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Phytochemistry 62 (2003) 959–969
PHYTOCHEMISTRY
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# Monitoring changes in anthocyanin and steroid alkaloid glycoside content in lines of transgenic potato plants using liquid chromatography/mass spectrometry

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Received 27 August 2002; received in revised form 15 November 2002

**Abstract**

Transgenic potato plants overexpressing and repressing enzymes of flavonoids biosynthesis were created and analyzed. The selected plants clearly showed the expected changes in anthocyanins synthesis level. Overexpression of a DNA encoding dihydroflavonol 4-reductase (DFR) in sense orientation resulted in an increase in tuber anthocyanins, a 4-fold increase in petunidin and pelargonidin derivatives. A significant decrease in anthocyanin level was observed when the plant was transformed with a corresponding antisense construct. The transformation of potato plants was also accompanied by significant changes in steroid alkaloid glycosides (SAG) level in transgenic potato tuber. The changes in SAGs content was not dependent on flavonoid composition in transgenic potato. However, in an extreme situation where the highest (DFR11) or the lowest (DFRa3) anthocyanin level was detected the positive correlation with steroid alkaloid content was clearly visible. It is suggested that the changes in SAGs content resulted from chromatin stressed upon transformation. A liquid chromatography/mass spectrometry (LC/MS) system with electrospray ionization was applied for profiling qualitative and quantitative changes of steroid alkaloid glycosides in tubers of twelve lines of transgenic potato plants. Except α-chaconine and α-solanine, in the extracts from dried tuber skin α-solamargine and α-solasonine, triglycosides of solasonine, were identified in minor amounts, triglycosides of solanidine dehydrodimers were also recognized.


*Keywords:* *Solanum tuberosum*; Steroid alkaloid glycosides; Anthocyanins; Transgenic potato; Liquid chromatography/mass spectrometry; Electrospray ionization

## 1. Introduction

Plant secondary metabolites play different roles in plant biochemistry and physiology. Many classes of natural products constitute a very important group of secondary metabolites involved in many physiological processes occurring in plants, particularly those responding to environmental stimuli. Moreover in agriculturally important species the composition of secondary metabolites in plant tissue may influence the quality of food or foodstuff produced for human and animals.

Steroid alkaloid glycosides are nitrogenous compounds synthesized via the triterpene pathway in plants of *Solanum* species. Cycloartenol and cholesterol, the product of this pathway, are both converted to solanidine and other steroidal alkaloids present in potato (*Solanum tuberosum*). The aglycone then is enzymatically glycosylated by specific glycosyltransferase. The two major steroid alkaloid glycosides (SAGs) in the potato are α-chaconine **3** and α-solanine **4** having a trisaccharide (chacotriose and solatriose, respectively) attached to the 3-hydroxy group of solanidine (Fig. 1).

Concomitant to the extensive structural studies the function and significance of steroid alkaloid glycosides for plant physiology were also investigated (Kutchan, 1995; Pazkowski et al., 2001; Roddick et al., 2001). It was found that several environmental factors affect glycoalkaloid

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doi:10.1016/S0031-9422(02)00720-3

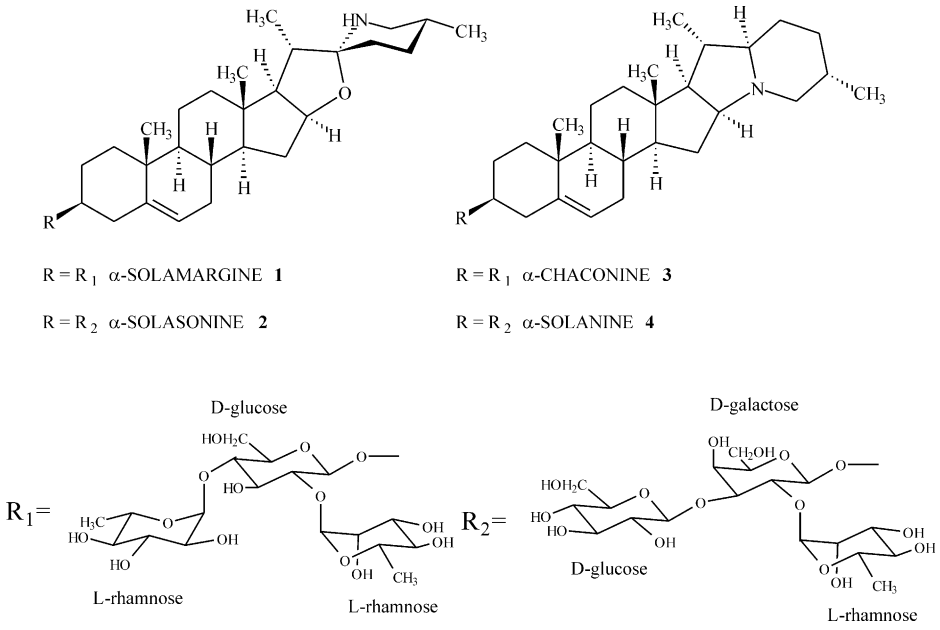


Fig. 1. Structures of steroid alkaloid glycosides.

synthesis. Exposure of tubers to light dramatically increases alkaloid content, interestingly under this condition also chlorogenic acid level increases significantly (Dao and Friedman, 1994). Conflicting data were obtained when the temperature effect on steroid alkaloid glycosides synthesis was studied. In some cases higher temperature and in others low temperatures caused the same effect, the increase in glycoalkaloids synthesis. It was then speculated that the increase in glycoalkaloids synthesis is not in response to a certain temperature but rather in response to stress in general. There is, however, no doubt, that glycoalkaloids level increases in response to tubers sprouting, mechanical damage and tuber storage time. The decrease in steroid alkaloid glycosides content was observed in tuber slices immersed in water. Ethylene and jasmonic acid inhibit the accumulation of glycoalkaloids in potato tubers. The glycoalkaloids protect plants from damage by insects while have little, if any, effect in resistance to bacterial and fungal attack (Valkonen et al., 1996; Friedman and McDonald, 1997).

In a low concentration the SAGs improve the taste and enhance potato flavor, however when higher than 200 mg per kg of fresh weight showed toxic effects on humans (Friedman and McDonald, 1997). α-Chaconine is the most toxic potato alkaloid but synergistic action of different glycoalkaloids was also reported. The poisoning effect of potato glycoalkaloids, including the death, on humans, was reported several times (Friedman and McDonald, 1997). It is important to note, that frying, baking and boiling have little effect on the glycoalkaloid content of potato products. Thus, it is of interest to develop new potato varieties producing alkaloids, that are nontoxic or minimally toxic to humans but would still protect the plants from insects and other pathogens (Esposito et al., 2002).

Recently we have observed that transgenic potato plants (14-3-3 repressed) showed significant reduction in SAGs level (Szopa, 2002). The 14-3-3 protein family is a class of adapter proteins and they are known to have multiple targets and to modulate a large range of processes. Since there is rather no relation between 14-3-3 content and glycoalkaloid synthesis, we speculate that plant transformation might be stressful and caused increase in steroid alkaloid glycoside presence. From other studies it is known, that wounding resulted in an increase of both total phenolic and glycoalkaloid synthesis (Friedman and McDonald, 1997). Thus, in this paper we have analyzed whether genetically engineered potato with the increase and decrease in anthocyanidin synthesis affects glycoalkaloid concentration in the tubers.

Due to high polarity of the potato steroid alkaloid glycosides only liquid chromatography ensures proper conditions for their separation without degradation. For this reason qualitative and quantitative profiling of SAGs was performed using liquid chromatography combined to mass spectrometry with electrospray ionization.

## 2. Results and discussion

### 2.1. Transgenic plant selection

The transgenic potato plants overexpressing and repressing key enzymes of flavonoid synthesis pathway were generated. With the aim to create plants with increased antioxidant properties (will be published elsewhere) the

plants were preselected by PCR reaction and selected by means of northern blot analysis. The preselection was carried out with the use of primers specific for neomycin phosphotransferase gene (*npt II*) and genomic DNA from tissue cultured plant as a template. The presence of *npt II* gene product (475 bp) in PCR reaction means that DNA fragment from binary vector containing cDNA of interest (CHS, CHI, DFR) and marker gene (*npt II*) is inserted into plant genome. Fig. 2 shows the result and plants exhibited expected 475 bp fragment were taken for further analysis. The final transgenic plants selection was based on mRNA analysis with the use of respective radiolabelled cDNA (CHS, CHI, DFR) as a probe. The data presented in Fig. 3 show that, as expected in contrast to repressed plants, those overexpressed cDNAs encoding enzymes of interest exhibited increased level of mRNAs.

### 2.2. Analysis of anthocyanin content in tuber extracts from transgenic potatoes

In order to characterize more precisely whether or not the selected plants reached the expected different level of anthocyanins, they were analyzed for the pelargonidin and petunidin derivative concentration, data presented in Fig. 4. The most abundant anthocyanins were a glycoside derivative of pelargonidin (pelargonidin 3-*O*-rutinoside-5-*O*-glucoside acylated with *p*-coumaric acid) and petunidin (petunidin 3-*O*-rutinoside-5-*O*-glucoside acylated with *p*-coumaric acid). These natural products were identified earlier in tubers of *S. tuberosum* (Lewis et al., 1998). In the samples low quantities of cyanidin derivatives were also detected. It was expected that at least in case of chalcone isomerase and dihydroflavonol reductase overexpressing plant, the content of anthocyanins increased. This was the case, when compared to control value, a significant increase in both pelargonidin (four fold) and petunidin (three fold) derivative content in tubers from DFR11 transgenic line was observed. Lower but still significant increase in all other DFR and

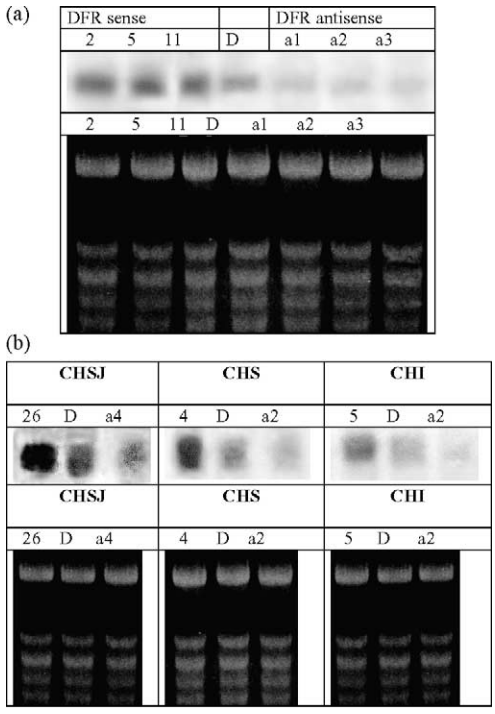


Fig. 3. Northern analysis of RNA isolated from tissue cultured plants of control (D) and transgenic potato plants (marked as in Fig. 2). Fifty μg total RNA from each sample was loaded in each lane. The blot was probed with $^{32}$P-labelled respective (CHS, CHI, DFR) cDNAs. Below northern blot is ribosomal RNA stained with ethidium bromide.

CHI transgenic lines was detected. Overexpressing chalcone synthase often caused co-supression of this enzyme and resulted in dramatic decrease in chalcone synthase content (Forkmann, 1989). This, however, did not occur when chalcone synthase cDNA from *Petunia hybrida* overexpressed in potato plant and the increase in both analyzed anthocyanins was the same as in other manipulated enzymes. Interestingly the overexpression of CHS cDNA from barley (CHSJ) cause only slight and not significant changes in anthocyanin content.

In order to verify that the changes in anthocyanin content resulted from gene construct expression plants

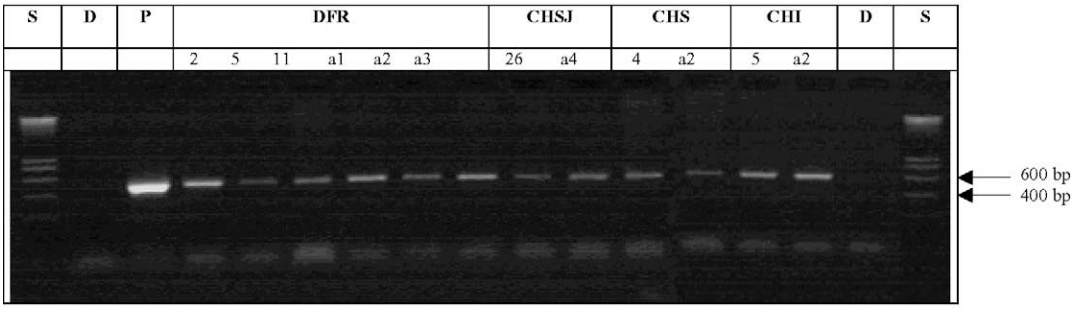


Fig. 2. Agarose gel electrophoresis of PCR product. *Npt II* gene was amplified with the use of specific primers and potato plant genomic DNA as a template. The plants were transformed with either sense or antisense (marked a) constructs of cDNAs encoding chalcone synthase (CHS), barley chalcone synthase (CHSJ), chalcone isomerase (CHI) and dihydroflavonol reductase (DFR). The different transgenic lines are numbered. D is a control nontransformed plant and P is a plasmid containing *npt II* gene (positive control). The length marker (S) is SmartLadder from Eurogenetec (Belgium).

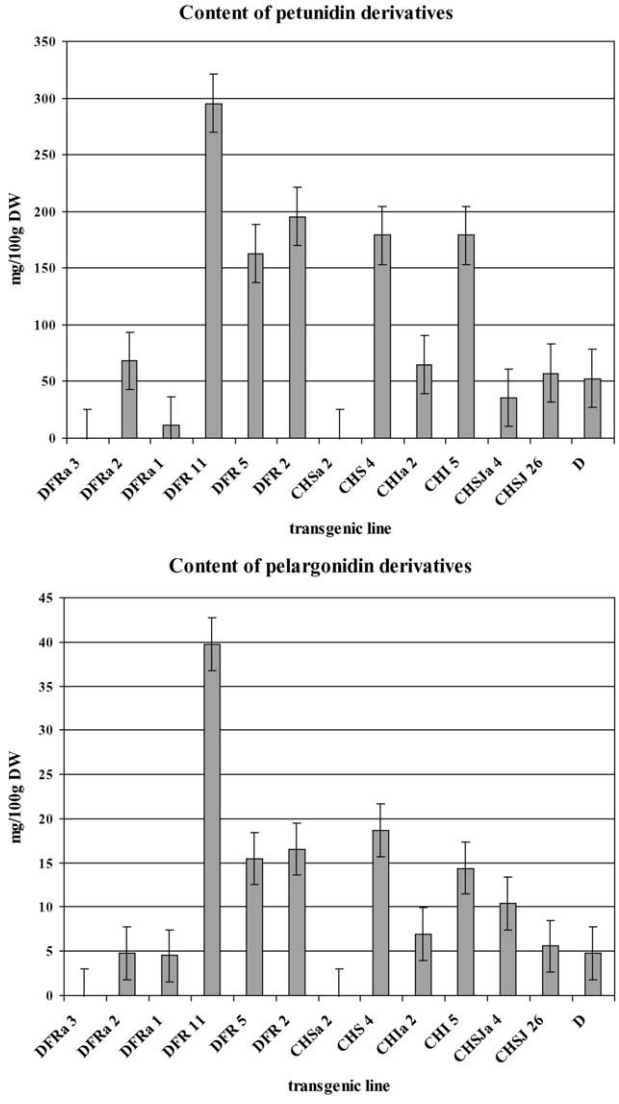


Fig. 4. Levels of pelargonidin and petunidin derivatives (mg/100 g DW) in epidermal tuber extracts from transgenic potato plants. The plants were transformed with either sense or antisense constructs of cDNAs encoding chalcone synthase (CHS), chalcone isomerase (CHI) and dihydroflavonol reductase (DFR). The respective antisense plants are marked CHSa, CHIa and DFRa. The mean value ($n=3$)±S.D. is presented.

repressing enzyme synthesis were created. The plants transformed with cDNAs encoding chalcone synthase, chalcone isomerase and dihydroflavonol reductase in antisense orientation were analyzed. In all cases repression of endogenous enzymes could be demonstrated and resulted in significant decrease in anthocyanin content. The experiments with chalcone isomerase gave different results and did not cause changes in anthocyanin concentration in transgenic plants, suggesting that petunia cDNA is not enough homologous to potato mRNA at the sequence level to cause repression of the latter. Also the barley CHS cDNA for chalcone synthase does not seem to interact with counterpart from potato plant. Thus, the result strongly suggest that the changes in anthocyanin content in transgenic potato plants upon expression of petunia cDNAs is the result of flavonoids metabolic pathway enzyme manipulation.

### 2.3. Identification of steroid alkaloid glycosides in tuber epidermis of transgenic plants

Most glycosidic conjugates of secondary metabolites present in plant tissues are polar compounds and thus they are not amenable for profiling with GC/MS even after derivatization of polar groups. In the last decade new efficient, low energy ionization methods at atmospheric pressure: electrospray (ESI) and atmospheric pressure chemical ionization (APCI) were introduced (Niessen, 1999). These ionization techniques are applied in mass spectrometric systems where liquid chromatographs or capillary electrophoresis instruments are hyphenated with mass spectrometers (LC/MS and CE/MS), for analysis of proteins, peptides or low molecular weight secondary metabolites with excellent results. The LC/MS technique has been successfully applied for analysis of flavonoid glycosides and saponins (Justesen, 1998; Lin et al., 2000; Bednarek et al., 2001; Klejdus et al., 2001; Huhman and Sumner, 2002).

For analysis of potato steroid alkaloid glycosides gas and liquid chromatography has been applied, both kind of instruments were often equipped in mass spectrometric detectors. In the case of gas chromatographic separation of derivatized alkaloid glycosides or free aglycones obtained after hydrolysis of extracts from plant material was used (Friedman and McDonald, 1997). During liquid chromatographic or capillary electrophoresis separation, except of UV detection of SAGs and their free aglycones, application of mass spectrometric detection of compounds with electrospray ionization has been also published (Kuronen et al., 1999; Cherkaoui et al., 2001). During our studies we applied LC/ESI/MS system for profiling SAGs in the extracts obtained from the tuber skin of 12 lines of transgenic potato.

The applied LC gradient of acetonitrile in water permitted to resolve all steroid alkaloid glycosides **1**–**6** during reasonable short time. Monitoring the elution profile of the compounds **1**–**6** was much more easier from single ion chromatograms of protonated molecules $[M+H]^+$, than in total ion chromatogram (Fig. 5). The resolution of the particular peaks of SAGs was satisfactory in the applied LC gradient and identification of the compounds on the basis of the registered mass spectra was unambiguous. In the extracts α-chaconine **3** and α-solanine **4** were identified as major glycoalkaloid components, their peaks were easily recognized in the total ion current. From the data collected except solanidine derivatives, solasodine 3-*O*-chacotrioside **1** and solasodine 3-*O*-solatrioside **2** were recognized. In the

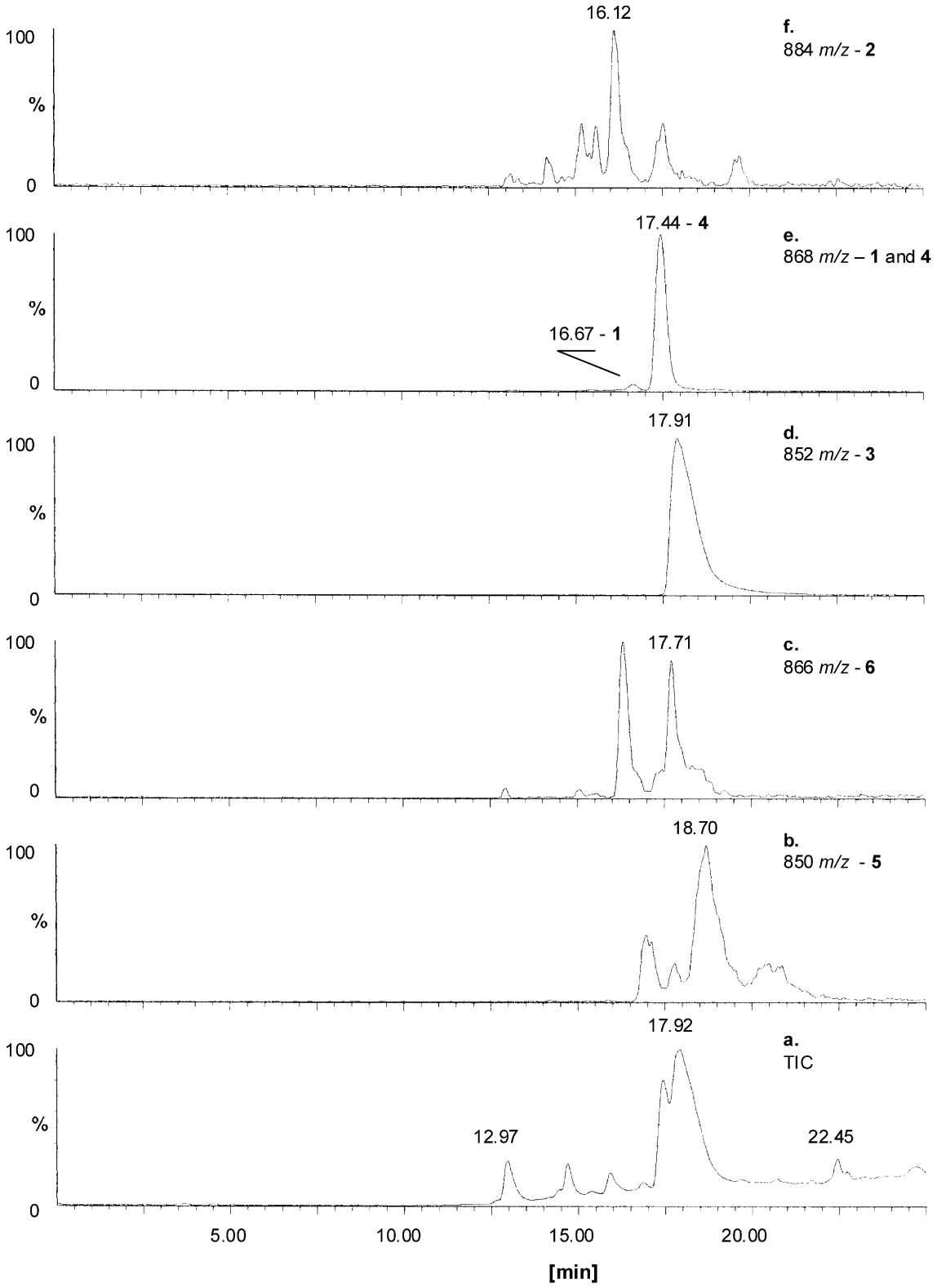


Fig. 5. Total ion current chromatogram (a) and single ion chromatograms of protonated molecular $[M+H]^+$ ions of steroid alkaloid glycosides **1**–**6** present in potato tuber epidermis extract (b–f).

mass spectra $[M+H]^+$ ions at $m/z$ 868 and 884 were observed. Two additional peaks of triglycosides described by us, as glycosidic conjugates of dehydrosolanidine **5** and **6** were also identified, their protonated molecular ions $[M+H]^+$ were detected at $m/z$ 850 and $m/z$ 866, respectively (Figs. 5 and 6). These two compounds were present in higher concentration than the solasodine conjugates. However, both pairs of the SAGs **1**, **2**, **5** and **6** were distinguishable only on the single ion chromatograms of protonated molecules $[M+H]^+$ (Fig. 5).

In the mass spectra of all triglycosides $[M+H]^+$ ions and fragments were observed, formed after consecutive

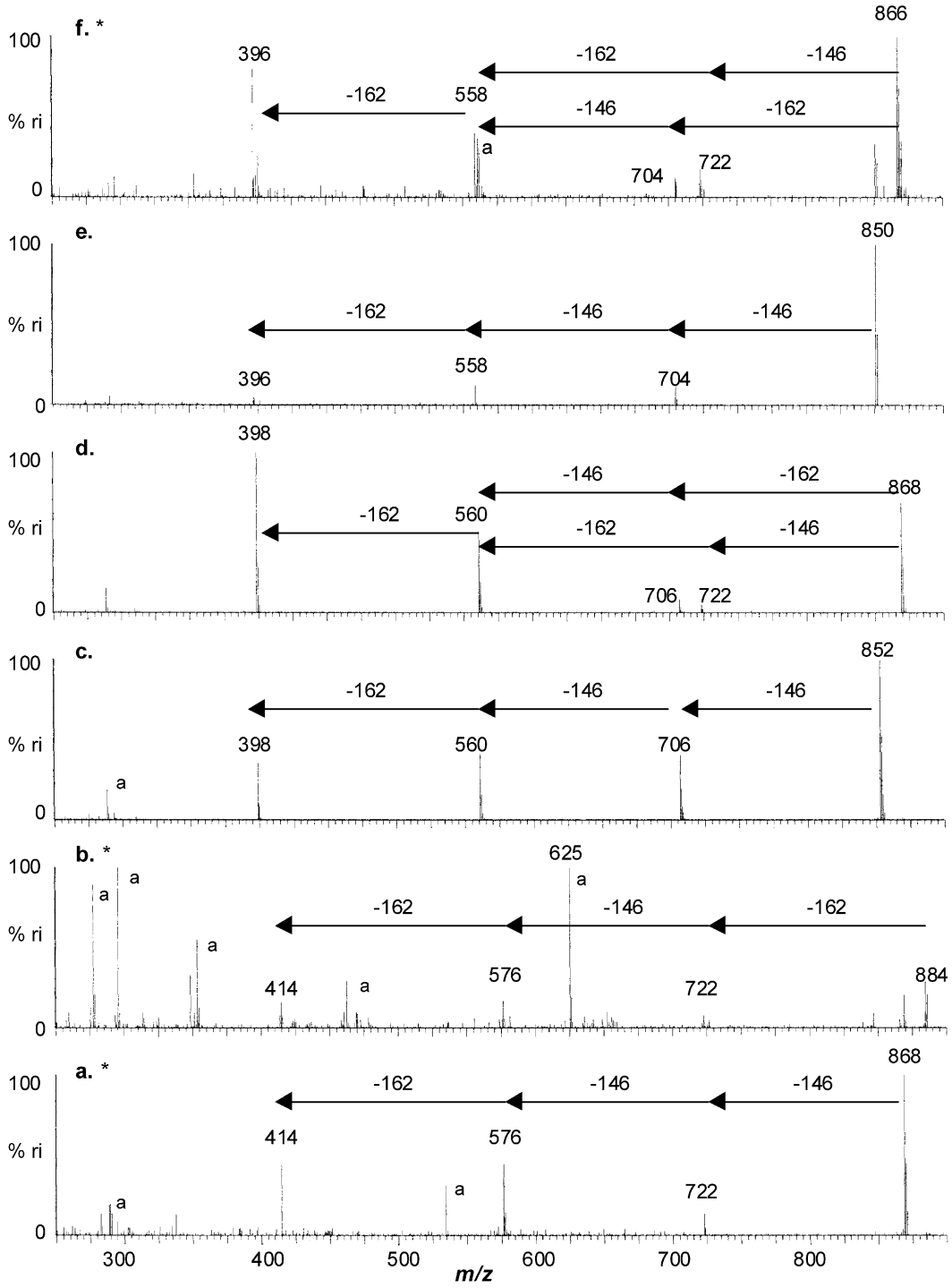


Fig. 6. Mass spectra of six steroid alkaloid glycosides present in potato tubers: (a) α-solamargine- **1**, (b) α-solasonine- **2**, (c) α-chaconine- **3**, (d) α-solanine- **4**, (e) solanidine dehydrodimer conjugated with chacotriose, (f) solanidine dehydrodimer conjugated with solatriose, (a—ions originating from compounds co-eluting with SAGs from LC column; *- background was substracted).

cleavage of glycosidic bonds between sugars and sugar and aglycone (Fig. 6). In the first step of the fragmentation pathway of studied compounds **1**–**6** cleavage of both sugar rings on the nonreducing end was observed. In the mass spectra of SAGs linked with solatriose **2**, **4** and **6**, in the first step of fragmentation elimination of glucose $\{[M+H]^+\text{-}162\}$ or rhamnose $\{[M+H]^+\text{- }146\}$ was recorded, however elimination of rhamnose was not observed in the case of solasonine (**2**) because of low concentration of this compound in the analyzed extracts. Due to simultaneous co-elution from LC column more than one secondary metabolite in the mass spectra were present but verification of the SAGs presence in the analyzed samples was possible. On the basis of single ion chromatograms for $[M+H]^+$ and fragment ions unambiguous identification of compounds of interest was achieved.

### 2.4. Quantification of glycoalkaloids in extracts form tuber epidermis of transgenic potatos

Glycoalkaloids may play different, unrecognized biological roles in plant cell, their content in plant is regulated by several biotic and abiotic stimuli and among environmental factors there are light, humidity, temperature and mechanical injury or wounding by insects (Friedman and McDonald, 1997). These metabolites are also actively regulated by physiological signals in plants, like tuber sprouting and storage time. SAGs are however mostly recognized as the compounds with toxicity. This feature is of special importance for potato for the common use of tubers as primary food source for humans and animals. Thus, it is useful to develop potato varieties with minimal toxicity and still able to protect the plants against insects and none suitable environment condition (Esposito et al., 2002).

During our studies quantitative analyses of SAGs content in the extracts of potato tuber skin were performed after integration of single ion chromatograms of protonated molecular ions $[M+H]^+$. The concentration of SAGs were estimated from the calibration curve prepared on the basis of LC/MS analyses run for different concentrations of solanidine used as standard for quantitative studies.

The analyses performed for 12 lines of transgenic potato plants with overexpressed or repressed genes of anthocyanin synthesis were repeated twice. The content of the sum of the steroid alkaloid glycosides **1**–**6** expressed as solanidine equivalents in the extracts from dried tuber skin of transgenic potato plants is presented in Fig. 7a. These results demonstrated that the amount of glycoalkaloids in the analyzed lines of transgenic potato differed from this of control plant. The content of the SAGs in the transgenic plants was not dependent on the transformed gene class of the phenylpropanoid pathway. The lowest quantity of glycoalkaloids was found in one of the potato line with repressed dihydroflavonol reductase gene (DFRa3). However, the biggest difference was observed between lines DFRa3 and DFR11, the quantity of the SAGs was about five times lower in DFRa3 than in one of the line overexpressing DFR gene. Moreover, the amount of glycoalkaloids in the line DFRa3 with the repressed gene was two times lower than in the control (desi).

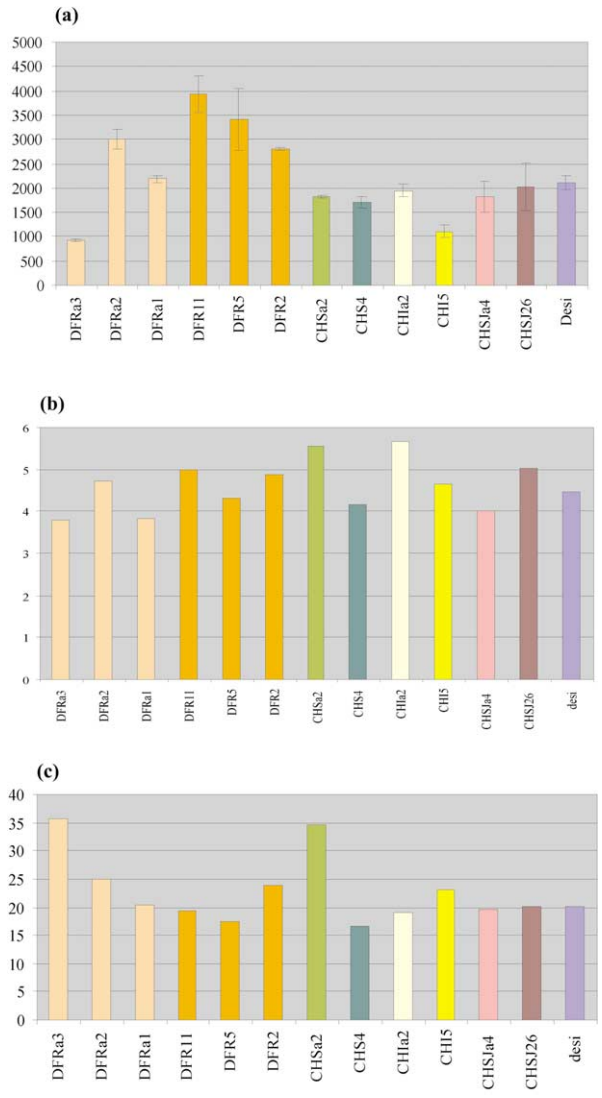


Fig. 7. Total steroid alkaloid glycosides content in twelve lines of potato tuber epidermis extracts obtained from transgenic potato plants with transformed genes of phenylpropanoid pathway: (a) total SGAs content in dried tuber epidermis in 12 lines of transgenic potato (μg/g DW). The data are derived from two independent experiments and represents the mean from two LC/MS analyses of two different samples, (b) relative ratio of α-chaconine to α-solanine in 12 lines of transgenic potato, data calculated from one set of LC/MS analysis, (c) relative ratio of total amounts of α-chaconine and α-solanine to the amounts of both glycosides of solanidine dehydrodimers (MW = 849 and 865) in 12 lines of transgenic potato, data calculated from one set of LC/MS analysis.

α-Chaconine and α-solanine constitute more than 90% of the total amounts of glycoalkaloids in the studied transgenic potato lines. Due to the possible synergistic toxic effect of different steroidal glycoalkaloides (Friedman and McDonald, 1997), the relative content of different groups of SAGs in the studied potato lines was also checked. The relative ratio of both the most abundant glycoalkaloids in the plant tissue of all lines was rather constant, its value ranged between 4 and 6 (Fig. 7b). Only in the case of two lines with the repressed genes DFRa3 and CHSa2 were observed higher relative ratio of α-chaconine and a-solanine to their dehydrodimers (Fig. 7c). Due to the alkaloids potential poisoning effect, it should be pointed out, that the decrease in SAGs content is treated as advantageous situation while significant increase in their level is a rather unwanted effect (Esposito et al., 2002).

To the best of our knowledge, no data have been published, that unequivocally demonstrated the changes of steroid alkaloid glycoside level in transgenic plants tissue by monitoring the target compounds by liquid chromatography/mass spectrometry. However the attempts to analyze SAGs concentration in potato tuber by matrix-assisted laser desorption time of flight mass spectrometry (MALDI TOF MS) has been undertaken (Driedger and Sporns, 1999).

### 2.5. The relation of glycoalkaloid to flavonoid content

The analysis of profiles of different classes of secondary metabolites in transgenic plants is important due to valuable knowledge about possible toxicity of plants used for food production (Kuiper et al., 2001).

It is generally accepted that changes in the steroid alkaloid glycosides content occurs in response to stress in general, the transformation of plant cell and plant regeneration might cause unpredictable fluctuation in glycoalkaloids qualitative and quantitative composition of SAGs.The concentration of phenolic compounds in plant is affected by environmental signals. Wounding and pathogen infection caused dramatic increase in total phenolics. Similar effect for glycoalkaloids level was detected. Thus the question arose whether there was an interplay between these compounds in plant physiology. We measured the glycoalkaloids level in the potato plant modified by introducing different gene constructs overexpressing and repressing enzymes of flavonoid synthesis. It was found the significant changes in glycoalkaloid content in transgenic potato tubers (Fig. 7a). The extreme situation in DFR plants was detected, repressed DFR enzyme in the transgenic line (DFRa3) showed two fold decrease in total SAGs level and enzyme overexpressed line (DFR11) two fold increase instead in the comparison to the control plant (desi). The data presented in Figs. 4 and 7a showed clearly that there is no correlation between anthocyanins and glycoalkaloids in transgenic plants. Thus, although both group of compounds are affected by the same signal the metabolic pathway of both are separately regulated. The steroid alkaloid glycosides concentration decrease was not dependent on a gene construct used for transformation and on orientation of coding sequence. Since the place of foreign gene incorporation into target chromatin was random, it might be suggested that the changes in glycoalkaloids level was dependent on the site of transgene incorporation. To verify this we have analyzed several transgenic lines (each line has its own site of gene incorporation) of the same transgene (DFR) and the results clearly showed that all transgenic lines with repressed DFR contained decreased concentration of SAGs in comparison to overexpressed DFR lines. Thus the genome site of foreign DNA incorporation did not affect glycoalkaloid content.

Recently in another study, where transgenic potato plants repressing 14-3-3 protein synthesis were analyzed, the steroid alkaloid glycoside quantity was dramatically decreased (Szopa, 2002). Thus the SAGs level was decreased upon chromatin stressed and the extent of compounds changes might depend on the number of genes incorporated into target chromatin DNA. This conclusion needs however further study and at the moment it is rather difficult to estimate the number of foreign gene incorporated into plant genome. Whatever the reason was, the decrease in the concentration of toxic compounds in potato tubers appears to be advantageous feature for new obtained varieties and consumers.

## 3. Experimental

### 3.1. Plant material

Potato plants (*Solanum tuberosum* L. cv. Desirée) were obtained from "Saatzucht Fritz Lange KG" (Bad Schwartau, FRG). Control and regenerated plants were cultivated in the greenhouse in soil under 16 h light (22 °C)–8 h dark (15 °C) regime. Plants were grown in individual pots and were watered daily. Tubers were harvested 3 months after transfer of the tissue culture plants to the greenhouse.

### 3.2. Construction of transgenic plants

In this study, four types of transgenic plants with genes in sense and antisense orientation were used: Type CHS—overexpressing (CHS) and repressing (CHSa) the *Petunia hybrida* cDNA encoding chalcone synthase (EMBL/GenBank database acc. no. X04080); CHI—overexpressing (CHI) and repressing (CHIa) the *Petunia hybrida* cDNA encoding chalcone isomerase (EMBL/GenBank database acc no. X14589); DFR—over-

expressing (DFR) and repressing (DFRa) the *Petunia hybrida* cDNA encoding dihydroflavonol 4-reductase (EMBL/GenBank database acc no. X15537); CHSJ—overexpressing (CHSJ) and repressing (CHSJa) the barley cDNA encoding chalcone synthase (EMBL/GenBank database acc. no. X58339). Petunia cDNAs and barley cDNA were provided by Dr. I Somssich and Dr. H. Hesse from Max Planck Institute, Cologne and Golm, Germany, respectively. For leaf explants transformation the pBin vector containing respective cDNA in sense and antisense orientation under the control of 35S promoter and OCS terminator was used. The vector was introduced into the *Agrobacterium tumefaciens* strain C58C1:pGV2260 as described before (Rocha-Sosa et al., 1989) and the integrity of the plasmid was verified by restriction enzyme analysis. Young leaves of wild-type potato *Solanum tuberosum* L. cv. Desirée were transformed with *A. tumefaciens* by immersing the leaf explants in the bacterial suspension. *A. tumefaciens*-inoculated leaf explants were subsequently transferred to callus induction and shoot regeneration medium (Rocha-Sosa et al., 1989). The selection marker was the neomycin phosphotransferase gene. The transgenic plants were preselected by PCR using primers (forward—5′CCGACCTGTCCGGTGCCC and backward- 5′CCGCCACACCCAGCCGGCC) specific for kanamycin resistance gene and then selected by means of northern blot analysis. Total RNA was prepared from frozen plant young leaves using the guanidinium hydrochloride method as described by Logemann et al. (1987). Following electrophoresis (1.5% [w/v] agarose, 15% [v/v] formaldehyde) RNA was transferred to nylon membranes (Hybond N, Amersham, UK). Membranes were hybridized overnight at 42 °C with radioactively labeled respective cDNA (CHS, CHI, DFR) as a probe. Filters were washed three times in 0.1× SSC and 0.1% SDS for 30 min at 65 °C (Wilczyński et al., 1998).

The following transgenic lines showed the highest respective mRNA level when compared to control plant and were used for further investigations, CHS 4, CHSJ 26, CHI 5, DFR 2, DFR 5 and DFR 11 overexpressing chalcone synthase (CHS, CHSJ), chalcone isomerase and dihydroflavonol reductase, respectively. The lowest mRNA signal from expressing chalcone synthase CHSa 2 and CHSJa 4, chalcone isomerase CHIa 2 and repressing dihydroflavonol reductase DFRa 1, DFRa 2 and DFRa 3 transgenic lines was detected.

### 3.3. Extraction of anthocyanins from tuber epidermis of transgenic potato

Vacuum dried tuber epidermis (150 mg) were extracted with 1 ml of methanol containing 1% HCl (v/v) in an ultrasonic bath for 15 min. After centrifugation the supernatants were dried in a speedvac and polyphenols resuspended in 1 ml of water. The solution was then applied to solid phase extraction (SPE) on silicagel RP C-18 column (Merck, Darmstadt, Germany), retained anthocyanins were eluted from the solid phase with 40% MeOH and analyzed using LC/UV.

### 3.4. HPLC analysis of anthocyanins in tuber extracts

HPLC system (Knauer, Germany) equipped with an autosampler 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 extracts were separated on a Superspher 100 RP-18 (5 μm) column (250×4 mm) preceded by a Superspher 100 RP-18 (5 μm) pre-column (4×4 mm) (Merck, Darmstadt, Germany). Detection was carried out by on-column measurement of UV absorption at 325 nm. The sensitivity was set at 0.04 a.u.f.s. The flow rate was adjusted to 1.0 ml $min^{-1}$. Anthocyanins were separated using an water-acetonitrile gradient. The following solvent system: A (90% $H_2O$ and 10% HCOOH, v/v) and B (90% MeCN and 10% HCOOH; v/v) was applied. After 2 min isocratic elution (90% A and 10% B), a linear gradient from 90% A to 70% A in (A + B) within 23 min was applied followed by 70% to 30% A in (A + B) within 2 min. The injection volume of the sample was 20 μl. The calibration graphs of polyphenols were prepared by measuring the peak area that were linear in the examined concentration range (between 0.4 and 2 μg per injection). All experiments were performed at room temperature (20 °C). The compounds were identified and quantified on the basis of standards analysis as described (Gabrielska et al., 1999). Pelargonidin chloride and petunidin chloride from Fluka GmbH (Switzerland) were used as standards during quantitative analysis.

### 3.5. Extraction of steroid alkaloid glycosides from tuber epidermis of transgenic potato

Samples of lyophilized tuber skin (150 mg) from each line of transgenic potato (12 lines) after homogenization were spiked with an internal standard. Genistein 7-*O*-glucoside was applied as standard, final concentration of standard in the extract sample injected on LC column was about 300 ng/μl. Genistein 7-*O*-glucoside was isolated from *Lupinus luteus* and characterized (Frański et al., 1999). Tissue samples were extracted with 10 ml of 80% methanol containing 1% HCl, in an ultrasonic bath for 30 min. The suspension was filtered through Büchner funnel and concentrated under vacuum at 40 °C to 1 ml volume. The extract samples were transferred to the screw capped vials evaporated to dryness in a stream of nitrogen. The dried samples were stored in a freezer prior to LC/MS analyses.

Before the analysis the extract was transferred to microvials in 100 μl volume of methanol/water mixture 1:1 (v/v).

### 3.6. LC/ESI/MS analysis of steroid alkaloid glycosides in tuber epidermis extracts

The LC/ESI/MS analyses were performed using a Waters/Micromass (Manchester, UK) ZQ mass spectrometer. The instrument was coupled with a Waters model 2690 HPLC pump (Milford, MA USA). The samples were injected using autosampler onto a Superspher (TM) 100 RP-18 (5 μm) column (250×2 mm) (Merck, Darmstadt, Germany). The flow rate was 0.2 ml $min^{-1}$. Steroid alkaloid glycosides were separated using an water- acetonitrile gradient. The following solvent system: A (95% $H_2O$, 4.5% MeCN, 0.5% HCOOH; v/v/v) and B (95% MeCN, 4.5% $H_2O$, 0.5% HCOOH: v/v/v) was applied. A linear gradient from 100% A to 100% B in (A + B) within 15 min was applied followed by isocratic elution during 10 min with 100% B. The injection on the LC column was 3 μl from 100 μl volume in which extract samples were dissolved. The ESI source potentials were following: capillary 3 kV, lens 0.5 kV, extractor 4 V and the cone voltage was changed during analyses from 30 to 65 V. Higher cone voltage value permitted to increase collision induced dissociation of protonated or molecules and fragmentation of protonated molecules was enhanced. Source temperature was 120 °C and desolvation temperature was 300 °C. Nitrogen was used as the nebulizing and desolvation gas at flow-rates of 100 and 600 l $h^{-1}$ respectively. Glycoalkaloids were identified on the basis of their MS spectra and retention times.

## Acknowledgements

This work was supported by grant No. 5P06A 02319 and PBZ/029/P06/2000 from National Research Committee (KBN).

## References

Bednarek, P., Frański, R., Kerhoas, L., Einhorn, J., Wojtaszek, P., Stobiecki, M., 2001. Profiling changes in metabolism of isoflavonoids and their conjugates in *Lupinus albu*s treated with biotic elicitor. Phytochemistry 56, 77–85.

Cherkaoui, S., Bekkouche, K., Christen, P., Veythey, J.-L., 2001. Non-aqueous capillary electrophoresis with diode array and electrospray mass spectrometric detection for analysis of selected steroidal alkaloids in plant extracts. J. Chromatogr. A. 922, 321–328.

Dao, L., Friedman, M., 1994. Chlorophyll, chlorogenic acid, glycoalkaloids, and protease inhibitor content of fresh and green potatoes. J. Agric. Food Chem. 42, 633–639.

Driedger, D.R., Sporns, P., 1999. Glycoalkaloid concentration in Alberta potatoes determined by matrix-assisted laser desorption/mass spectrometry. J. AOAc Int. 82, 908–914.

Esposito, F., Fogliano, V., Cardi, T., Carputo, D., Filippone, E., 2002. Glycoalkaloid content and chemical composition of potatoes improved with nonconventional breeding approaches. J. Agric. Food Chem. 50, 1553–1561.

Friedman, M., McDonald, G.M., 1997. Potato glycoalkaloids: chemistry, analysis, safety, and plant physiology. Crit. Rev. Plant Sci. 16, 55–132.

Forkmann, G., 1989. Gene-enzyme relations and genetic manipulation of anthocyanin biosynthesis in flowering plants. In: Styles, E.D., Gavazzi, G.A., Racchi, M.L. (Eds.), The Genetics of Flavonoids. Edizioni Unicopli, Milan, pp. 49–60.

Frański, R., Bednarek, P., Siatkowska, D., Wojtaszek, P., Stobiecki, M., 1999. Application of mass spectrometry to structural identification of flavonoid monoglycosides isolated from shoot of lupin (*Lupinus luteus* L.). Acta Biochim. Pol. 46, 459–473.

Gabrielska, J., Oszmianski, J., Komorowska, M., Langner, M., 1999. Anthocyanin extracts with antioxidant and radical scavenging effect. Z. Naturforsch. C. 54, 319–324.

Huhman, D.V., Sumner, L.V., 2002. Metabolic profiling of saponins in *Medicago sativa* and *Medicago truncatula* using HPLC coupled to an electrospray ion-trap mass spectrometer. Phytochemistry 59, 347–360.

Justesen, U., Knuthesen, P., Leth, T., 1998. Quantitative analysis of flavonols, flavones and flavanones in fruits vegetables and beverages by high-performance liquid chromatography with photodiode array and mass spectrometric detection. J. Chromatogr. A. 799, 101–110.

Klejdus, B., Vitamvasova-Sterbova, D., Kuban, V., 2001. Identification of isoflavone conjugates in red clover (*Trifolium pratense*) by liquid chromatography-mass spectrometry after two-dimensional solid phase extraction. Anal. Chim. Acta 450, 81–97.

Kuiper, H.A., Kleter, G.A., Noteborn, H.P.J.M., Kok, E.J., 2001. Assessment of the food safety issues related to genetically modified foods. Plant J. 27, 503–528.

Kuronen, P., Väänänen, T., Pehu, E., 1999. Reversed-phase liquid chromatographic separation and simultaneous profiling of steroidal glycoalkaloids and their aglycones. J. Chromatogr. A. 863, 25–35.

Kutchan, T.M., 1995. Alkaloid biosynthesis— the basis for metabolic engineering of medicinal plants. Plant Cell 7, 1059–1070.

Lewis, C.E., Walker, J.R.L., Lancaster, J.E., Sutton, K.H., 1998. Determination of anthocyanins, flavonoids and phenolic acids in potatoes. I: Coloured cultivars of *Solanum tuberosum* L. J. Sci. Food Agric. 77, 45–57.

Lin, L.Z., He, X.G., Lindenmaier, M., Yang, J., Cleary, M., Qiu, S.X., Cordell, G.A., 2000. LC/ESI/MS study of the flavonoid glycoside malonates of red clover (*Trifolium pratense*). J. Agric. Food Chem. 48, 354–356.

Logemann, J., Schell, J., Willmitzer, L., 1987. A rapid method for the isolation of RNA from plant tissues. Anal. Biochem. 163, 21–26.

Niessen, W.M.A., 1999. 2nd Ed. Liquid Chromatography–Mass Spectrometry, second ed. Marcel Dekker, New York.

Pazkowski, C., Kalinowska, M., Wojciechowski, Z.A., 2001. Phospholipids modulate the substrate specificity of soluble UDP-glucose:steroid glucosyltransferase from eggplant leaves. Phytochemistry 58, 663–669.

Rocha-Sosa, M., Sonnewald, U., Frommer, W., Stratmann, M., Schell, J., Willmitzer, L., 1989. Both developmental and metabolic signals activate the promoter of a class I patatin gene. EMBO J. 8, 23–29.

Roddick, J.G., Weissenberg, M., Leonard, A.L., 2001. Membrane disruption and enzyme inhibition by naturally-occuring and modified chacotriose-containing *Solanum* steroidal glycoalkaloids. Phytochemistry 56, 603–610.

Szopa, J., 2002. Transgenic 14-3-3 isoforms in plants: the metabolite profiling of repressed 14-3-3 protein synthesis in transgenic potato plants. Biochem. Soc. Trans. 30, 405–410.

Valkonen, J.P.T., Keskitalo, M., Vasara, T., Pietila, L., 1996. Potato glycoalkaloids: a burden or a blessing? Crit. Rev. Plant Sci. 15, 1–20.

Wilczyński, G., Kulma, A., Szopa, J., 1998. The expression of 14-3-3 isoforms in potato is developmentally regulated. J. Plant Physiol. 153, 118–126.