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Antioxidant Capacity Manipulation in Transgenic Potato Tuber by Changes in Phenolic Compounds Content

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The main goal of this study was to generate potato tubers with increased levels of flavonoids and thus modified antioxidant capacities. To accomplish this, the vector carrying multigene construct was prepared and several transgenic plants were generated, all overexpressing key biosynthesis pathway enzymes. The single-gene overexpression or simultaneous expression of genes encoding chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR) resulted in a significant increase of measured phenolic acids and anthocyanins. The increase in phenolic compounds synthesis is accompanied by decreases in starch and glucose levels in transgenic plants. The flavonoids-enriched plants showed improved antioxidant capacity; however, there is a complex relationship between antioxidant capacity and flavonoids content, suggesting the great participation of other compounds in the antioxidant potential of the plants. These other compounds are not yet recognized.

KEYWORDS: Flavonoids; DFR; CHS; CHI; antioxidants; potato; transgenic plants

INTRODUCTION

Secondary metabolites are important constituents of plant cells. They play different roles in plant biochemistry and physiology. Many classes of natural products responding to environmental stimuli may influence the quality of food or foodstuff produced for human and animals.

Flavonoids are synthesized in the phenylpropanoid biosynthesis pathway, and anthocyanins constitute one of the most abundant classes of flavonoids. The pathway is initiated by deamination of phenylalanine to cinnamic acid and following reactions lead to the formation of hydroxycinnamoyl CoA thioester, which is then the substrate for branch pathways of lignin monomers, anthocyanins, coumarins, and simple esters such as chlorogenic acid synthesis (*1*). Naringenin, the product of this pathway, is converted to pelargonidin, petunidin, and other anthocyanins present in potato, by several steps of hydroxylation. The aglycon then is enzymatically glycosylated by specific glycosyltransferase. The two major anthocyanins in potato, pelargonidin and petunidin, have a trisaccharide side chain attached to the 3-hydroxy group of the aglycon.

Concomitant to the extensive structural studies, the function and significance of anthocyanins for plant physiology were also investigated. It was found that their synthesis in petals is intended to attract pollinators; they can also be important as feeding deterrents, as protection against damage from UV irradiation, and as metal chelators (*2*). Pelargonidin 3-glucoside in seed coats of *Phaseolus vulgaris* was shown to inhibit the growth of pathogenic microbes and to stimulate the growth of symbiotic bacteria (*3*), whereas cyanidin, delphinidin, and pelargonidin glucosides and their aglycons had potent antioxidant activities (*4*). It was found that several environmental factors affect flavonoids synthesis. It was reported that the wounding of potato tubers and their exposure to illumination and diseases stimulate the various phenylpropanoid pathways (*5*). The recently published data on changes of anthocyanins content in potato upon repression of 14-3-3 protein synthesis suggest that these natural compounds respond also to stress upon transformation (*6*). There is no indication for direct interaction of anthocyanins synthesis pathway and 14-3-3 protein content.

In addition, there is a large body of evidence from both in vitro and in vivo studies to suggest that flavonoids are beneficial for human health. Results from epidemiological studies suggest that due to their antioxidant properties flavonoids may help to protect against chronic diseases, such as cardiovascular disease and cancer (*7*).

All of these data strongly suggest that the increase of anthocyanins content in crop plants may result in their higher self-protection against biotic and abiotic stresses and help to obtain food with increased antioxidant capacity, lengthening its storage time and benefitting consumer health.

The powerful approach for obtaining new, improved organisms is the generation of transgenic plants. The primary goal

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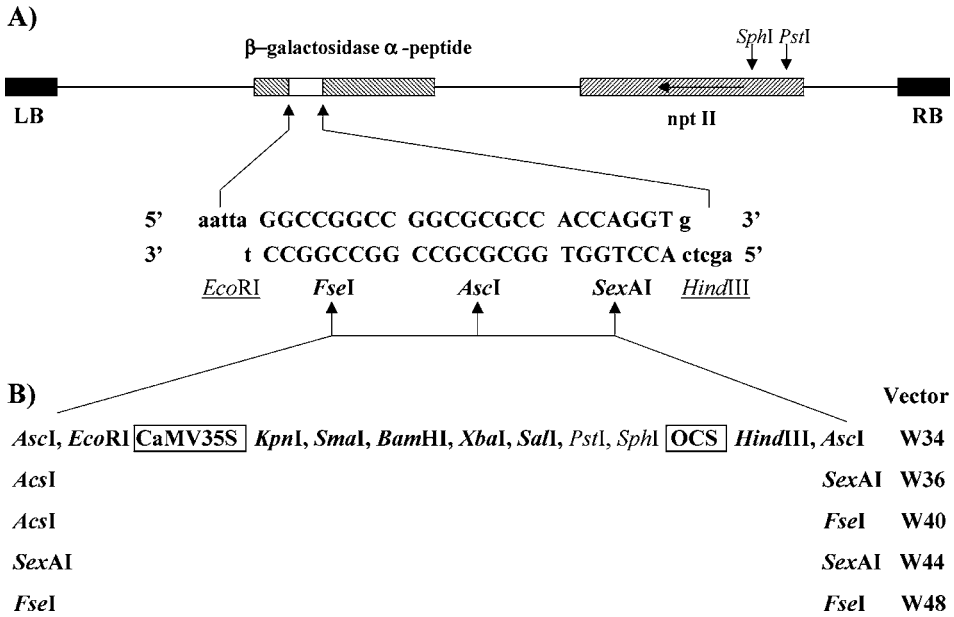

Figure 1. (**A**) T-DNA region of modified plant binary vector pBinAR. In the plasmid pBinW88 the plant expression cassette *Eco*RI–*Hin*dIII was replaced by polylinker with restriction sites for *Fse*I, *Asc*I, and *Sex*AI enzymes and the *Eco*RI and *Hin*dIII sites were destroyed. (**B**) Expression cassette *Eco*RI–*Hin*dIII from pBinAR was introduced into SK+ plasmid and flanked with five combinations of *Fse*I, *Asc*I, and SexAI restriction enzymes enabling introduction into pBinW88 of up to five different constructs. LB, left border; RB, right border; nptII,kanamycin resistance gene with nopaline synthase promoter and terminator; CaMV35-35S promoter of cauliflower mosaic virus; OCS, octopine synthase terminator. Unique restriction sites are in bold.

of this study was to modulate the anthocyanin level in potato plants by manipulating the flavonoid biosynthesis pathway. The manipulated genes were those encoding key enzymes of this pathway such as chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR). We have generated transgenic plants transformed with single (CHS, CHI, or DFR), double (CHI and DFR), and triple (CHS, CHI, and DFR) gene constructs and in either of two orientations, sense or antisense. The transgenic plants were preselected by PCR and selected by means of northern blot (*8*). The selected plants were then analyzed for anthocyanin content and antioxidant capacity. It was found that the most effective in anthocyanins production in potato is the single construct containing a DFR cDNA in a sense orientation. The antisense construct of the DFR gene was also the most effective in repression of anthocyanin biosynthesis. The changes in anthocyanin level in DFR plants perfectly correlated with the antioxidant capacity of transgenic potato.

MATERIALS AND METHODS

Plant Material. Potato plants (*Solanum tuberosum* L. cv. Desiree) were obtained from Saatzucht Fritz Lange KG (Bad Schwartau, Germany). Control and regenerated plants were cultivated in the greenhouse in soil under 16 h of light (22 °C) and 8 h of darkness (15 °C). 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 and were used mainly for transgenic selection. For biochemical analysis field-grown potatoes were used. The field trial took place at Experimental Station Swojec in the vicinity of Wroclaw. Tubers were harvested in September 2001 after 5 months of growth.

Construction of the Transgenic Plants. In this study, five types of transgenic plants were used: type CHS, overexpressing (CHS) the barley cDNA encoding chalcone synthase (EMBL/GenBank database accession no. X58339); CHI, overexpressing (CHI) the *Petunia hybrida* cDNA encoding chalcone isomerase (EMBL/GenBank database accession no. X14589); DFR, overexpressing (DFR) and underexpressing (DFRa) the *P. hybrida* cDNA encoding dihydroflavonol 4-reductase (EMBL/GenBank database accession no. X15537); type W95, overexpressing CHI and DFR; type W92, overexpressing all three, barley and petunia cDNAs. Petunia cDNAs and barley cDNA were provided by Dr. I. Somssich and Dr. H. Hesse, respectively. For leaf explants transformation the pBin vector containing respective cDNA in sense and reverse 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 (*9*), and the integrity of the plasmid was verified by restriction enzyme analysis. Young leaves of wild-type potato *S. tuberosum* L. cv. Desiree 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 (*9*). The selection marker was the neomycin phosphotransferase gene (npt II). The transgenic plants were preselected by PCR using primers specific for the kanamycin resistance gene and then selected by means of northern blot analysis (*8*). Total RNA was prepared from frozen young plant leaves using the guanidinium hydrochloride method as described (*10*). Following electrophoresis [1.5% (w/v) agarose, 15% (v/v) formaldehyde) RNA was transferred to nylon membranes (Hybond N, Amersham, U.K.). Membranes were hybridized overnight at 42 °C with radioactively labeled respective cDNA (CHS, CHI, and DFR) as probes. Filters were washed three times in 0.1× SSC and 0.1% SDS for 30 min at 65 °C.

The following transgenic lines showed the highest respective mRNA level when compared to control plant and were used for further investigations: CHS 26, 32, and 62; CHI 3, 5, and 26; and DFR 2, 5, and 11, overexpressing chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR), respectively. Of triple (W92) and double transformants (W95) the transgenic lines W92.4, W92.5, W92.21, W95.11, W95.21, and W95.43 were selected. The lowest mRNA signal was detected from underexpressing dihydroflavonol reductase DFRa 1, DFRa 2, and DFRa 3 transgenic lines.

Extraction of Anthocyanins from Tuber Epidermis of Transgenic Potato. One hundred and fifty milligrams of dried tuber epidermis was extracted with 1 mL of methanol/0.1% HCl solution in an ultrasonic bath for 15 min. After centrifugation, supernantant was filtered through a Millipore (0.2 μm) and then dried in a speedvac; polyphenols were resuspended in 1 mL of water. The solution was then applied onto the SPE column (Merck), and retained anthocyanins were eluted from the solid phase with 40% MeOH and analyzed on LC-UV.

HPLC Analysis of Anthocyanins in Tuber Extracts. An HPLC system (Knauer) equipped with an automated sample injector and a UV detector (Knauer variable wavelength monitor type 87.00) con-

nected to a personal computer (HPLC software/hardware package version 2.21A) was used. The sample extract was separated on a LiChroCART 250-4 100 RP-18 (5 μm) column preceded by a LiChroCART 4-4 100 RP-18 (5 μm) precolumn (Merck). Compound detection was carried out by on-column measurement of UV absorption at 325 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 program I (table HPLC). The volume of the sample injected was 20 μL. The calibration graphs of polyphenols were prepared by measuring the peak area, and they were linear in the examined range (0.02−0.10 mg/mL). For the HPLC analyses gradient grade acetonitrile was used. Water was glass distilled and deionized. Solvent solutions were vacuum degassed with sonication prior to usage. All experiments were performed at room temperature (20 °C). The compounds were identified and determined on the basis of standards analysis as described (*11*). Pelargonidin 3-rut-5-glu and petunidin 3-rut-5-glu both acylated with *p*-coumaric acid were used as standards.

Determination of Starch and Soluble Sugars Content. Potato tuber slices 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 (*12*). 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 amount of released glucose was determined enzymatically.

Antioxidant Activity of Potato Tuber Extracts. An acidic methanol extract of potato tuber epidermis was diluted in the range from 2500 to 35000 times with water and directly analyzed. The chemiluminescence method (*13*) was used to determine the antioxidant activity of the extracts. 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 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), freshly prepared. The luminol solution (100 μM) and diluted extracts were automatically injected. The photons produced in the reaction were counted on an EG&G Berthold LB96P microplate luminometer at 30 °C. The antioxidant potential (IC_{50}) was defined as the amount of tuber skin that inhibits luminol chemiluminescence by 50%.

RESULTS

Construction of Multigene Vector. The main goal of this paper was to obtain potato plants with increased content of anthocyanins. To achieve this, multigene as well as single-gene constructs were prepared. For multigene constructs the new multiple cloning site (rare cutting sites) was inserted into the pBinAR vector, resulting in a new pBinW88 multigene vector. By this modification up to five genes can be easily cloned into one binary vector.

The backbone for new construction was the binary vector pBinAR (*14*), a derivative of pBin19 (GenBank U09365 and U12540) (*15*) obtained by inserting the *Eco*RI−*Hin*dIII expression cassette comprising the cauliflower mosaic virus 35S promoter (CaMV 35S), multiple cloning site, and octopine synthase (OCS) 3′ transcription terminator (**Figure 1A**). The *Eco*RI−*Hin*dIII fragment of pBinAR was replaced by a short polylinker containing restriction sites for three rare cutting enzymes, *Asc*I, *Fse*I, and *Sex*AI (**Figure 1B**), resulting in plasmid vector designed pBinW88.

Similarly, the set of five auxiliary vectors was constructed in two steps. First, the multicloning site of pBluescriptSK+ (Stratagene; GenBank X52328) was replaced by five polylinkers each containing *Eco*RI−*Hin*dIII sites located within different combinations of *Asc*I, *Fse*I, and *Sex*AI sites. Next, to each of those polylinkers the *Eco*RI−*Hin*dIII expression cassette, previously excised from pBinAR, was inserted. The resulting vectors W34, W36, W40, W44, and W48 retain typical features of plasmid pBluescriptSK$^+$. Its size and copy number in *Escherichia coli* offer excellent efficiencies in all recombination

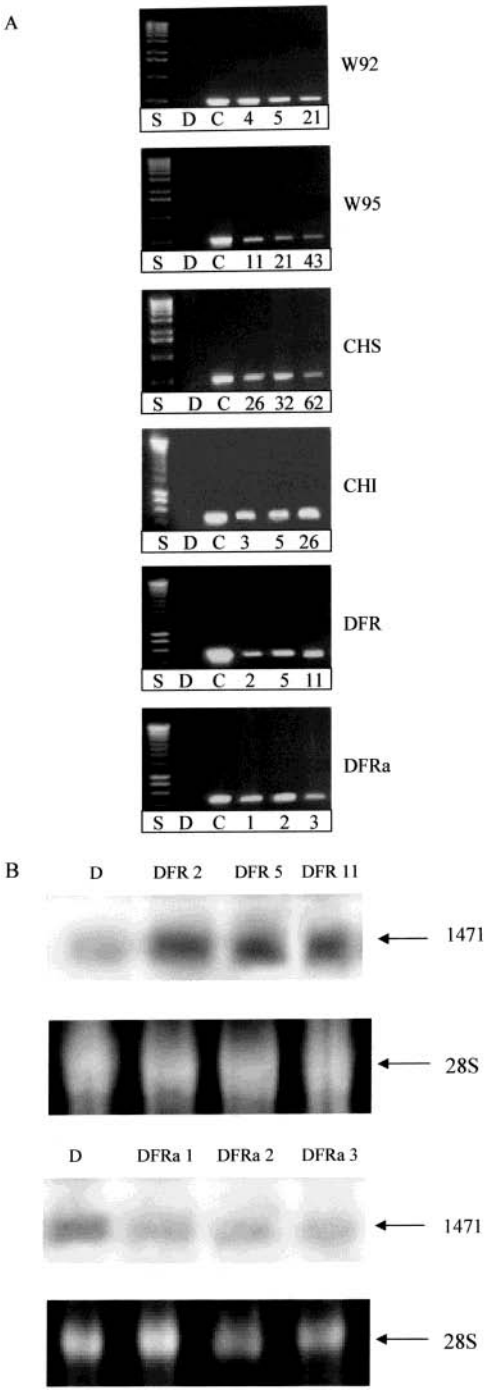

Figure 2. Verification of transgenic plants by agarose gel electrophoresis of PCR product and northern blot. (**A**) *Npt II* gene was amplified with the use of specific primers and potato plant genomic DNA as a template. The plants were transformed with either sense or antisense constructs of cDNAs encoding chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR). W95 and W92 are plants transformed with two (CHI, DFR) and all three genes, respectively. The respective antisense plants are marked DFRa; s, DNA length marker (DNA ladder; Gibs, Eurogentec); D, nontransformed plant; c, positive control (pBin W88 vector); numbered transgenic plants. (**B**) Northern analysis of RNA isolated from transgenic plants transformed with DFR from *P. hybrida* in sense and antisense orientation. Fifty micrograms of total RNA was loaded in each lane, and the blot was probed with ^{32}P-labeled DFR cDNA. Lower blot is ribosomal RNA stained with ethidium bromide.

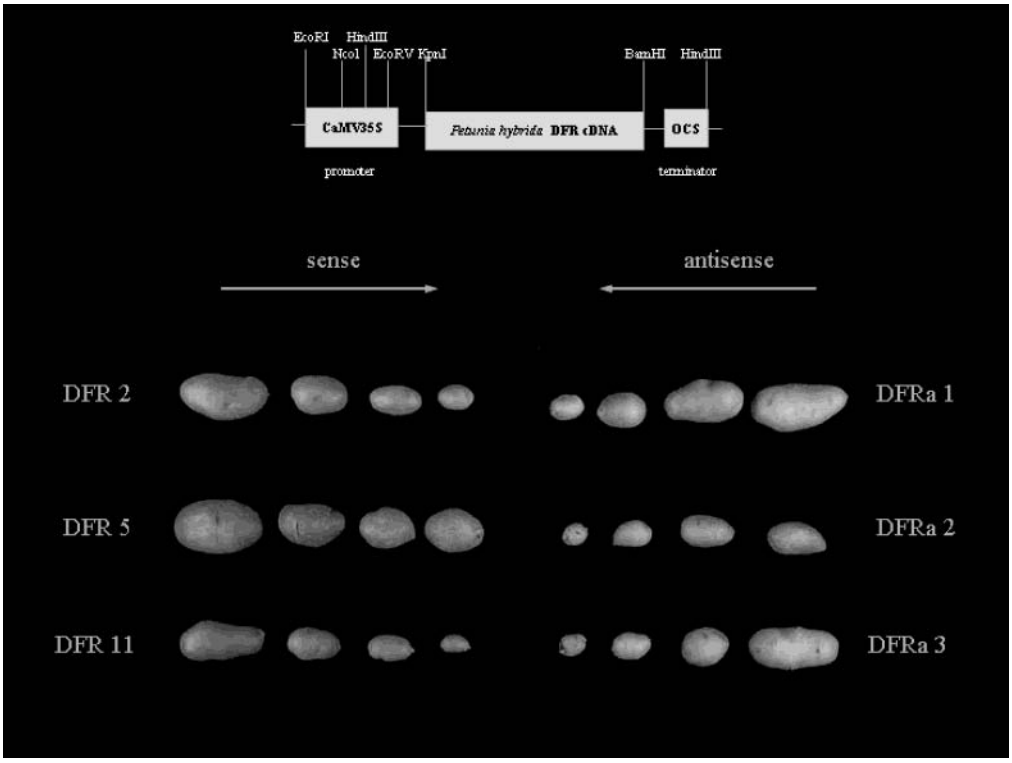

Figure 3. Gene construction for DFR overexpressed (sense) and repressed (antisense) potato plant (upper panel) and greenhouse-grown tubers from different transgenic lines (four from each). The different transgenic lines are numbered.

procedures. Any cloned gene can be easily inserted into the multicloning site between the CaMV 35S promoter and OCS terminator and then transferred as an entire expression cassette to the pBinW88 capable of accepting up to five such cartridges.

Transgenic Plant Generation and Selection. The transgenic potato plants overexpressing (CHS, CHI, and DFR) and repressing (DFR) the key enzymes of the flavonoid synthesis pathway were generated to modulate antioxidant properties. As described under Materials and Methods, the plants from the greenhouse were preselected by PCR and selected by means of northern blot analysis (*8*).

The greenhouse-selected tubers were then grown in a field. Transgenicity of harvested tubers was verified by PCR (**Figure 2A**) and northern blot (**Figure 2B**). All analyzed plants showed clearly the presence of the selection gene marker (npt II), and thus they are all stable transformants. High identity (90% at nucleotide level) between native DFR from *S. tuberosum* (EMBL/GenBank database accession no. AF449422) and *P. hybrida* enabled repression by antisense constructs in DFRa1−3 plants (**Figure 2B**).

Phenotype Analysis. Obtained transgenic plants maintained in a greenhouse were visually indistinguishable from the nontransformed control plants. When grown in the field, there was also no visible change in the phenotype of the aerial parts. However, underground parts of the potatoes differed significantly. The color of DFR-overexpressing plants become deep red (**Figure 3**) and repressed plants showed light-yellow tuber skins. The differences between control plants and other transgenic lines were less visible. Thus, the DFR single construct appears to be more effective in anthocyanin manipulation than other enzymes and multigene constructs.

Chlorogenic and Caffeic Acids Contents. In comparison to control plants significant differences have been found in the content of other flavonoids abundant in potato tubers (**Figure 4**). Chlorogenic and caffeic acids are both highly synthesized in potato tubers. The highest increase (1.5−1.7-fold) in the quantity of both compounds in tubers from DFR-overexpressed plants was detected. Also, the significant increase in chlorogenic content in tubers from triple-construct transformants (W92) was found; however, the content of caffeic acid in those plants was significantly lower than the control value. From among double-construct transformants (W95) two transgenic lines showed significant increases in chlorogenic acid level but very slight changes in caffeic acid content. Of CHS and CHI plants only single transgenic line (CHS 62 and CHI 26) revealed increases in chlorogenic and caffeic acids quantities. Thus, the most effective transformants in phenolic acid synthesis are those from the DFR construct.

Analysis of Anthocyanins Content in Tuber Extracts from Transgenic Potato. To characterize more precisely whether the selected plants reached the expected different levels of anthocyanins, they were analyzed for the pelargonidin and petunidin quantities (data presented in **Figure 4**). The most abundant among anthocyanins were trisaccharide derivatives of pelargonidin (pelargonidin 3-rut-5-glu acylated with *p*-coumaric acid) and petunidin (petunidin 3-rut-5-glu acylated with *p*-coumaric acid); a far lower quantity of cyanidin was detected (not shown). It was expected that at least in the case of chalcone isomerase and dihydroflavonol reductase overexpressing plants, the contents of anthocyanins would increase. This was the case, when compared to control values; significant increases in both pelargonidin (4-fold) and petunidin (3-fold) contents in tubers from the DFR11 transgenic line were observed. Lower but still significant increases in all other DFR and CHI transgenic lines were detected. Overexpression of chalcone synthase often caused cosupression and resulted in a dramatic decrease in enzyme content. This, however, did not occur when chalcone synthase cDNA from barley was overexpressed in potato plant. However, the overexpression of cDNA from barley causes only slight and not significant changes in anthocyanins content. The exception

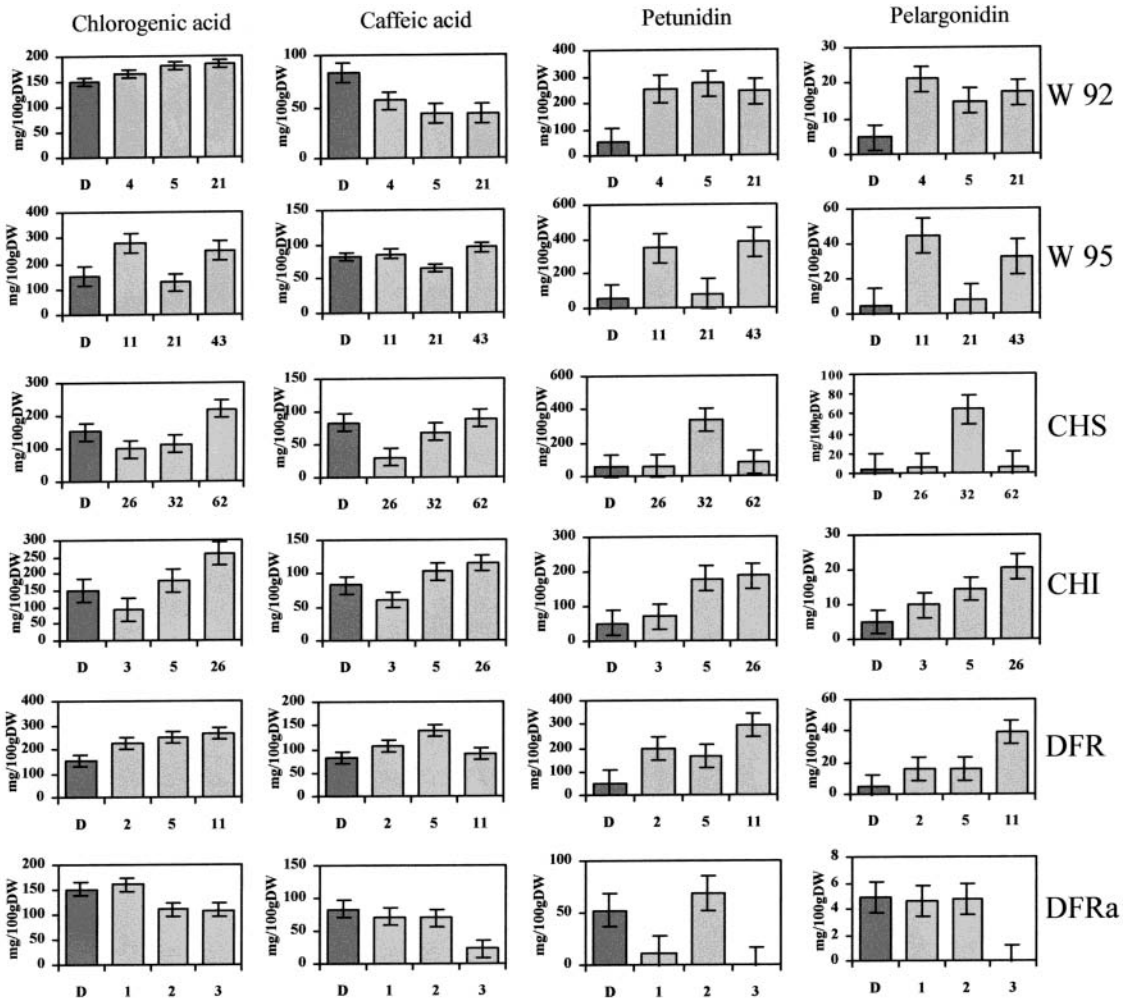

Figure 4. Levels of phenolic compounds, chlorogenic and caffeic acids, and anthocyanins, pelargonidin, and petunidin derivatives in epidermal tuber extracts from transgenic potato plants. The phenolic compounds content was measured in field-grown transgenic potato tubers. The plants were transformed with triple (W92, containing all three cDNAs), double (W95, containing CHI and DFR cDNAs), and single (CHS, CHI, DFR) gene constructs in sense orientation. The cDNAs encoding chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR) were used for constructs preparing. The single-DFR antisense plants are marked DFRa. Tuber material was frozen in liquid nitrogen and examined in enzymatic assay and compared to the control (D). The value ± SD represents three determinations for each transgenic line (numbered). Control and each transgenic line were represented by at least three plants.

is line CHS 32, in which the anthocyanins content reached the value of other transformants.

Analysis of Antisense Transformants. To verify that the changes in anthocyanins content resulted from gene construct expression, plants with repressed enzyme synthesis were created. Plants transformed with cDNAs encoding chalcone synthase, chalcone isomerase, and dihydroflavonol reductase in reverse orientation were analyzed. In all cases repression of endogenous enzymes was effective and resulted in a significant decrease in phenolic compounds content (not shown). Because DFR transformants were most highly effective in phenolic compound synthesis, the DFR antisense plants (DFRa) were created and analyzed in detail. Almost all transgenic lines showed decreases in chlorogenic and caffeic acids content (**Figure 4**), and the lowest amount was detected for the DFRa3 transgenic line. Line DFRa3 showed also a very high in reduction in anthocyanins synthesis.

Carbohydrate Level in Transgenic Plants. Because the carbohydrate provides a carbon skeleton for flavonoids, we decided to analyze transgenic tubers for soluble sugars and starch content.

In almost all transgenic tubers the starch quantity (**Figure 5**) was significantly reduced when compared to control tuber. Only a few selected lines (CHS 32, CHI 5, and DFR 2) showed starch levels similar to control. In contrast, the sucrose content in most transgenic tubers was significantly higher than in the control; in only a few cases was the level the same as in the control tubers. Similarly to starch, the glucose content in most cases was significantly lower than in control plants. Interestingly, both DFR and DFRa showed decreases in glucose content, which suggests that gene orientation does not affect carbohydrate metabolism.

Thus, there is no clear correlation between flavonoids level and carbohydrate metabolism.

Antioxidant Capacity of Tuber Extract from Transformed Plants. It is known that phenolic compounds are involved in the maintenance of the redox status of the cell and also in the response to cold, UV irradiation, wounding, and pathogen attack. Thus, we analyzed the total antioxidant status of tuber epidermis extracts from transgenic plants.

To evaluate the antioxidant properties of transgenic tuber extracts, we used a rapid and sensitive chemiluminescence (CL) method. It is based on the chemiluminescent response of luminol to oxidation. Antioxidants in the test sample scavange the reactive oxygen species, which prevents them from oxidizing the luminol, thus reducing the chemiluminescent light response

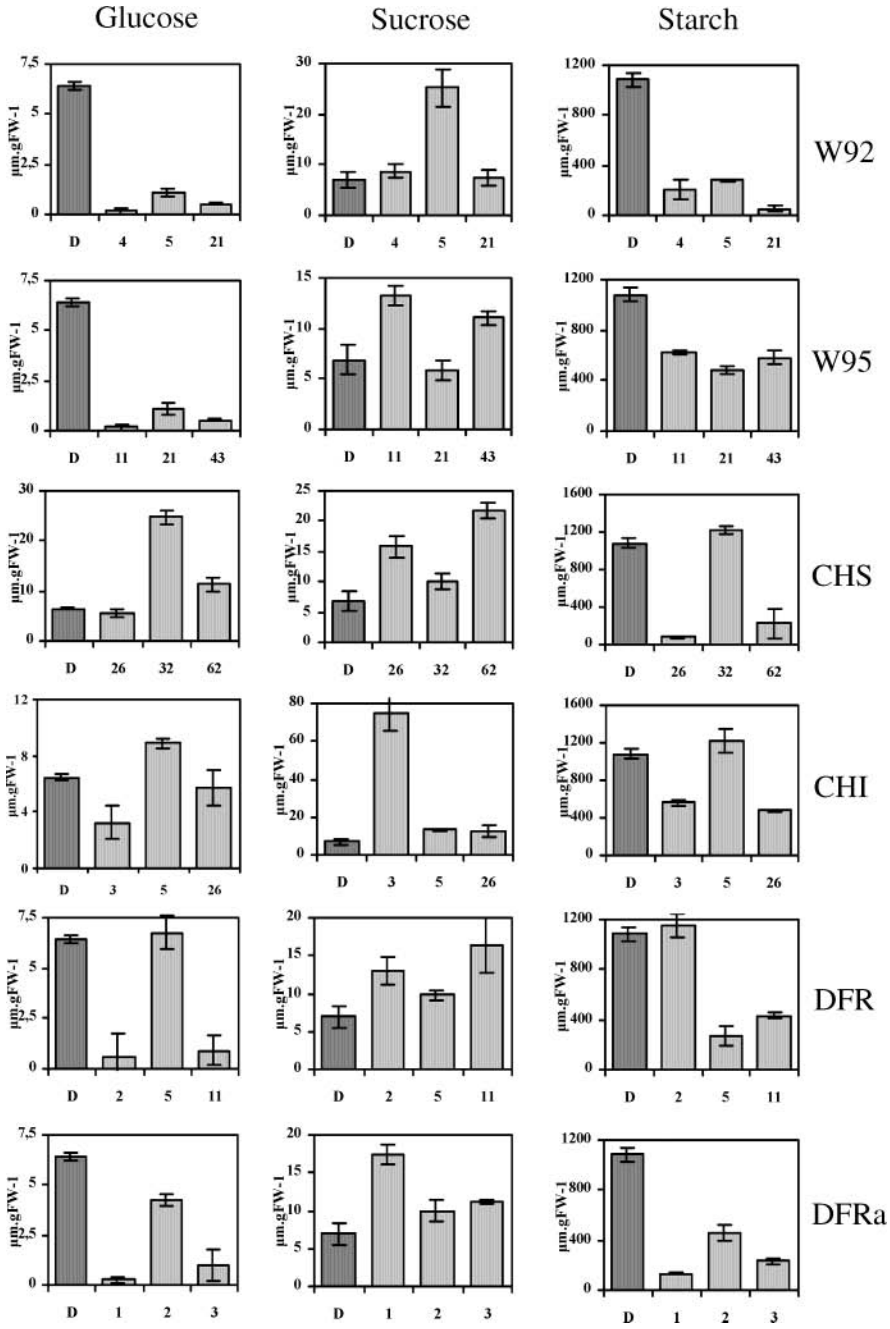

Figure 5. Level of carbohydrates in flavonoids-enriched potato. The sugars content was measured in field-grown transgenic potato tubers. The plants were transformed with triple (W92, containing all three cDNAs), double (W95, containing CHI and DFR cDNAs), and single (CHS, CHI, DFR) gene constructs in sense orientation. The cDNAs encoding chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR) were used for constructs preparing. The single-DFR antisense plants are marked DFRa. Tuber material was frozen in liquid nitrogen and examined in enzymatic assay and compared to the control (D). The value ± SD represents three determinations for each transgenic line (numbered). Control and each transgenic line were represented by at least three plants.

(*13*). Results expressed as IC_{50} values, the amount of extract inhibiting luminescence by 50%, are presented in **Figure 6**. Almost all extracts from flavonoid-overexpressing plants showed significant decreases in IC_{50} relative to the control. The best antioxidant ability was obtained for the DFR transgenic lines. This increase in antioxidant potential correlates with the increase of anthocyanins content.

The W92 plants showed rather unchanged antioxidant potential, and for these plants a decrease in caffeic acid and an increase in anthocyanins content were observed. Thus, in the antioxidant capacity of plants all phenolic compounds content and the level of possibly other compounds (vitamins) take part.

For transgene with repressed DFR expression three independent transgenic lines were tested. The antioxidant potential of mutants was 2-fold lower than that of the wild type plant. The data suggest that repression of the DFR gene may reflect diminished synthesis of antioxidant compounds.

DISCUSSION

The complexity of the flavonoid pathway has been revealed in past decades by a great deal of studies. The biochemical studies first characterized the enzymatic steps involved in flavonoid production and the wide range of compounds produced by this pathway in wild type plants. First, in our study

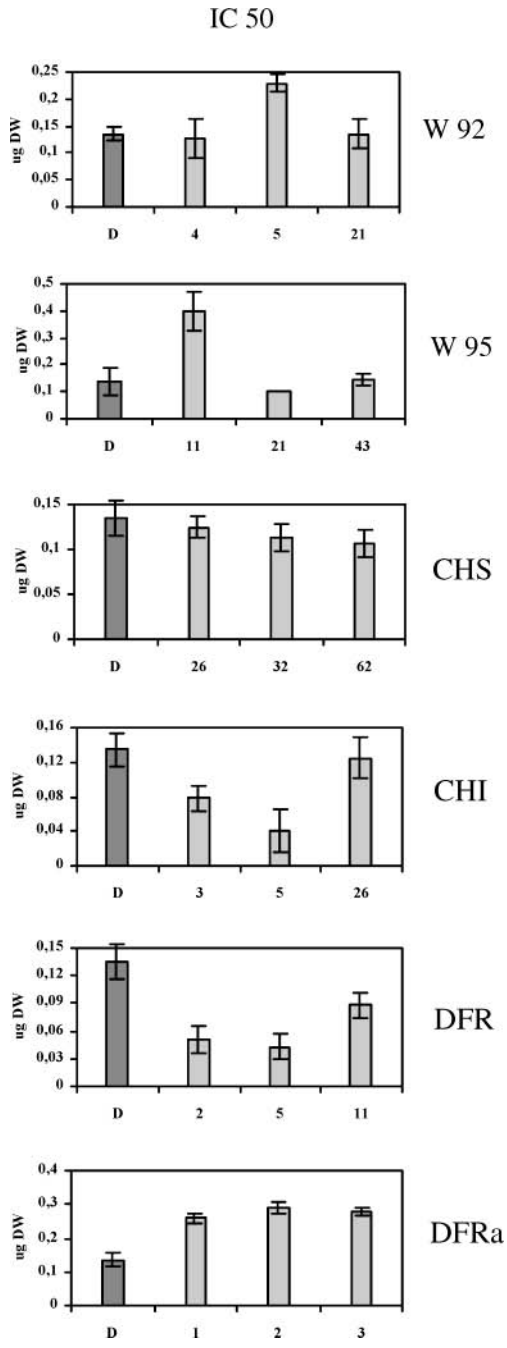

Figure 6. Antioxidant potential of control (D) and transgenic plant overexpressed flavonoids biosynthesis enzymes. The analysis of tuber extract was performed as specified under Materials and Methods. The plants were transformed with triple (W92, containing all three cDNAs), double (W95, containing CHI and DFR cDNAs), and single (CHS, CHI, DFR) gene constructs in sense orientation. The cDNAs encoding chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR) were used for constructs preparing. The single-DFR antisense plants are marked DFRa. The mean value ($n = 3$) ± SD is presented.

we tried to increase our knowledge about the limiting steps in anthocyanin synthesis and the influence of these compounds on antioxidant activity in transgenic potato plants. For this reason we have constructed a new vector capable of transfer of up to five genes. The vector is obtained by introducing three rare cutting restriction sites into pBinAR, thus creating five gene sites. For cloning improvement, five compatible pBluescript vectors containing a gene cassette with a promoter, a multiple cloning site, and a terminator were generated. The whole system (binary and bacterial vectors) is very simple and easy to use for multigene construction.

Second, our goal was to create plants with increased levels of anthocyanins using genetic background. Because of the flavonoid function we could produce potato plants more resistant to biotic and abiotic stress, UV-B radiation, and infectious agents. The overexpression of either any single gene or all three genes simultaneously resulted in significant increases of phenolic acids content and anthocyanins as well. The most effective in chlorogenic increase were W92 and DFR plants. All transgenic lines from among these constructs revealed a significant increase in chlorogenic acid content. Of other transformants two transgenic lines of double-gene transformant (W95) and one transgenic line of CHS and CHI plants with chlorogenic acid content increase have also been detected. For an as yet unknown reason the triple-gene transformants showed a significant decrease in caffeic acid content, and the other transformants showed slight increases or decreases in acid content depending on the transgenic line.

The stimulation of phenolic compound synthesis in transgenic plants is more clearly visible in the case of anthocyanins. In all transgenic lines of triple-gene transformants and CHI and DFR plants the increase in anthocyanins content is well seen. The interesting fact is that mostly the content of petunidin and pelargonidin in transgenic plants ranged from 20 to 30 mg/100 g of dry weight (DW) and from 6 to 15 mg/100 g of DW, respectively. This might suggest existence in the potato of a mechanism that controls the overall synthesis of anthocyanins, and it is independent of CHS, CHI, and DFR genes. In other words there is a certain, maximal level of anthocyanins in potato that can be tolerated and cannot be surpassed. This maximal value can be reached by overexpression of any of the analyzed genes. This hypothesis could be supported by a recent finding that in some cases development is controlled by reactive oxygen species (*16*), which are scavenged by antioxidants such as flavonoids. On the other hand, this suggestion is to some extent in contradiction with the recent finding that tomato anthocyanins content can be most strongly increased by CHI overexpression (*17*). In view of our results, however, the degree of anthocyanins synthesis stimulation does not depend on the gene used but it does for the yield of transgenic plant generation.

It is interesting that all transgenic plants showed a decrease in starch level, which ranged from a few percent to >10-fold (W92.21) of control value. This is accompanied by a significant decrease in glucose and an increase in sucrose level. It can be suggested that the limitation of flavonoid synthesis might result from the carbohydrate level available for phenolic compounds synthesis.

To investigate the physiological significance of a flavonoids level increase, the antioxidant properties of transgenic tuber extracts were measured. The results are expressed as IC_{50} values and indicate the amount of extract inhibiting luminol luminescence by 50%. Almost all extracts from flavonoid-overexpressed plants showed significant decreases in IC_{50} relative to the control. The best antioxidant ability was obtained for the DFR transgenic lines. This increase in antioxidant potential correlates with the increase of anthocyanins content.

The W92 plants showed rather unchanged antioxidant potentials, and for these plants the decrease in caffeic acid and increase in anthocyanins content were observed. The total quantity of measured phenolic acids for W92 plants is in the same range (220−230 mg/100 g of DW) as for control plants (233 mg/100 g of DW) but for DFR plants is significantly higher

(330−390 mg/100 g of DW). The repressed DFR plants with low antioxidant capacity showed significant decreases in total phenolic acids content. Thus, in the antioxidant capacity of plants all phenolic acids content and the quantity of possibly other compounds (vitamins) may take a reasonable part.

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