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Ectopic Expression of Anthocyanin 5-*O*-Glucosyltransferase in Potato Tuber Causes Increased Resistance to Bacteria

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The principal goal of this paper was to investigate the significance of anthocyanin 5-*O*-glucosyltransferase (5-UGT) for potato tuber metabolism. The ectopic expression of a 5-UGT cDNA in the tuber improved the plant's defense against pathogen infection. The resistance of transgenic lines against *Erwinia carotovora* subsp. *carotovora* was about 2-fold higher than for nontransformed plants. In most cases the pathogen resistance was accompanied by a significant increase in tuber yield. To investigate the molecular basis of transgenic potato resistance, metabolic profiling of the plant was performed. In tuber extracts, the anthocyanin 3,5-*O*-substituted level was significantly increased when compared to that of the control plant. Of six anthocyanin compounds identified, the highest quantity for pelargonidin 3-rutinoside-5-glucoside acylated with *p*-coumaric acid and peonidin 3-rutinoside-5-glucoside acylated with *p*-coumaric acid was detected. A significant increase in starch and a decrease in sucrose level in transgenic tubers have been detected. The level of all other metabolites (amino acids, organic acids, polyamines, and fatty acids) was quite the same as in nontransformants. The plant resistance to bacterial infection correlates with anthocyanin content and sucrose level. The properties of recombinant glucosyltransferase were analyzed in in vitro experiments. The enzyme kinetics and its biochemical properties were similar to those from other sources.

KEYWORDS: anthocyanin 5-*O*-glucosyltransferase; pathogen resistance; metabolic profiling; *Solanum tuberosum* var. Desiree

INTRODUCTION

Glucosylation is a prominent modification reaction in plant metabolism. The secondary metabolites are frequent targets of this modification (*1*). In many cases glucosylation is the last step in the biosynthesis of a number of secondary plant products including flavonoids, cyanohydrins, steroidal alkaloids, and saponins (*2*). To date more than 6000 different glucosidic derivatives have been identified and described (*3*).

It has been reported that flavonoids have multiple biological activities. It was found that they have anti-allergenic, anti-viral, and anti-inflammatory activities. They act in plants as antioxidants, photoreceptors, visual attractors, and antimicrobials (*4*). Most of the known flavonoids occur as glucosides in plants. The transfer of a nucleotide-diphosphate activated monosaccharide unit (glucose, rhamnose, galactose, xylose, rutinose, and neohesperidose) (*5*) to an acceptor molecule is catalyzed by glucosyltransferases (UGTs). The modification often leads to changes in the activity of the acceptor molecule and its subcellular localization (*1*).

Glucosyltransferases are found in many organisms, and their particular importance for plants has been suggested. They belong to a superfamily of more than 100 members. In plants where a variety of enzymes has been detected, they exhibited diverse functions and anthocyanin metabolism (anthocyanin glucosyltransferases and rhamnosyltransferases) regulation, auxin metabolism (indole-3-acetic acid glucosyltransferase) modulation, and an unknown function induced by methyl jasmonate and salicylic acid (*6*) are among them. They have been isolated and characterized from many plant sources including cell suspensions, seedlings, flower parts, and roots; they were not however detected in fruits (*7*). Flavonoid glucosyltransferases, including those that catalyze *O*-glucosylation at ring position 3 (3-UGT), 5 (5-UGT), 7 (7-UGT), and 4′ (4′-UGT), generally have optimum pHs in the alkaline range (i.e., 7−9) (*7, 5*) and have a molecular mass ranging from 45 to 60 kDa (*8*). In contrast to mammalian UGTs, most of the plant enzymes are soluble and therefore thought to be cytosolic (*8*).

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Most of the data on the characteristics of glucosyltransferase enzyme derive from studies of recombinant enzyme produced in yeast or bacteria cells. Almost all UGTs have an affinity for a variety of compounds serving as acceptors of sugar moieties (*8*). Flavonols, anthocyanidins and anthocyanins, are the substrate for glucosylation reaction catalyzed by UGT. However the data accumulated so far clearly indicate the regiospecificity of glucosyltransferase. The position of the B-ring accepting sugar molecule clearly distinguishes the enzyme involved in the modification. Glucosylation of anthocyanidins at the 3-*O*-position is the reaction that leads to the formation of the first stable anthocyanin compound. The subsequent modification of 3-glu-anthocyanidins is their glucosylation at the 5-position of the B-ring. This modification is an important step in the anthocyanin biosynthesis pathway producing stable anthocyanin complexes involved in flower copigmentation (*9*). Thus, 5-UGT is the key enzyme modifying the color of flowers.

For the first time, anthocyanidin 5-*O*-glucosyltransferase was detected in *Silene dioica* by Kamsteeg et al. (*10*). To date the enzyme in flowers from *Iris ensata*, *Perilla frutescens*, *Verbena hybrida*, *Petunia hybrida*, and *Torenia hybrida* (*11*) was found and analyzed.

In this paper, the recombinant anthocyanin 5-*O*-glucosyltransferase isolation and enzyme biochemical analysis are described. Also, the transgenic tuber overexpressing the respective cDNA has been generated and investigated for the enzyme function in plant metabolism and pathogenicity. The broad substrate specificity was a characteristic feature of the analyzed enzyme, and transgenic plants overproducing 5-UGT were highly resistant to *Erwinia carotovora* infection.

MATERIALS AND METHODS

Plant Material. Potato plants (*Solanum tuberosum* L. cv. Desiree) were obtained from "Saatzucht Fritz Lange KG" (Bad Schwartau, Germany). Control and transgenic plants were grown in a greenhouse under a 16 h light (21 °C)/8 h dark (16 °C) regime. The plants in soil were grown in individual pots and were watered daily. Harvested tubers were then used for plant cultivation in the field. The field trial was conducted in the vicinity of Wrocław between May and September 2003. All data presented in this paper concern the tubers from field-grown plants.

Recombinant 5-UGT Protein Preparation. The amplified coding region of the 5-UGT gene from *Solanum sogarandinum* (EMBL/GenBank account no. AY033489) (*12*) was digested with EcoRI-XhoI enzymes and ligated into the same site of pBluescript SK^+. Then the BamHI-KpnI fragment was excised and inserted into the same site of the pQE 30 (Qiagen) expression vector resulting in the pQE 30-UGT construct. *Escherichia coli DH5*α cells were transformed with pQE 30-UGT and precultured in LB medium supplied with ampicillin (100 μg/mL). The LB medium (500 mL) was inoculated with 10 mL of precultured bacteria cells. Following incubation at 37 °C for 3 h, isopropyl β-D-thiogalactoside (IPTG) was added to final 1 mM concentration. After an additional 4 h of incubation, the suspension was centrifuged (4000*g*, 20 min, 4 °C). The cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol) with lysozyme (1 mg/mL) and incubated on ice for 30 min. Then the cells were sonicated on ice and the suspension was centrifuged (12000*g*) at 4 °C for 10 min. The pellet was resuspended in lysis buffer with 1% Triton X-100 and left on ice. After 15 min, the lysate was centrifuged (12000*g*, 10 min, 4 °C) and the pellet was resuspended in lysis buffer with 4 M urea, incubated on ice for 15 min, and centrifuged (12000*g*, 10 min, 4 °C). The supernatant was discarded, and the pellet was resuspended in 8 M urea. After 15 min, the suspension was centrifuged (12000*g*, 10 min, 4 °C); the supernatant was used as the enzyme source and after 30 min of incubation with Ni-NTA resin at 4 °C was loaded onto the column. The purification of recombinant protein was conducted according to the manufacturer's procedure (Qiagen). The last step of purification was dialysis of enzyme against stepwise decreasing concentration of urea. The purified recombinant protein was used for 5-UGT enzyme assays and injection of rabbits for antibody preparation.

The Enzyme Assay. The substrate specificity of recombinant 5-UGT protein was examined using different anthocyanidin molecules (malvidin, peonidin, pelargonidin, delfinidin, cyanidin, and petunidin) as acceptor substrates and UDP-Glc and ADP-Glc as donors of sugar moieties. The reaction mixture (total volume, 75 μL) used for the assay of glucosyltransferase activity consisted of increasing content of aglycones (from 2 to 15 μg per sample, 1 mg/mL stock solution in ethylene glycol monomethyl ether) as the substrate, PBS buffer (pH 8.0), and 9 μL of UDP-Glc (10 nmol/μL) or 9 μL of ADP-Glc (10 nmol/μL). The reaction (16 min at 30° C) was started by addition of 50 μL (1 mg/mL) of enzyme solution. The anthocyanins were then extracted with 500 μL of methanol, dried, and redissolved in methanol (50 μL) and subjected to HPLC analysis.

A HPLC system (Knauer, Germany) equipped with automated sample injector and UV detector (Knauer variable wavelength monitor type 87.00) connected to a personal computer (HPLC software/hardware package version 2.21A) was used. The sample was separated on a BetaBasic-18 250 × 4.6 mm (5 μm) column (Thermo Hypersil, U.K.). Compound detection was carried out by on-column measurement of UV absorption at 275 nm. The sensitivity was set at 0.04 aufs. The flow rate was adjusted to 1.0 mL/min. Anthocyanins were separated using an acetonitrile−water gradient with formic acid addition according to the following solvent program: solvent A, 5% formic acid in acetonitrile; solvent B, 5% formic acid in water; starting from 15% solvent A in solvent B, up to 50% solvent A in solvent B in 10 min. A 20 μL volume of analyzed samples was injected. For the HPLC analyses, gradient grade acetonitrile was used (Merck, Germany). Water was glass distilled and deionized. Solvent solutions were vacuum degassed with sonication prior to usage. The calibration graphs of analyzed compounds were prepared by measuring the peak area, and they were linear in the examined range (0.02−0.10 mg/mL). All experiments were performed at room temperature (20 °C). The compounds were identified and determined based on standards analysis.

Potato Transformation. Plasmid pQE 30-UGT containing cDNA sequence coding for 5-*O*-glucosyltransferase was digested with Bam HI-Sal I, and the fragment was ligated into the same site of vector pBinAR B33 (*13*), under tuber specific B33 promoter. Two-week-old leaves of wild type plant were transformed by submerging the leaf explants in a suspension of *Agrobacterium tumefaciens* containing binary vector. *A. tumefaciens* inoculated leaves were subsequently transferred to callus induction and shoot regeneration medium.

Transgenic Plant Selection. The transformants were preselected by polymerase chain reaction (PCR) and then selected by means of Northern and Western blot analysis. PCR was carried out with the use of specific primers for neomycine phosphotransferase (npt II) gene (forward, CCGACCTGTCCGGTGCCC; reverse, CGCCACACCCAGCCGGCC) and glucosyltransferase gene (forward, GTCCTCTTGGTGACATTTCCCACAC; reverse, TGAGGAAATGCCACCACAGGTACAC) on genomic DNA isolated from 3-week-old tissue cultured plants as a template. Transgenic plants that contain predicted npt II (475 bp fragment) and 5-UGT (1100 bp fragment) gene products were further analyzed. The final selection was carried out by means of Northern and Western blot analysis. Total RNA was prepared from frozen potato tubers using the guanidinium hydrochloride method (*14*). RNA was separated on agarose gel (1.5% [w/v], 15% [v/v] formaldehyde) and blotted onto Hybond N+ (Amersham) filter. The membrane was hybridized overnight at 42 °C with radiolabeled 5-UGT cDNA as a probe and then washed three times with SSPE buffer containing 0.1% SDS for 30 min at 42 °C. Fourteen transgenic lines that showed the highest 5-UGT mRNA level were finally selected by immunodetection.

The protein extracts prepared from the potato tubers were run on 12% SDS−polyacrylamide gel and blotted electrophoretically onto nitrocellulose membrane (Schleicher and Schuell). Following transfer, the membrane was incubated with blocking buffer (5% dry milk) and then with antibody against 5-UGT-recombinant protein (1:2000 dilution). Alkaline phosphatase-conjugated goat anti-rabbit IgG was used as a secondary antibody at 1:1500 dilution.

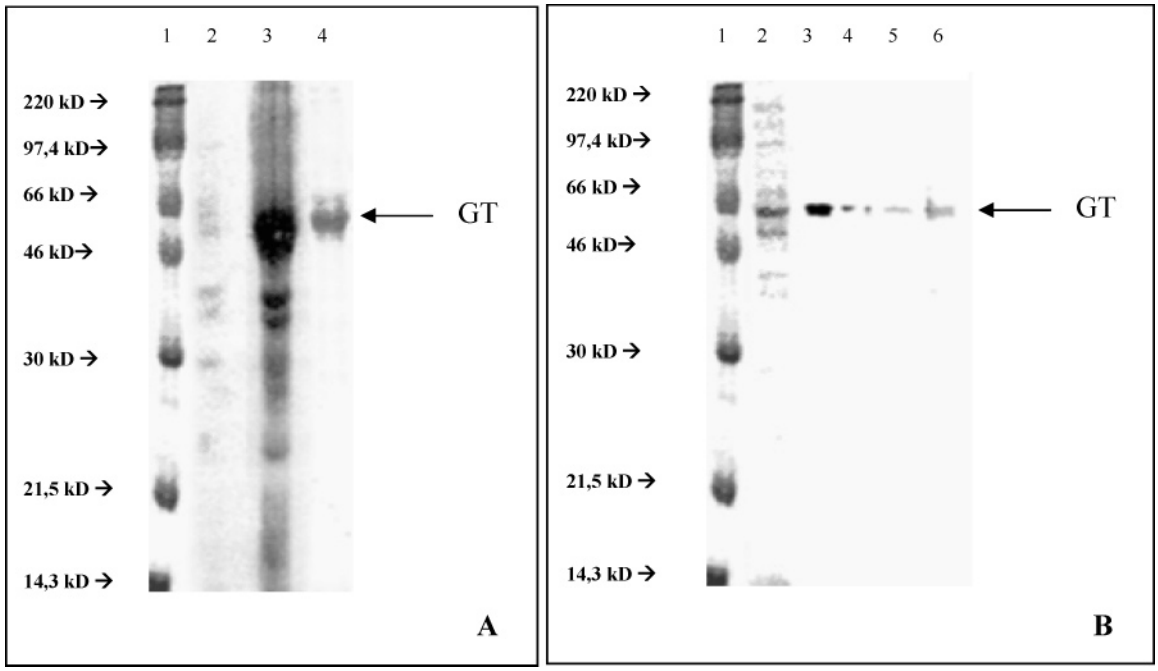

Figure 1. Gel electrophoresis of recombinant protein Coomassie Blue stained (**A**) and probed with IgG anti-UGT (**B**). (**A**) *E. coli* protein extracts (20 μL culture) were analyzed on 12% SDS–polyacrylamide gel. Lane 1, rainbow molecular weight marker RPN 756 (Amersham); lanes 2 and 3, bacterial cell SDS extracted before and after induction with IPTG, respectively; lane 4, purified (3 μg) recombinant UGT. (**B**) Lane 1, rainbow molecular weight marker RPN 756 (Amersham); lanes 2–6, *E. coli* protein extracts (10 μL culture) Coomassie Blue stained or probed with antibody against UGT at 1:100, 1:500, 1:1000, and 1:2000 dilution, respectively.

Table 1. Substrate Specificity of Recombinant 5-UGT[a]

anthocyanidin	Km (μM)	Vmax (μmol/s X mg)	Vmax/Km
peonidin	25.25 ± 1.37 (80.58 ± 6.11)	523.00 ± 178.12 (2812.27 ± 349.64)	20.71 (34.90)
malvidin	32.41 ± 1.50 (208.11 ± 19.78)	991.00 ± 292.49 (5424.61 ± 497.98)	30.58 (26.07)
cyanidin	40.63 ± 1.34 (68.32 ± 6.78)	1372.60 ± 476.19 (1869.49 ± 685.83)	33.78 (27.36)
pelargonidin	144.10 ± 32.34 (267.97 ± 37.63)	3722.60 ± 563.94 (8196.43 ± 1938.64)	25.83 (30.59)
petunidin	149.82 ± 29.68 (103.12 ± 5.12)	3126.60 ± 268.45 (3680.66 ± 458.84)	20.87 (35.69)
delfinidin	208.21 ± 20.38 (3869.54 ± 245.00)	6582.20 ± 1789.98 (20745.22 ± 5897.58)	31.61 (5.36)

[a] The substrate specificity was measured as described in the materials and methods section with the use of UDP-Glc and ADP-Glc (in parentheses) as a glucose donor. The mean value ($n = 4$) ± SE is presented.

Tuber Infection Test. *E. carotovora* subsp. *carotovora* 3193 (*15*) strain from a laboratory collection was grown in liquid LB medium at 27 °C for 18 h with shaking. The culture (1.5 mL) was centrifuged (Eppendorf) at 8000 rpm for 2 min. The supernatant was discarded, and the pellet was resuspended in 1 mL of 0.85% NaCl and spun down. Washing of bacteria was repeated. In the final step, the pellet was resuspended in 1 mL of 0.85% NaCl and the bacterial concentration was adjusted to 2×10^8 cfu/mL ($OD_{600} = 0.1$). The suspension was then used for inoculation of potato tubers. Potato tubers were washed and immersed twice in 10% sodium hypochloride (Clorox) for 15 min and rinsed in sterile water followed by spraying with ethanol and air-drying (*16*). Bacterial suspension (50 μL, 2×10^8 cfu mL^{-1}) was inserted with sterile pipet tips into the tuber parenchyma to the depth of 10 mm (*16*). Three series of experiments were performed for at least five tubers of each line in each experiment. The tubers were incubated in a moist (98%) chamber. Disease severity was estimated by the progression of rot. Tubers were cut vertically through each inoculation point, and the diameter of rotting tissue was measured 72 h after the inoculation.

HPLC Analysis of Anthocyanins in Tuber Extract. Vacuum-dried tuber epidermis (100 mg) was extracted with a 1 mL methanol−1% CH_3COOH solution in an ultrasonic bath for 15 min. After centrifugation, the supernatant was filtered through a Millipore (0.2 μm) filter and then dried in a Speedvac and the polyphenols were resuspended in 100 μL of methanol. The HPLC system consisted of a pump (L-7100), photodiode array detector (Merck-Hitachi L-7455), and D-7000 HSM multisolvent delivery system. Separation of anthocyanins was done by a prepacked LiChroCART 125-3 Purospher RP-18 (5 μm) column (Merck) chromatograph. Compound detection was carried out by on-column measurement of absorption at 200−600 nm. Anthocyanins were separated using an acetonitrile gradient with formic acid addition according to the following solvent program: solvent A, 4.5% formic acid in acetonitrile; solvent B, 80% acetonitrile in 20% solvent A; starting from 100% solvent A, up to 20% solvent A in 16 min, and to 100% solvent B in 24 min. A 100 μL volume of analyzed sample was applied. The compound identification and quantitation were based on standards analysis. Pelargonidin 3-rut-5-glu and peonidin 3-rut-5-glu both acylated with *p*-coumaric acid were used as standards. Standards were obtained according to the method described previously (*17, 18*).

The Antioxidant Capacity of Potato. The chemiluminescence method was used to determine the antioxidant activity of the extracts. A methanol extract of potato was diluted in the range from 1000 to 15 000 times with water and directly analyzed. The experiments were performed in a final volume of 250 μL on white microplates in a solution containing 0.1 M Tris−HCl buffer, pH 9.0, and 4 mM AAPH freshly prepared. The luminol solution (100 μM) and diluted extracts were automatically injected. The photons produced in the reaction were

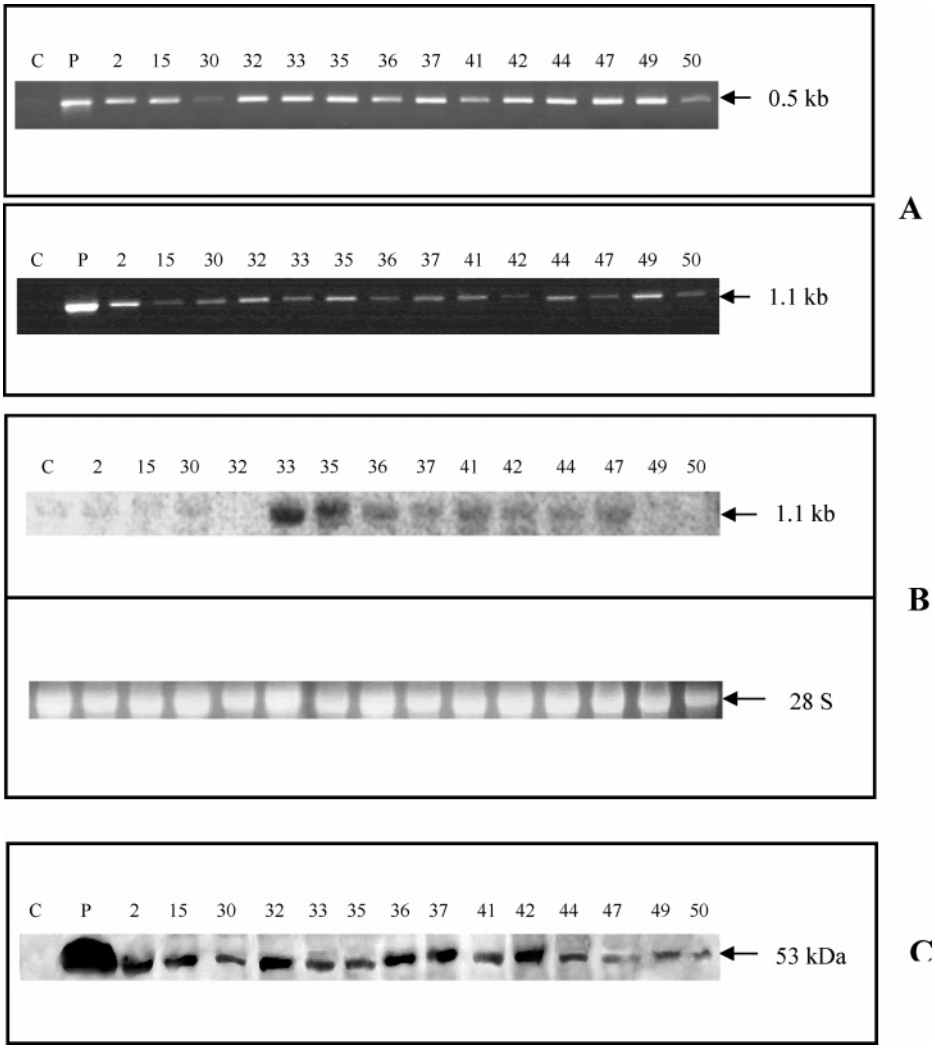

Figure 2. (**A**) Agarose gel electrophoresis of *npt II* gene (upper panel) and *GT* gene (bottom panel) PCR product (500 and 1100 bp, respectively). *npt II* and *GT* gene were amplified with the use of specific primers; genomic DNA isolated from tissue-cultured potato plants was used as a template; C, negative control (nontransformed plant); P, positive control (plasmid containing a *npt II* gene, upper panel; plasmid containing a *GT* gene, bottom panel); the different transgenic lines are numbered. (**B**) Northern analysis of RNA isolated from potato tubers of control (C) and independent transgenic lines (numbered). Thirty micrograms of total RNA from each sample was loaded in each lane. The blot was hybridized with ^{32}P-labeled *GT* (1.1 kb) cDNA. A ribosomal RNA stained with ethidium bromide was used as a control of equal amounts of RNA applied onto the gel (bottom panel). (**C**) The transgenic plant protein extract isolated from the potato tuber was run on 12% SDS–polyacrylamide gel and blotted electrophoretically onto nitrocellulose membrane (Schleicher and Schuell). Following transfer, the membrane was incubated with blocking buffer (5% dry milk) and then with antibody against GT-recombinant protein (1:2000 dilution). Alkaline phosphatase-conjugated goat anti-rabbit IgG was used as a secondary antibody at 1:1500 dilution. C, nontransformed plant; P, positive control (*S. sogarandinum*); the different transgenic lines are numbered.

counted on an EG&G Berthold LB96P microplate luminometer at 30 °C. The antioxidant potential (IC50) was defined as the amount of potato extract which inhibits luminol chemiluminescence by 50%.

Determination of Starch and Soluble Sugar Content. Potato tuber slices and leaf disks were extracted with 100% ethanol−50 mM HEPES−KOH, pH 7.4, at 70 °C. The supernatant was used for enzymatic analysis of glucose, fructose, and sucrose (*19*). For starch measurement, extracted plant material was homogenized in 0.2 M KOH and, following incubation at 95 °C, adjusted to pH 5.5 with 1 M acetic acid. Starch was hydrolyzed with amyloglucosidase, and the released glucose was determined enzymatically.

Tuber Metabolite Profiling by GC−MS. Frozen tuber disks (100−150 mg) were powdered in liquid nitrogen and extracted with MeOH (14 mL/g FW). The samples were heated for 15 min at 70 °C and centrifuged for 10 min at 14 000 rpm (Eppendorf). Then, 1500 μL of water was added to the sample, which was then extracted with 750 μL of $CHCl_3$. A portion of the water phase was dried under vacuum and used for derivatization. An internal standard (ribitol, 120 μg/g FW) was added to the sample homogenate. Dried extract was mixed with methoxyamine hydrochloride (20 mg/mL) and incubated for 120 min at 37 °C. Then the sample was derivatized with 70 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide at 37 °C for 30 min and analyzed by GC−MS (*20*). The GC−MS system consisted of a gas chromatograph GC 8000, an autosampler AS 2000, and a Voyager quadropole mass spectrometer (ThermoQuest, Manchester, U.K.). The chromatograms and mass spectra were evaluated using the MASSLAB program (ThermoQuest). Retention time and mass spectral library for peak quantification of metabolite derivatives was implemented within the MASSLAB method format.

Determination of Mineral Contents in Potato Tubers. The Mg, Fe, Zn, and Cu ion contents in tubers were determined by means of atomic absorption spectroscopy (*21*). The calcium content was measured by means of atomic emission spectroscopy using an AAS 1N apparatus (Carl Zeiss Jena, Germany). Three medium-sized tubers were taken as the sample for analysis. The tuber samples were peeled and homogenized and then dry-mineralized at 450 °C. The ash was dissolved in 0.64 M HNO_3 for analysis. Spectrally pure reagents and standards (Merck, Germany) were used.

Table 2. Yield of Field-Cultivated Tubers from the Control and Transgenic Potato Lines[a]

line	number of tubers per plant	FW of tubers per plant (g)	FW of a single tuber (g)
C	18.00 ± 10.03	421.51 ± 291.12	23.42
UGT 2	25.67 ± 3.98	824.93 ± 129.37	32.14
UGT 15	23.67 ± 14.21	714.67 ± 380.94	30.20
UGT 30	22.50 ± 5.13	844.87 ± 204.23	37.55
UGT 32	28.17 ± 9.79	973.77 ± 390.40	34.57
UGT 33	26.17 ± 9.83	1123.13 ± 358.93	42.92
UGT 35	15.20 ± 9.23	616.54 ± 457.08	40.56
UGT 36	16.00 ± 4.97	577.83 ± 204.92	36.11
UGT 37	22.67 ± 6.62	628.32 ± 199.10	27.72
UGT 41	16.17 ± 4.54	869.98 ± 444.06	53.75
UGT 42	28.83 ± 13.09	1393.80 ± 466.15	48.34
UGT 44	14.00 ± 2.37	427.67 ± 138.17	30.55
UGT 47	14.00 ± 7.90	339.88 ± 189.15	24.28
UGT 49	15.20 ± 4.32	590.88 ± 162.38	38.87
UGT 50	17.83 ± 5.00	667.13 ± 190.59	37.41

[a] Tubers from independent transgenic lines that overexpressed glucosyltransferase were analyzed and compared to the control (C) from field trials performed in 2003. The mean value ($n = 25$) ± SE is presented.

RESULTS AND DISCUSSION

The Isolation and Analysis of 5-UGT cDNA from *S. sogarandinum*. Rorat et al. (*12*) have isolated several cold-induced clones from the cDNA library of cold resistant potato (*S. sogarandinum*). The inspection of databases revealed that 5-UGT full-length clone (GenBank account no. AY033489) encoded protein with 84% homology to anthocyanin-5-*O*-glucosyltransferase from *Nicotiana tabacum* GT2 (NTGT2). The lower identity was found for *P. hybrida* (68%), *P. frutescens* (59%), *V. hybrida* (57%), and *T. hybrida* (53%) plants.

The Characteristic of Recombinant Enzyme. To analyze the expression of 5-UGT protein, the cDNA encoding UGT was inserted into the pQE 30 vector, and recombinant protein was isolated. The purified recombinant protein showed a single band when stained with Coomassie Blue (**Figure 1A**) and used for boosting of rabbits and antibody preparation. The bacterial extract analyzed by Western blot and probed with polyclonal antibody showed also a single band (**Figure 1B**) with the expected molecular mass (53 kDa). The recombinant protein was further used for enzyme analysis.

Numbers of biochemical properties were similar to those reported for other 5-UGTs. The calculated molecular mass of protein was 53 kDa, which coincides well with the molecular mass of 5-UGTs from *P. hybrida* (52 kDa) and *P. frutescens* and *V. hybrida* (51 kDa). We have investigated the substrate specificity of recombinant protein, and similarly to other UGTs, it exhibited 5-UGT activity transferring the glucose moiety from UDP-glucose to the 5-position of various anthocyanidins. Although peonidin is the best substrate (Km = 25.25 μM) among the examined sugar acceptor molecules, the enzyme showed broad substrate specificity. Km values for other examined substrates are shown in **Table 1**. Interestingly, the enzyme also accepts ADP-glucose as a source of carbohydrate moiety; however this form of glucose is less effective (**Table 1**).

The best substrate for recombinant 5-UGTs from *P. frutescens* and *V. hydrida* was cyanidin 3-glucosides; however, the enzyme modifies effectively the whole spectrum of anthocyanidin 3-glucosides (*6*). Also, the 5-UGTs from *P. frutescens* and *V. hybrida* showed broad substrate specificity toward several anthocyanidin 3-glucosides, but the enzyme from *P. hybrida* exhibited strict substrate specificity toward anthocyanidin 3-acylrutinoside (*9*).

In conclusion, UGTs are highly regioselective enzymes and specific in respect to the sugar donor molecule.

Generation of Transgenic Plants. Although the functions of glucosides in plant cells are largely unclear, they are assumed to represent the accumulation form of the flavonoids and the defense compounds against potential pathogens such as fungi and bacteria (*2*). The adsorption, metabolism, and antioxidant activity of flavonoids differ significantly after glucosylation, and the properties vary with the position and nature of glucosylation (*22*, *23*). The antioxidant properties of flavonoids result from the presence of the phenolic OH group. It has been reported that the flavonoid aglycones are more potent antioxidants than their glucosides. It was also suggested that increased water solubility due to glucosylation might act as a vacuolar trapping mechanism for small lipophilic aglycones that otherwise may diffuse through the tonoplast (*5*, *24*).

To verify the in vivo function of flavonoid glucosylation, the transgenic plant approach has been used. A binary vector containing glucosyltransferase cDNA under the control of tuber specific B33 promoter was prepared and used for potato plant transformation. Transformed cells were regenerated, and the regenerants were prescreened using the PCR method with specific primers for neomycin phosphotransferase and UGT genes. Plants that exhibited 475 bp (npt II) and 1100 bp (5-UGT) DNA fragments were used for further selection by

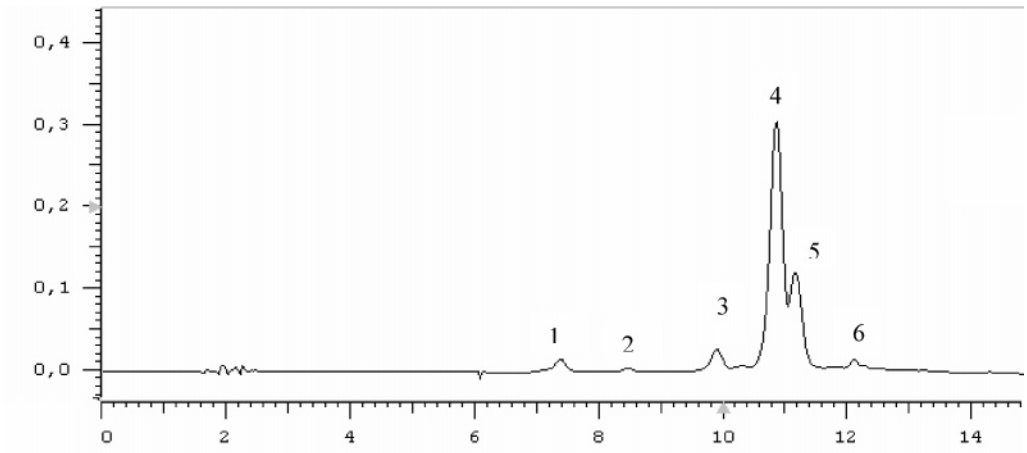

Figure 3. The chromatogram of the control tuber and peak identification based on the retention time of anthocyanin standards: pelargonidin-3-rut-5-glu (1), peonidin-3-rut-5-glu (2), malvidin-3-rut-5-glu (3), pelargonidin-3-(p-coumaroyl-rut)-5-glu (4), peonidin-3-(p-coumaroyl-rut)-5-glu (5), and malvidin-3-(p-coumaroyl-rut)-5-glu (6).

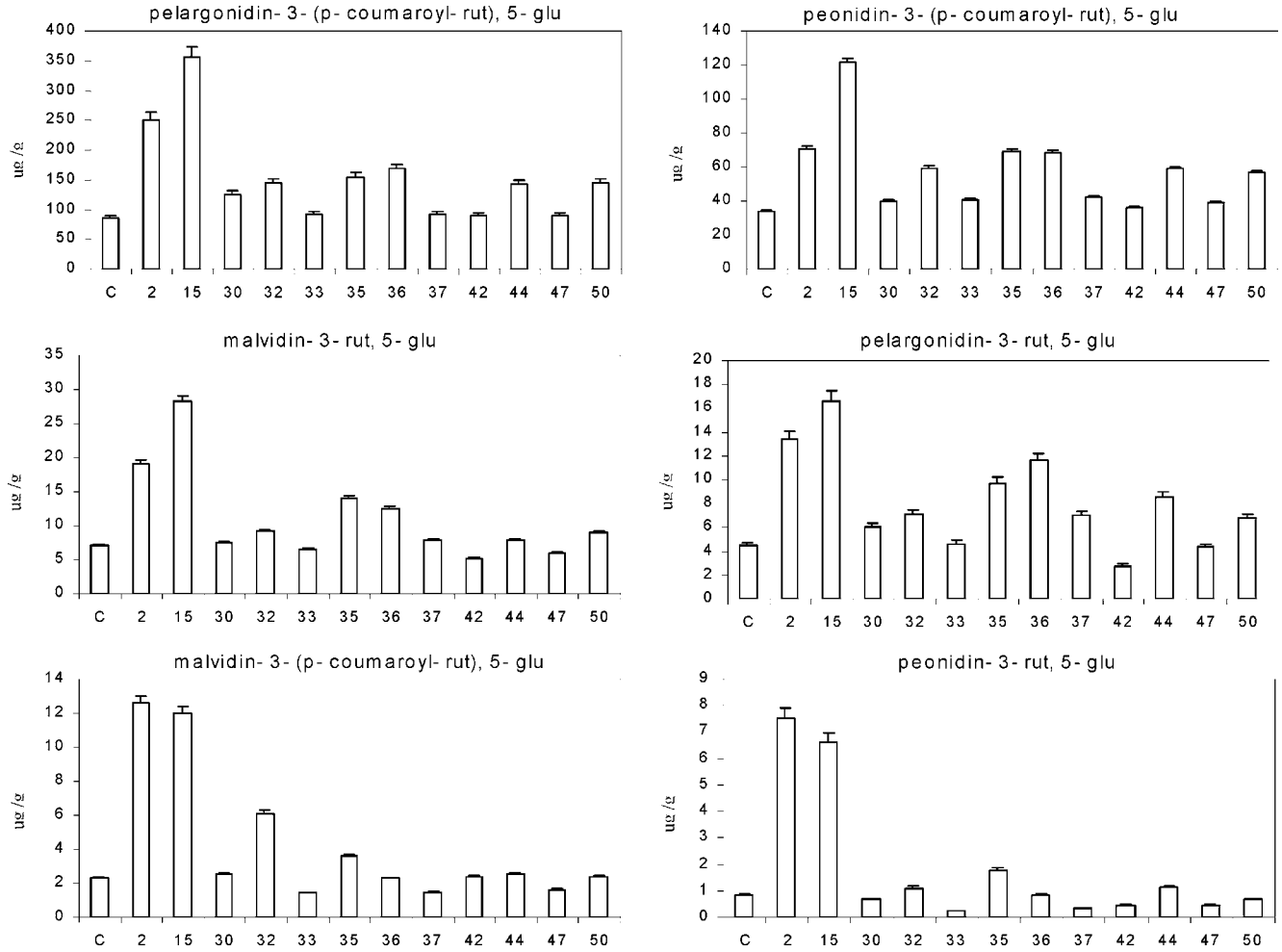

Figure 4. HPLC determination of anthocyanins in tuber extracts from control (C) and transgenic lines (numbered). Anthocyanins were separated on a prepacked LiChroCART 125-3 Purospher RP-18 (5 μm) column, Merck. The mean value ($n = 6$) ± SE is presented.

Northern and Western blot analysis (**Figure 2**). From within several positive transgenic lines, those with the highest UGT protein content were used for further analysis. In several cases, the high level of UGT mRNA measured in Northern does not coincide with protein synthesis detected in Western.

Phenotype Analysis. The obtained transgenic plants were visually indistinguishable from nontransformants. Transgenic plants however showed ca. 3 weeks earlier flowering compared to the control plants. In most transgenic lines, the yield of tubers was significantly higher and the tuber numbers and their size were also higher compared to those of nontransformants (**Table 2**).

Analysis of Anthocyanin Content in Tuber Extracts. The potato anthocyanidin is enzymatically glucosylated by specific glucosyltransferase and resulted in anthocyanin compound synthesis. The major two anthocyanins in potato, pelargonidin and peonidin, have a trisaccharide side chain and monoglucosidic residue attached to the 3-hydroxy and 5-hydroxy groups of aglycone, respectively.

To characterize more precisely whether the selected plants reached the expected different levels of anthocyanin derivatives, they were analyzed for the anthocyanin quantity. Based on retention times of standards, six anthocyanidin derivatives have been identified (**Figure 3**) and the results agreed well with published data (*25*).

The data in **Figure 4** showed that the most abundant were carbohydrate derivatives of pelargonidin (pelargonidin 3-rutinoside-5-glucoside acylated with *p*-coumaric acid) and peonidin (peonidin 3-rutinoside-5-glucoside acylated with *p*-coumaric acid). The quantity of the other four anthocyanidin derivatives (pelargonidin 3-rutinoside-5-glucoside, malvidin 3-rutinoside-5-glucoside, peonidin 3-rutinoside-5-glucoside, malvidin 3-(*p*-coumaroyl-rutinoside)-5-glucoside) was far lower and ranged from 1 to 7 μg/g DW. Most of the transgenic lines showed an increase in anthocyanin content, and in the UGT2 and UGT15 transgenic lines over 2-fold increase has been detected. The calculated correlation coefficient between the UGT protein content measured by Western (**Figure 3C**) densitometry and the anthocyanin level was +0.61. Thus, as expected, the overexpression of glucosyltransferase enzyme resulted in an increased quantity of 3,5-*O*-substituted derivative of anthocyanidin in potato tubers.

Determination of Antioxidant Potential. Antioxidants have protective functions against infection by microorganisms. Since flavonoids belong to these compounds and affect the oxidative status of tubers (*26*), the antioxidant potential of tuber extract from UGT overexpressed plant was determined. Most transgenic lines showed an increase in IC50 value and thus a decrease in antioxidant potential, and the other lines appeared to be very similar to the control level (**Figure 5**). Thus the increase in anthocyanidin glucoside content resulted in an increase of IC50 parameter and for seven (UGT33, UGT35, UGT37, UGT42, UGT44, UGT47, and UGT50) transgenic lines the correlation coefficient for this parameter reached the reasonable 0.5 value.

It has been reported that the flavonoid aglycones are more potent antioxidants than their glucosides for the reason that there

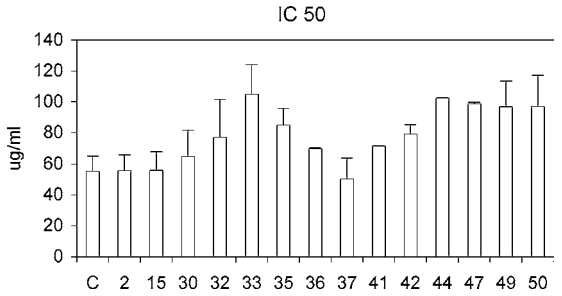

Figure 5. The antioxidant potential (IC50) of potato extract from the control (C) and transgenic plants (numbered) that overexpressed 5-UGT. The analysis of potato extracts was performed as specified in the materials and methods section. The mean value ($n = 4$) ± SE is presented.

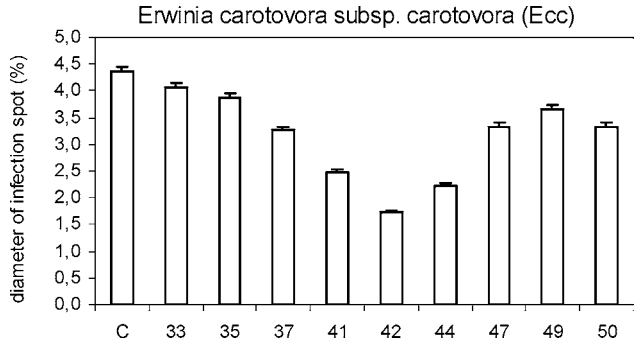

Figure 6. Tuber infection test. Evaluation of resistance of control (C) and transgenic lines to *Ecc*. Disease severity was estimated by the progression of rot. The mean value ($n = 15$) ± SE is presented.

is strong correlation between the number and position of the OH group and their activity (*21*, *22*). Data from our in vivo experiments confirmed this finding.

Transgenic Tubers are Resistant to *Erwinia* Infection. Phenolic glucosides are involved in plant−microbe interactions. Phenolic glucosides accumulated by *Prunus avium* can induce phytotoxin synthesis in *Pseudomonas syringae*, while their corresponding aglycones lack such activity (*27*). Glucosylation also affects inter-plant-to-plant signaling within the same species (*27*).

Thus it was expected that constitutive overexpression of UGT will affect the resistance of transgenic plants to pathogen infection. For infection experiments, we have used only those transgenic lines that showed the highest correlation coefficient between the IC50 value and the anthocyanin content. The transgenic lines with the highest anthocyanin level (UGT2 and UGT15) were omitted for the reason that they showed IC50 close to the control value and also were characterized by a very low correlation coefficient for IC50 and anthocyanin content.

Potato tuber infection by *E. carotovora* subsp. *carotovora* was measured, and the data are presented in **Figure 6**. All analyzed transgenic lines were more resistant than the control, line UGT42 showing the highest resistance to *Erwinia*.

Sugar Content in Transgenic Plants. Flavonoid glucosyltransferases are widely distributed in plant tissue and have been implicated in a number of biological processes of plant growth. Many different roles have been ascribed to the UGTs, and stabilization, detoxification, and solubilization of different cellular metabolites are among them. The UGTs convert reactive or toxic aglycones into the stable and nonreactive storage forms (*6*) that are easily conveyed through physiological fluids (*28*) to the vacuole (*27*). Glucosylation increases the polarity of compounds (*1*) and affects the action of plant hormones (*27*).

Table 3. Metabolite Profiling of the Selected Compound Content in Potato Tubers from Control and Transgenic Plants[a]

	line			
metabolite	C	UGT 33	UGT 35	UGT 42
fucose	1 ± 0.01	2.24 ± 0.21	0.81 ± 0.01	0.94 ± 0.21
maltose	1 ± 0.13	2.24 ± 0.27	0.53 ± 0.04	0.72 ± 0.08
trehalose	1 ± 0.03	2.78 ± 1.28	1.06 ± 0.05	0.36 ± 0.04
arabinose	1 ± 0.03	0.97 ± 0.04	0.90 ± 0.14	0.65 ± 0.06
galactose	1 ± 0.04	0.85 ± 0.02	1.14 ± 0.02	0.73 ± 0.04
rhamnose	1 ± 0.05	1.01± 0.07	1.02 ± 0.05	0.84 ± 0.06
glutamine	1 ± 0.02	1.47 ± 0.48	2.08 ± 0.82	1.24 ± 0.57
asparagine	1 ± 0.05	2.78 ± 0.07	1.29 ± 0.02	0.57 ± 0.17
ornithine	1 ± 0.09	1.82 ± 0.01	1.45 ± 0.03	1.31 ± 0.04
methionine	1 ± 0.10	1.29 ± 0.15	1.17 ± 0.14	1.17 ± 0.07
arginine	1 ± 0.30	0.33 ± 0.11	0.31 ± 0.13	0.86 ± 0.29
cysteine	1 ± 0.07	0.90 ± 0.05	0.65 ± 0.04	0.97 ± 0.03
proline	1 ± 0.17	1.20 ± 0.16	1.58 ± 0.08	1.33 ± 0.09
mannitol	1 ± 0.03	1.17 ± 0.04	1.04 ± 0.02	0.75 ± 0.04
myo- inositol	1 ± 0.01	0.80 ± 0.01	0.60 ± 0.00	0.55 ± 0.02
glycerol	1 ± 0.10	0.60 ± 0.03	0.62 ± 0.03	0.61 ± 0.02
ononitol	1 ± 0.15	0.94 ± 0.10	0.47 ± 0.01	0.55 ± 0.06
galacturonate	1 ± 0.10	4.79 ± 0.19	3.30 ± 0.13	3.56 ± 0.10

[a] Transgenic tubers from UGT overexpressed plants (numbered) were frozen in liquid nitrogen and then extracted as described in the materials and methods section and compared with the control (C). The results are expressed as relative response ratio per g FW. The mean value ($n = 6$) ± SE is presented.

Thus in the first instance primary metabolites, glucose, fructose, sucrose, and starch have been determined enzymatically in all transgenic lines, and the results are presented in **Figure 7**. The quantity of sucrose in most cases was decreased, and interestingly, a high negative correlation (−0.83) was established between resistance to *Ecc* and sucrose content. The content of glucose in several cases and starch in all UGT transgenic lines significantly increased, and a high positive correlation (0.67) between glucose level and resistance to *Ecc* was calculated.

Interestingly, the routinely measured enzyme activity of carbohydrate metabolism (sucrose phosphate synthase, ADP-glucosepyrophosphorylase) was unchanged in transgenic tubers (not shown).

Metabolic Profiling of Transgenic Tubers. To study the molecular basis of transgenic plant resistance to pathogens in more detail, analysis of more than 150 compounds was conducted and the data were compared to the control. For this study, three transgenic lines with low (UGT33), moderate (UGT35), and high (UGT42) resistance to *Erwinia* infection have been chosen. We are presenting however only those data which differentiate substantially the transgenic plant from the control.

Carbohydrates. The sugar content in the three analyzed transgenic lines showed significant changes. The quantity of fucose, maltose, and trehalose in most cases was decreased (the only exception is the UGT33 transgenic line where an increase in sugar content was detected); however the quantity of arabinose, galactose, and rhamnose was unchanged (**Table 3**).

Since carbohydrates provide the carbon skeleton for the biosynthesis of other compounds, we have measured the content of amino acids.

Amino Acids. It was expected that the amino acid content would stay at the same level in these plants. This was mainly the case; however, in a few cases (glutamine, asparagine, ornithine, and methionine) the content of amino acids was increased, and only the arginine and cysteine level decreased in transgenic tubers (**Table 3**).

Other Metabolites. There were no changes in the content of polyamines, fatty acids, and most metabolites affected by stress conditions. However, the levels of two osmoprotectants, proline

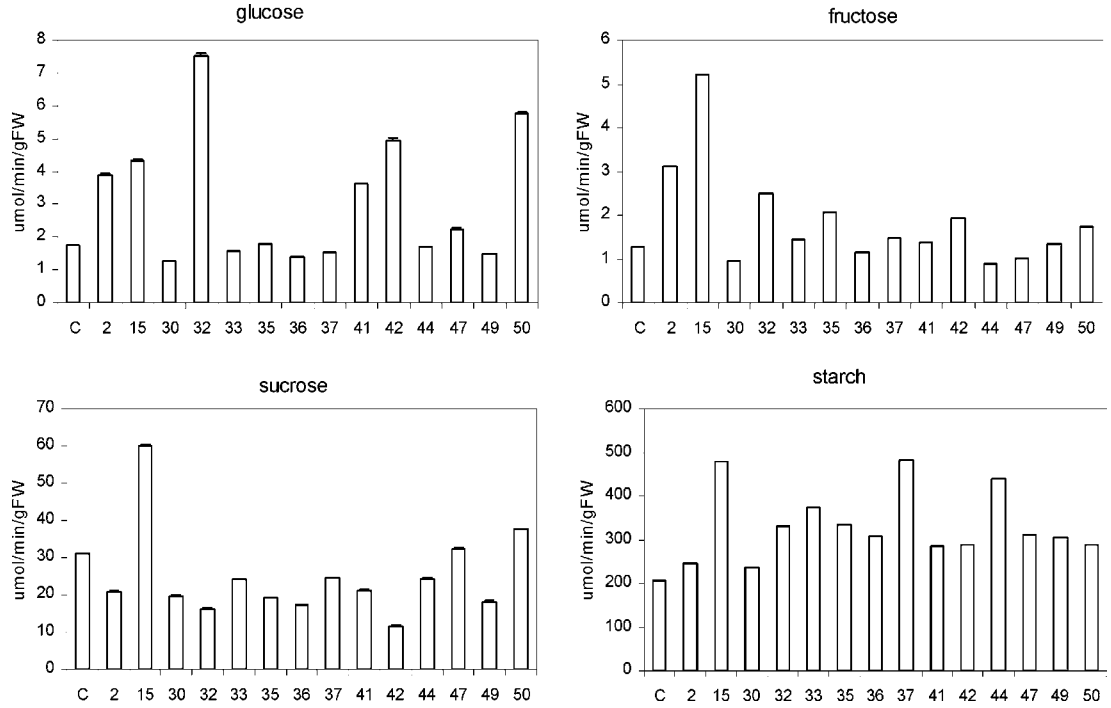

Figure 7. Determination of starch and soluble sugar content. Potato tuber slices were frozen in liquid nitrogen and then extracted as described in the materials and methods section and compared with the control (C). Results are mean ± SE ($n = 6$).

Table 4. Determination of Metal Ion Contents in Potato Tubers[a]

line	Mg	Ca	Fe	Co	Zn
C	97.36 ± 3.85	59.63 ± 4.57	0.90 ± 0.02	0.75 ± 0.05	1.92 ± 0.05
UGT 2	101.55 ± 4.29	57.24 ± 5.22	1.64 ± 0.24	1.01 ± 0.04	2.19 ± 0.12
UGT 15	79.56 ± 6.87	47.70 ± 1.85	1.64 ± 0.45	0.48 ± 0.09	1.55 ± 0.56
UGT 30	87.94 ± 4.96	27.03 ± 3.69	1.47 ± 0.28	0.73 ± 0.04	1.81 ± 0.32
UGT 32	83.75 ± 7.36	32.60 ± 4.37	1.47 ± 0.11	0.75 ± 0.06	1.89 ± 0.11
UGT 33	87.94 ± 2.59	40.94 ± 2.97	1.80 ± 0.59	0.69 ± 0.09	2.51 ± 0.66
UGT 35	96.31 ± 3.57	77.91 ± 5.98	1.06 ± 0.34	0.99 ± 0.01	2.19 ± 0.34
UGT 36	92.13 ± 6.12	77.12 ± 2.01	1.06 ± 0.89	0.89 ± 0.16	1.76 ± 0.48
UGT 37	87.94 ± 4.67	55.65 ± 6.35	1.31 ± 0.47	0.83 ± 0.05	2.00 ± 0.51
UGT 41	85.85 ± 1.69	34.98 ± 6.30	0.82 ± 0.65	0.63 ± 0.03	1.60 ± 0.60
UGT 42	78.52 ± 2.11	30.61 ± 4.25	0.90 ± 0.13	0.46 ± 0.01	1.12 ± 0.04
UGT 44	79.57 ± 1.09	49.29 ± 1.66	1.06 ± 0.10	0.71 ± 0.23	1.89 ± 0.02
UGT 47	104.69 ± 6.23	66.78 ± 2.09	0.74 ± 0.03	0.79 ± 0.05	2.29 ± 0.63
UGT 49	81.66 ± 1.69	69.96 ± 5.46	1.64 ± 0.19	0.73 ± 0.046	1.76 ± 0.01
UGT 50	74.33 ± 2.23	35.78 ± 1.78	2.29 ± 0.65	0.77 ± 0.056	1.68 ± 0.01

[a] The magnesium (Mg), calcium (Ca), iron (Fe), copper (Co), and Zn (zinc) ion contents in tubers were determined by means of atomic absorption spectroscopy. The calcium content was measured by means of atomic emission spectroscopy using an AAS 1N apparatus. Transgenic tubers from UGT overexpressed plants (numbered) were extracted as described in the materials and methods section and compared with the control (C). The results are expressed as mg/100 g of DW. The mean value ($n = 6$) ± SE is presented.

and mannitol, were slightly increased in all transgenic lines (**Table 3**), which may indicate that for an unknown reason the transgenic plants are under osmotic stress. On the other hand, myo-inositol and glycerol, also osmoprotectants, were slightly decreased in the analyzed lines. Ononitol, the content of which depends on the concentration of myo-inositol and might provide tolerance to drought and salt (*29*), was significantly decreased in all transgenic lines.

Interestingly, the level of galacturonic acid was significantly (4-fold on average) increased in all transgenic lines. Since naturally occurring galacturonate from pectin is a source of energy for bacteria and polygalacturonases contribute to the *Erwinia* virulence (*30*), it is possible that an increased quantity of galacturonate in the UGT plant resulted in delay of bacterial expansion and produced the effect of resistance.

Contents of Minerals in Potato Tubers from the Field Trial. Concomitant to the extensive structural studies, the function and significance of anthocyanins for plant physiology are also under great attention. It was found that among others, anthocyanin serves as a metal chelator (*31*). Thus it was of special interest to measure the content of metal ions in plants with modified content of anthocyanin derivatives. As expected, the decrease in anthocyanin reactivity by glucosylation resulted in a decrease of metal ion accumulation in transgenic tubers. The decrease in calcium, copper, and zinc content has been detected in transgenic tubers (**Table 4**). Interestingly, the calculated correlation coefficient for metal ion content and plant defenses against *Erwinia* is high and negative and for calcium, copper, and zinc is −0.79, −0.73, and −0.67, respectively. Thus the metal ion content in host cells might contribute to *Erwinia* virulance; the reason for it however is as yet unknown.

Conclusion. In summary, in this paper we have described the properties of recombinant anthocyanin 5-*O*-glucosyltransferase and the manipulation of the flavonoid biosynthesis

pathway via introduction of cDNA encoding 5-UGT into the potato plants. The recombinant enzyme showed broad substrate specificity similarly to those isolated and characterized from other plant sources. A binary vector containing glucosyltransferase cDNA under the control of tuber specific B33 promoter was prepared and used for the potato plant transformation. A significant increase in anthocyanin diglucoside level characterized the created transgenic lines as expected. The transgenic plant revealed earlier flowering (ca. 3 weeks) when compared to the control. The increase in anthocyanin content resulted in an IC50 increase and thus decreases in the tuber's antioxidant potential. It is thus suggested that overexpression of UGT resulted in an increase of the pool of anthocyanin's storage form which does not change IC50 but is mobilized in case of pathogen attack. Transgenic plants produced up to 60% more tubers per plant compared to the control. Interestingly, the yield of the tubers was 60% higher and the fresh weight of tubers per plant was more than 3-fold higher than in the control plants. The increase in tuber yield was accompanied by a significant increase of starch content in transgenic lines. Additionally, resistance to *E. carotovora* subsp. *carotovora* was significantly improved in transgenic plants and the resistance was about 60% higher when compared to control plants. Since the metabolic profile of transgenic plants does not differ substantially from that of nontransformants, the accumulation of a higher quantity of anthocyanin diglucoside in transgenic tubers is the main reason for tuber resistance to pathogen infection.

Interestingly, overexpression of 5-UGT caused a decrease in carbohydrate level. It is known that carbohydrates of infected cells serve as a carbon source for fungi. Thus we speculate that the tuber protection against pathogen infection might also derive from carbohydrate content decrease in host cells.

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