaciens*-mediated transformation. While no alterations were observed in the pattern and quantity of cell wall-bound phenolic compounds in transgenic plants, the soluble fraction contained several new compounds. The major one was isolated and identified as tyrosol glucoside by liquid chromatography–electrospray ionization–high resolution mass spectrometry and NMR analyses. Our results indicate that expression of a tyrosine decarboxylase in potato does not channel tyramine into the hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)-transferase reaction but rather unexpectedly, into a different pathway leading to the formation of a potential storage compound.

Keywords: *Solanum tuberosum*; *Solanaceae*; Pathogen response; Salidroside

1. Introduction

Infection of potato plants with the oomycete *Phytophthora infestans*, the causal agent of late blight disease, initiates a multicomponent defense response that includes the accumulation of secondary phenolic compounds such as hydroxycinnamic acid tyramine amides (Schmidt et al., 1998). Since these amides are also incorporated into the cell wall, they are generally believed to play a role in the defense against pathogen infection by reinforcing and thereby decreasing the digestibility of the cell wall. Upon infection of potato plants with *P. infestans* or upon treatment of potato cells with *P. infestans*-derived elicitor, activities of tyrosine decarboxylase (TyrDC, EC 4.1.1.25) as well as those of enzymes leading to the formation of hydroxycinnamic acids, phenylalanine ammonia lyase (PAL, EC 4.3.1.5) and 4-coumarate:CoA ligase (4CL, EC 6.2.1.12) increase (Schmidt et al., 1998; Keller et al., 1996), followed by the accumulation of tyramine, hydroxycinnamic acids and amides thereof, such as 4-coumaroyltyramine and feruloyltyramine, as soluble and cell wall-bound phenolics (Schmidt et al., 1998).

The role of phenolic compounds in defense responses has been analyzed using functional approaches in transgenic plants. In particular, modulation of the expression of amino acid decarboxylases, which act at the interface of primary and secondary metabolism, has been used to analyze the role of secondary metabolites. Thus, in canola (Facchini et al., 1999) and tobacco

* Corresponding author. Tel.: +49-345-55821440; fax: +49-345-55821409.
E-mail address: srosahl@ipb-halle.de (S. Rosahl).

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plants (Guillet et al., 2000) transformed with a TyrDC gene, increases in the activity of TyrDC led to higher amounts of cell wall-bound tyramine and to a decreased digestibility of the cell wall (Facchini et al., 1999), as well as to higher levels of soluble tyramine (Guillet et al., 2000). Increased levels of tyramine were also detected in transgenic tobacco plants expressing a tryptophan decarboxylase (TrpDC, 4.1.1.28; Songstad et al., 1991), presumably due to a general stimulation of aromatic amino acid biosynthesis (Facchini et al., 2000). In potato, expression of a TrpDC did not only lead to decreased levels of the aromatic amino acids tryptophan and phenylalanine, but also to impaired accumulation of phenolic compounds such as chlorogenic acid in response to pathogen infection (Yao et al., 1995). The higher susceptibility of these transgenic tubers to infection with *P. infestans* suggests that synthesis and accumulation of phenolics is crucial for successful defense. The importance of aromatic amines has been demonstrated with TrpDC-overexpressing tobacco plants, where reproduction of white flies was drastically reduced, suggesting that the high levels of tryptamine present in the transgenic plants exert a negative effect on feeding insects (Thomas et al., 1995).

To understand the role of tyramine in the response of potato to infection with *P. infestans*, particularly with respect to the synthesis of cell wall-bound hydroxycinnamic acid tyramine amides, we analyzed whether expression of a parsley TyrDC in transgenic potato leads to higher levels of soluble and cell wall-bound tyramine-derived phenolic compounds. Since the enzyme catalyzing the formation of hydroxycinnamic acid tyramine amides, hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)-transferase (THT, 2.3.1.110), has a high affinity towards tyramine in the presence of the acyl donor feruloyl-CoA (Schmidt et al., 1999), increasing the levels of tyramine might stimulate the rate of synthesis of hydroxycinnamic acid tyramine amides. Expression of the parsley TyrDC, however, did not lead to increased levels in tyramine nor hydroxycinnamic acid tyramine amides, but rather to accumulation of a new compound, tyrosol glucoside, which has not previously been shown to occur in potato.

2. Results

2.1. Generation of transgenic potato plants expressing a parsley TyrDC

TyrDC cDNAs have been isolated from parsley as pathogen elicitor-induced genes (Kawalleck et al., 1993). Of the three isoforms identified, TyrDC-2 is the best characterized and exhibits, as a recombinant protein, the highest specific activity with tyrosine and 19–28% of this activity with dopa as a substrate (Kawalleck et al., 1993). In order to attempt to increase the levels of tyramine in potato plants, we cloned the insert of the cDNA-clone PcTyrDC-2 encoding parsley TyrDC-2 into the binary vector pBinKan-TX behind the 35S-TX-promoter (Fig. 1; Gatz et al., 1991). Agrobacteria transformed with this construct were used for leaf-disk transformation of potato plants. Nine kanamycin-resistant plants were regenerated and analyzed for the presence and expression of the PcTyrDC-2 sequences. DNA was isolated from seven kanamycin-resistant plants and one non-transformed wild type plant, digested with the restriction enzymes EcoRI and HindIII and subjected to Southern analysis (Fig. 2). In all samples, a 2.5 kb fragment specifically hybridizing to the PcTyrDC-2 probe was present, indicating the presence of a complete PcTyrDC-2 cDNA in the potato genome.

2.2. Expression of TyrDC in transgenic potato plants

RNA was isolated from leaves of transgenic potato plants grown in soil for 4 weeks and subjected to Northern analyses using the radioactively labelled insert of PcTyrDC-2 as a probe (Fig. 3). In all six plants analyzed, transcripts hybridizing to PcTyrDC-2 were detectable. The highest transcript levels were observed in plant K, while plants B, G and M had intermediate and plants D and H lower levels of PcTyrDC transcripts. Apart from the bands of the expected size of around 1.7 kb, we consistently observed larger transcripts in transgenic plants. Since these bands were not detected in untransformed wild type potato plants, they appear to be transgene-specific.

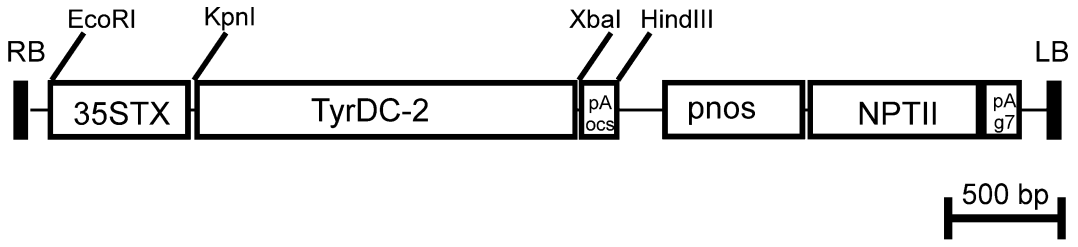

Fig. 1. Structure of the binary vector containing the parsley TyrDC-2 cDNA. The cDNA of TyrDC-2 was cloned between the 35STX promoter (Gatz et al., 1991) and the polyadenylation signal of the octopine synthase gene (pAocs). Recognition sites for restriction enzymes EcoRI, KpnI, XbaI and HindIII are indicated. The bar represents 500 bp. RB: right border, LB: left border, pnos: promoter of the nopaline synthase gene, NPTII: neomycinphosphotransferase, pAg7: polyadenylation signal of gene 7.

2.3. Identification of tyramine-derived compounds

In order to detect any changes in the level of phenolic compounds in PcTyrDC-transgenic plants compared to untransformed wild type plants, HPLC analyses were performed for nine transgenic potato plants. No hydroxycinnamic acid tyramine amides, such as 4-coumaroyltyramine or feruloyltyramine were detectable in cell wall extracts from either transgenic plants or wild type plants (data not shown), suggesting that expression of PcTyrDC-2 in potato does not lead to qualitative nor quantitative changes in cell wall-bound phenolics. In contrast, HPLC profiles of methanolic extracts (Fig. 4) revealed the presence of several new soluble compounds, one of which (compound **1**) representing the major phenolic in leaves of all transgenic plants. Compound **1** was not detectable in leaves of untransformed potato plants but was present in different transgenic plants at concentrations of 9.2 (plant B), 4.5 (C), 6.6 (H), 7.1 (H-2), 3.6 (I), 8.0 (J), 16.8 (K), 4.1 (K-1) and 5.0 (M) μmol tyramine equivalents per g fr. wt. Tyramine itself was not detectable in any of the extracts.

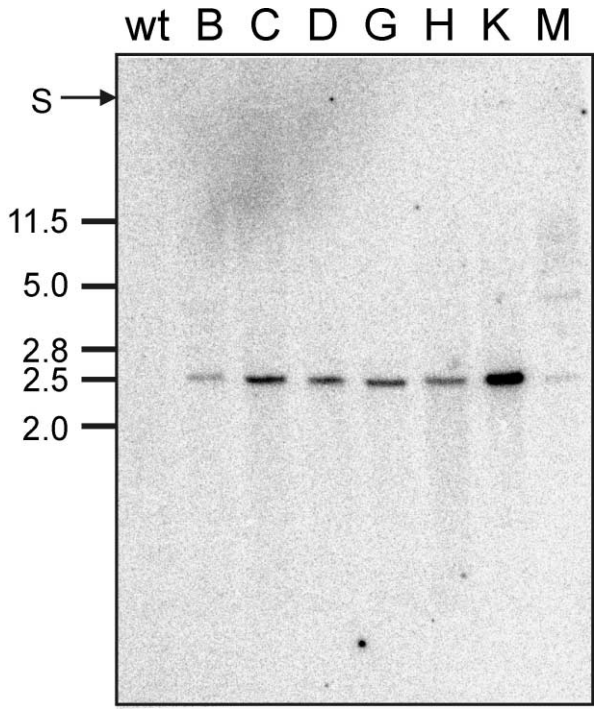

Fig. 2. Southern analysis of DNA of transgenic potato plants. DNA from regenerated kanamycin-resistant plants (B, C, D, G, H, K, M) and untransformed wild type potato plants (wt) was digested with EcoRI and HindIII and subjected to Southern analysis. The radioactively labeled 1.7 kb fragment of PcTyrDC-2 was used as a probe. Numbers at the left indicate the size (kb) of markers. S indicates the start point.

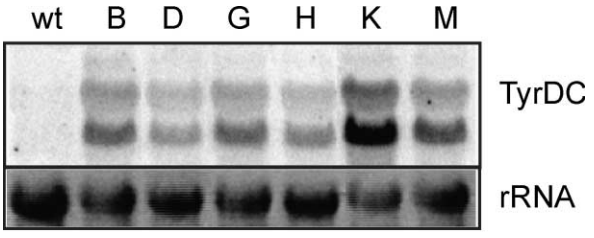

Fig. 3. RNA analysis of transgenic plants. RNA was isolated from transgenic (B, D, G, H, K, M) and untransformed wild type potato plants (wt) and subjected to Northern analysis. The radioactively labeled 1.7 kb fragment of PcTyrDC-2 was used as a probe ("TyrDC"). The lower panel shows rRNA stained with ethidium-bromide ("rRNA") as a loading control.

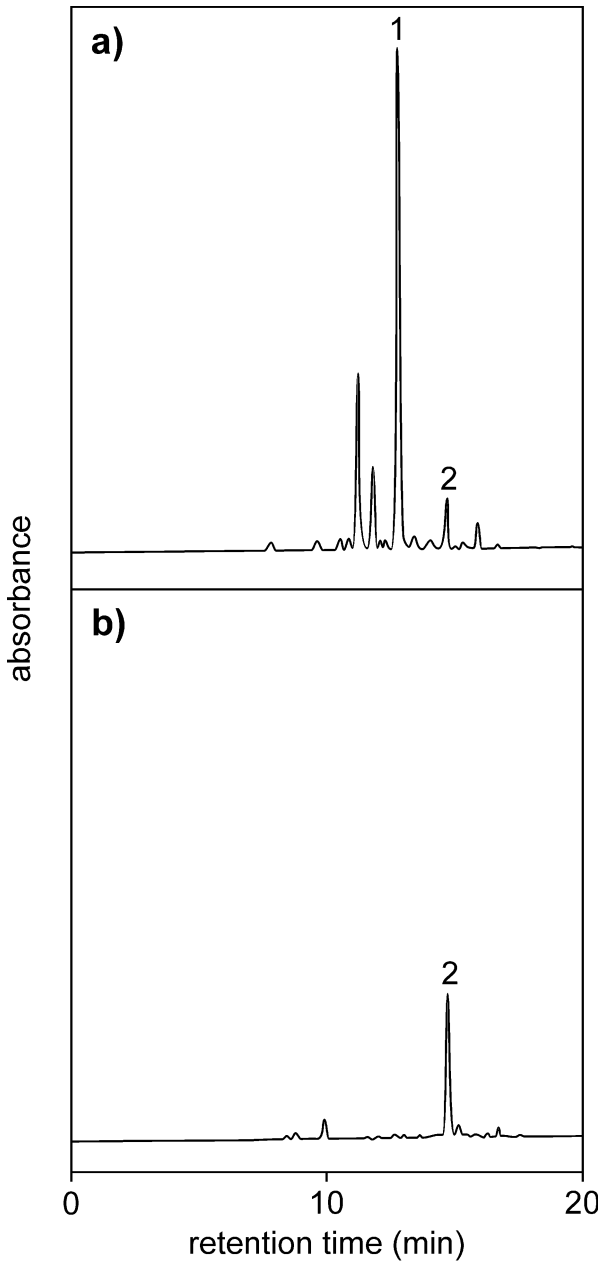

Fig. 4. HPLC patterns of methanolic extracts from leaves of the transgenic plant K (**a**) and a wild-type potato plant (**b**) at maxplot detection between 220 and 400 nm. Peak identification: **1**, tyrosol 8-*O*-β-D-glucopyranoside (R_t 12.6 min; λmax 274.6); **2**, tryptophan (R_t 14.6 min). Tyramine (R_t 7.7 min) was not detected in the leaf material. The pattern is representative for all transgenic plants analyzed.

Compound **1** could be identified as tyrosol 8-*O*-β-D-glucopyranoside (**1**) by LC-high resolution electrospray MS and ^{1}H-NMR studies. The positive ion electrospray MS of **1** displays a $[M+NH_4]^+$ ion at *m/z* 318. The elemental composition ($C_{14}H_{20}O_7$) of the molecular ion was deduced from the HR–MS (ESI-QqTOF system) of the $[M+NH_4]^+$ and the $[M+H]^+$ peaks. Key ions in the CID spectrum appear at *m/z* 180 (**a**), 163 (**b**) representing the glucosyl moiety ($C_6H_{11}O_5$) and 121 (**c**) as a part of the tyrosol unit (Fig. 5).

The structure of the compound was established from the combined 1D and 2D NMR data. The identity of the β-glucopyranose moiety was evident from the correlations in the 2D COSY spectrum and from the magnitudes of the chemical shifts and vicinal coupling constants. The same spectra indicated the presence of a *p*-disubstituted benzene and 1,2-disubstituted ethane system. The arrangement of these units and their linkages was evident from the correlations in the heteronuclear multiple-bond ^{1}H–^{13}C correlation (HMBC). Unambiguous correlations were observed between the anomeric proton of the sugar moiety and C-7 of the ethane unit and the reverse correlations of H-7A and B with C-1, and between H-7A/B and H-8 with carbons of the aromatic ring system and appropriate reverse correlations. Finally the chemical shifts of C-6 and C-12 indicated the terminal position of the sugar unit and, in combination with the MS data, the presence of a hydroxyl group in the *p*-position of the aromatic ring.

3. Discussion

Manipulation of metabolic pathways is usually achieved by modulating the expression of endogenous enzymes or by introducing new enzyme activities. Here, we have transferred the cDNA encoding parsley TyrDC-2 into potato plants to increase the amounts of tyramine, a substrate for the THT reaction. The ectopic expression of a TyrDC under the control of the 35S promoter has been achieved in transgenic canola and tobacco plants and led to increased levels of free and cell wall-bound tyramine as well as a decreased digestibility of the cell wall (Facchini et al., 1999). Since incorporation of hydroxycinnamic acid tyramine amides into cell walls is observed as a response of plants against pathogens (Schmidt et al., 1998), it would be of interest to analyze these transgenic tobacco and canola plants with respect to increased resistance against pathogens.

In contrast, in the potato plants described here, expression of the parsley TyrDC did not result in altered amounts of either free tyramine or cell wall-bound tyramine. Also, we were unable to detect changes in the level of hydroxycinnamic acid tyramine amides. The presence of the novel compound, tyrosol glucoside, suggests that overexpression of TyrDC leads to increases in tyramine which is channeled into a different metabolic route. Tyrosol glucoside has not been reported to occur in potato before, and its synthesis in response to overexpression of a TyrDC appears to be specific for potato, since we did not detect tyrosol glucoside in transgenic tobacco plants expressing the parsley TyrDC-2 (data not shown) nor has the presence of this compound been reported for TyrDC-expressing transgenic canola plants (Facchini et al., 1999).

We propose that the synthesis of tyrosol glucoside might proceed from tyramine *via* the formation of 4-hydroxyphenylacetaldehyde and the subsequent reduction to 4-hydroxyphenylethanol to its glycosylation (Fig. 6). Indeed, high activity of tyramine oxidase

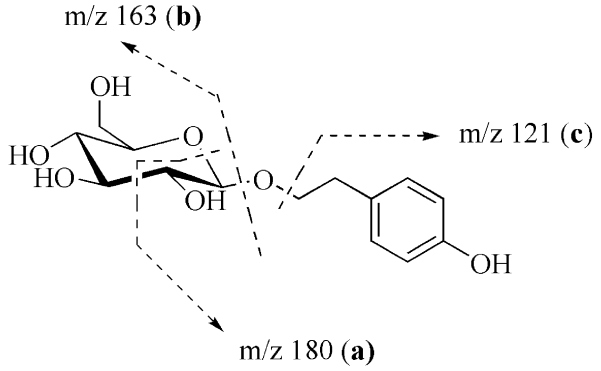

Fig. 5. Scheme of the collision-induced fragmentation of the $[M+NH_4]^+$ ion of compound **1** obtained by LC-ESIMS.

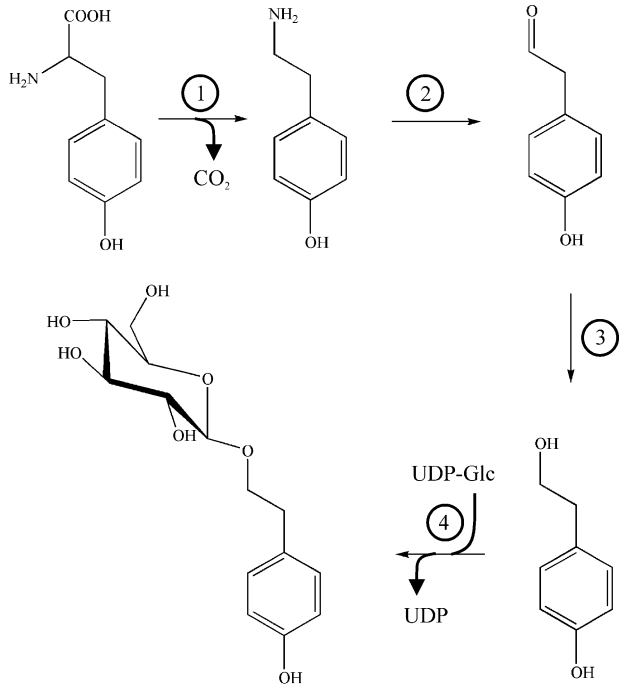

Fig. 6. Proposed biosynthetic pathway of tyrosol glucoside formation. The possible route of tyrosol glucoside formation in transgenic potato plants expressing the parsley TyrDC-2 is shown. 1: TyrDC, 2: monoamine oxidase, 3: reductase, 4: tyrosol glucosyltransferase.

(EC 1.4.3.4), the enzyme catalyzing the conversion of tyramine to 4-hydroxyphenylacetaldehyde, and of tyrosol glucosyltransferase is found in suspension cultured cells of tyrosol glucoside-producing plant species (Rueffer and Zenk, 1987; Xu et al., 1998a).

Tyrosol glucoside is also known as salidroside and has been identified in a number of plants such as *Linaria japonica* (Otsuka, 1993), birch (Shen et al., 1999) and olive (Maestroduran et al., 1994). As one of the active components of the arctic root (*Rhodiola rosea*), salidroside is suggested to play a role as an adaptogen, i.e. a biologically-active compound that is supposed to increase resistance in humans to different stress-related disorders and a variety of ailments or diseases. For example, salidroside has been reported to act as an anti-ageing, anti-inflammatory and anti-cancer compound. Biotechnologically, salidroside is produced from cell suspension cultures from *Rhodiola sachalinensis* (Xu et al., 1998a) which can be manipulated to accumulate salidroside up to 15% of the dry weight (Xu et al., 1998b).

The synthesis of tyrosol glucoside in transgenic potato expressing the parsley TyrDC most likely represents a mechanism to withdraw excess tyramine from the cell metabolism. Although tyramine itself is not deleterious when added exogenously to tobacco cells (Negrel et al., 1993) and can accumulate to high levels in transgenic plants expressing a TrpDC (Songstad et al., 1991), extreme toxicity is observed in the presence of tyramine-oxidizing enzymes. Thus, in tobacco callus cells, the toxicity of tyramine may be due to the formation of indolequinones after oxidation by polyphenol oxidases (Negrel et al., 1993). This notion is supported by the observation that suppression of toxicity, induced by cytokinins, correlates with decreased polyphenol oxidase activity and with increased THT activity (Negrel et al., 1993). Apparently, in tobacco callus cells treated with cytokinin, the incorporation of tyramine into cell walls as hydroxycinnamic acid tyramine amides acts as a mechanism for removal of tyramine (Negrel et al., 1993). As shown in the present study, a different mechanism appears to take place in potato. Possibly, in contrast to tobacco and canola, tyramine does not reach the site of THT action and tyramine is instead converted into tyrosol glucoside. The absence of tyramine in transgenic potato tissue furthermore indicates that conversion via this metabolic route is rapidly and efficiently stimulated.

4. Experimental

4.1. Cloning of TyrDC into binary vectors

The parsley cDNA PcTyrDC-2, contained in pBluescript SK+ (Kawalleck et al., 1993), was isolated by digesting plasmid DNA with NotI. After filling-in of the protruding ends, the 1.7 kb fragment representing the TyrDC cDNA was isolated and ligated to the SmaI-digested and dephosphorylated binary vector pBinKan-TX. This vector was constructed by cloning the 0.8 kb EcoRI/HindIII fragment from pBinHygTX (kindly provided by Christiane Gatz, Göttingen), containing the 35S-TX promoter and the polyadenylation signal of the octopine synthase gene, into the EcoRI/HindIII-digested binary vector pPGTV-Kan (Becker et al., 1992), eliminating the GUS coding region and the polyadenylation signal of the nopaline synthase gene. Recombinant plasmids (pBinKanTX - PcTyrDC) were identified by restriction analysis of purified plasmid DNA and used to transform *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983).

4.2. Transformation of potato, regeneration and analysis of transgenic plants

Plant transformation and regeneration of transgenic plants was carried out as described (Feltkamp et al., 1995). Regenerated, kanamycin-resistant plants were subjected to Southern analysis as described (Schmidt et al., 1999). RNA was isolated and analyzed by Northern blots as described (Geerts et al., 1994). Hybridization of Southern and Northern blots was carried out as described (Schmidt et al., 1999) using the radioactively labelled 1.7 kb PcTyrDC-2 cDNA as a probe.

4.3. HPLC analysis

Leaf material (100 mg fresh weight) was extracted with $MeOH/H_2O$ (4:1) in 2 ml-safe-lock tubes in the presence of zirconia beads (1 mm i.d.) using a bead beater (Bio Spec Products, Inc., Bartlesville, OK, USA). The extracts were centrifuged, and 20 μl-samples were injected onto a 5 μm Nucleosil C18 column (250×4 mm i.d.; Macherey-Nagel, Düren, Germany). Separation was achieved using a 20-min linear gradient at 1 ml min^{-1} from 0 to 35% MeCN in 1.5% H_3PO_4 followed by a gradient from 35 to 70% MeCN within 5 min. Compounds were photometrically detected (maxplot between 220 and 400 nm) by a Waters 996 photodiode array detector.

For preparative HPLC, 10 g freshly harvested leaf material was extracted with $MeOH/H_2O$ (4:1), centrifuged and concentrated. The extract was repeatedly chromatographed on a preparative HPLC column (10 μm-Nucleosil 100–10 C_{18} Vario Prep, 250×40 mm i.d.; Macherey-Nagel, Düren, Germany) using the liquid chromatograph (System Gold; Beckman Instruments, München, Germany). The separation was performed with a linear gradient within 80 min from solvent A (aq. HOAc, pH 3) to 60% solvent B (MeCN) in A at a flow rate of 10 ml min^{-1} (2-ml injection volume).

Compounds were photometrically detected (maxplot 210–500 nm). Pure tyrosol 8-*O*-β-D-glucopyranoside was obtained in a yield of approximately 11 mg.

4.4. LC/MS

The positive ion electrospray (ES) mass spectra were obtained from a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV; heated capillary temperature 220 °C; sheath and auxillary gas gas: nitrogen) coupled with a Micro-Tech Ultra-Plus MicroLC system equipped with a RP18-column (4 μm, 1×100 mm, Ultrasep). For the HPLC a gradient system was used starting from H_2O:MeCN 4:1 (each of them containing 0.2% HOAc) to 1:9 within 15 min; flow rate 70 μl min^{-1}.

4.4.1. Tyrosol 8-O-β-D-glucopyranoside (*1*)

RT (LC–MS), 6.33 min, positive electrospray MS *m/z* (rel. int.): 318 ($[M+NH_4]^+$, 100), 301 ($[M+H]^+$, 5).

Positive ion HRESI mass spectra (electrospray voltage: 5.5 kV, source temperature: ambient, curtain/nebulizer gas: N_2) were recorded on an API QSTAR Pulsar Hybrid Quadrupole TOF LC MS/MS instrument (Perkin Elmer Sciex, Foster City USA, Analyst QS software, MDS Sciex Concord Ontario, Canada) coupled to an UltiMate Micro HPLC/Famos autosampler system (UltiChrom 3.1 software, LC Packings Amsterdam, The Netherlands) equipped with a Fusica C18 column (3 μm, 150×3 mm, PepMap). For LC/MS, a linear gradient system starting from 90% H_2O/10% MeCN to 10% H_2O/90% MeCN was used at a flow rate of 4 μl min^{-1}. Each of the solvents contained 0.05% HCOOH. For HRESI MS/MS spectra a collision energy of 15 eV was applied, and N_2 was used as collision gas.

LC-ESIQqTOF MS: RT (LC/MS), 13.33 min; positive high-resolution electrospray MS (*m/z*, rel. int.): 318.1503 ($[M+NH_4]^+$, calc. for $C_{14}H_{24}NO_7$, 318.1547, 43); 301.1276 ($[M+H]^+$, calc. for $C_{14}H_{21}O_7$, 301.1281, 17); 265.1056 ($[M+H-2H_2O]^+$, calc. for $C_{14}H_{17}O_5$, 265.1070, 49); 247.0925 ($[M+H-3H_2O]^+$, calc. for $C_{14}H_{15}O_4$, 247.0964, 35); 229.0864 ($[M+H-4H_2O]^+$, calc. for $C_{14}H_{13}O_3$, 229.0859, 27); 205.0818 (calc. for $C_{12}H_{13}O_3$, 205.0859, 64); 187.0738 ($[m/z\ 205-H_2O]^+$, calc. for $C_{12}H_{11}O_2$, 187.0753, 25); 180.0855 (**a**, calc. for $C_{10}H_{12}O_3$, 180.0780, 38); 163.0596 (**b**, calc. for $C_6H_{11}O_5$, 163.0601, 85); 156.0988 ($[tyrosol+NH_4]^+$, calc. for $C_8H_{14}NO_2$, 156.1019, 12); 145.0483 (**b**-H_2O, calc. for $C_6H_9O_4$, 145.0495, 76); 127.0367 (**b**-$2H_2O$, calc. for $C_6H_7O_3$, 127.0389, 11); 121.0631 (**c**, calc. for C_8H_9O, 121.0647, 100); 85.0276 (calc. for $C_4H_5O_2$, 85.0284, 3).

4.5. NMR analysis

NMR: 1D (^1H, ^{13}C and DEPT-135) and 2D (COSY and HMBC) NMR spectra were recorded at 300 K on a Bruker AVANCE DMX 600 NMR spectrometer locked to the deuterium resonance of the solvent, D_2O. Chemical shifts are in ppm and couplings constants in Hz.

^1H NMR $\delta = 7.26$ ["*d*", H-10/H-14, AA' part of AA'BB' system, $J(10–11) + J(10–13)$ 8.5], 6.90 ["*d*", H-11/H-13, BB' part of AA'BB' system], 4.49 [*d*, H-1, *J*(1–2) 8.1], 4.13 [*dt*, H-7A, *J*(7A-7B) 10.1, *J*(7A-8) 7.0], 3.93 [*dd*, H-6A, *J*(6A-6B) 12.3, *J*(6A-5) 2.2], 3.91 [*dt*, H-7B, *J*(7B-8) 7.0], 3.74 [*dd*, H-6B, *J*(6B-5) 5.9], 3.50 [*dd*, H-3, *J* (2–3) 9.4, *J*(3–4) 9.3], 3.46 [*ddd*, H-5, *J*(4–5) 9.8], 3.40 [*dd*, H-4], 3.27 [*dd*, H-2], 2.92 [*t*, H-8].

^{13}C NMR $\delta = 154.4$ (*s*, C-12), 130.8 (*s*, C-9), 130.7 (*d*, C-10/C-14), 115.8 (*d*, C-11/C-13), 102.6 (*d*, C-1), 76.3, 76.1 (*d*×2, C-3, C-5), 73.5 (*d*, C-2), 71.4 (*t*, C-7), 70.0 (*d*, C-4), 61.1 (*t*, C-6), 34.7 (*t*, C-8).

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