JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ADP Ribosylation Factor Regulates Metabolism and Antioxidant Capacity of Transgenic Potato Tubers

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In our recent studies we have evidenced that repression of ADP-ribosylation factor (ARF) in potato plants results in 14-3-3 gene activation. The significant alteration in plant phenotype and in carbohydrate content clearly indicates that there may also be changes in other metabolite syntheses. In this paper we present the data on contents of compounds, occurring in transgenic potato tubers from field trial, known to be important for the human diet. We also determine which of the ARF-antisense plant features resulted from ARF repression. This determination was accomplished by the analysis of ARF-antisense plants transformed with cDNA encoding 14-3-3 protein in reverse orientation. The sucrose accumulation and the decrease in glycoalkaloids level were found to be characteristic features of all transgenic plants. The increase in antioxidant capacity of transgenic potato tubers should also be pointed out. The analysis of fat from modified potato tubers revealed a nutritionally valuable composition of fatty acids, including the significant increase of linoleic acid level.

KEYWORDS: ARF; carbohydrates; 14−3−3 protein; transgenic plants; antioxidant potential

INTRODUCTION

ADP-ribosylation factor (ARF) is a small, about 21 kD, GTP-binding protein, originally identified in mammalian cells as a protein required for ADP-ribosylation of the α subunit of the heterotrimeric G protein G_S, thereby leading to activation of adenylate cyclase (*1*). ARF functions as an activator of the enzyme phospholipase D and is required in coated vesicle assembly of the Golgi apparatus (*2*, *3*). In recent years many isoforms of ARF protein, obtained from different sources including mammals, higher plants, and fungi, were cloned and sequenced (*1*, *4*, *5*). ARFs are supposed to participate in protein trafficking pathways (*6*) and may be involved in the regulation of ADP− ribosyltransferase. To analyze ARF significance in potato plants, transgenic organisms were constructed. From among several obtained transgenic lines, three lines (J1.7; J1.11; J1.38) were selected and analyzed (*7*).

Differences in the pattern of ADP− ribosylated polypeptides were observed in ARF−antisense transgenic potatoes in comparison with un-transformed control plants. The transformants showed slight differences in the process of tuber formation. The reduction of stolons and appearance of knobby tubers are characteristic features of these potato plants. It is interesting to note that leaves of the upper half of this transgenic plant were smaller and narrower than in controls and strongly resembled the very young (sink) leaves appearing at the top of the stem of the control plant (*7*). The ARF-antisense plants showed also differences in carbohydrate metabolism and remarkable increase in starch accumulation in tubers as well as a high increase in glucose synthesis in sink organs. Since all these features were detected for plants grown in a greenhouse, the aim of this paper is to verify transgenic properties of plants grown in a field. For the field trial the transgenic line J1.11 was chosen, which completely repressed ARF synthesis. The exceptional feature of the transgene was the increase of 14-3-3 (29G isoform) protein content. The 14-3-3 is now recognized as an adaptor protein, which controls nitrate reductase and sucrose phosphate synthase activities (*8*). Hence another aim of this paper is to indicate whether any of the ARF-antisense transgenic plant features result from the increased content of 14-3-3 protein. Therefore, the double antisense plants with repression of both ARF and 14-3-3 protein synthesis were generated and analyzed.

MATERIALS AND METHODS

Plant Material. Potato plants (*Solanum tuberosum* L. cv. Desiree) were obtained from Saatzucht Fritz Lange KG (Bad Schwartau, FRG). Plants were cultivated in a 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 the transfer of tissue culture plants to the greenhouse. Field trials were performed in the vicinity of Wrocław (Poland) between April and September 2001.

Construction of Transgenic Plants. In this study three lines of transgenic plants were used: line J1.11−underexpressing the potato

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10.1021/jf020779r CCC: $25.00
Published on Web 12/03/2002

ADP−ribosylation factor cDNA (EMBL/GenBank Database Account No. X74461)—and lines G2.18 and G2.25—the potato double antisense plants underexpressing both ADP-ribosylation factor and 14-3-3 isoform cDNA 29G (EMBL/GenBank Database Account No. X97724). For leaf explant transformation a binary vector, containing respective cDNA in reverse orientation under the control of 35S promoter and Nos terminator, was used.

The selection marker was the neomycin phosphotransferase gene, except for G2 plants which were transformed using two vectors, one containing kanamycin (ARF antisense, J1) and the other containing hygromycin resistance gene. The transgenic plants were preselected by PCR using primers specific for respective phosphotransferase (Kan/Hyg) genes and then selected by means of northern and western blot analysis as described previously (*7*, *9*). From among G2 transformants the G2.18, G2.25, and G2.42 transgenic lines showed the most similar to the wild-type level of 14-3-3 proteins, and G2.18 and G2.25 were used for detailed investigations.

Protein Extraction and Determination. The tissues were powdered in liquid nitrogen and extracted using 50 mM HEPES−NaOH buffer, pH 7.4, containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 10% glycerol, 0.1% Triton X-100, and 0.2% 2-mercaptoethanol (buffer E) (*10*). Following the 20 min long centrifugation at 13 000 rpm (HERAEUS minifuge) at 4 °C, the supernatant was either used immediately or frozen in liquid nitrogen and stored at −70 °C for later usage. The protein content in the tissue, extracted for western analysis, was determined with the routine Bradford method.

Western Blot Analysis. The assessment of the expression of ARF and 14-3-3 genes by means of western blot analysis, with the rabbit IgG anti-recombinant ARF and 14−3-3 proteins, was conducted as described previously (*9*). Briefly, a solubilized protein was separated in 12% SDS−polyacrylamide gel and blotted electrophoretically onto nitrocellulose membranes (Schleicher and Schuell). Following transfer the membrane was sequentially incubated with blocking buffer (5% dry milk) and antibody directed against ARF or the 14-3-3 recombinant proteins (1:2000 dilution). Formation and detection of immune complexes were performed as previously described (*11*). Alkaline phosphatase-conjugated goat anti-rabbit IgG served as a second antibody and was used at a dilution of 1:1500.

Tissue Extraction for Amino Acids Analysis. The frozen plant tissue was powdered in liquid nitrogen and extracted with 10% trichloroacetic acid (TCA). The TCA extract was processed basically as described by Steiner (*12*). TCA supernatant was extracted six times with 10 volumes of ethyl ether. The extracted phase was evaporated in a vacuum.

Determination of Crude Protein in Potato Tuber from Field Trial. The crude protein content in tuber extracts was determined by the standard Kjeldahl procedure (*13*) with the use of Kjeldahl apparatus type K-416 and B-426 (Büchi, Germany). Both transgenic lines and wild-type potato plants were represented by at least three samples of tuber each weighing 3 kg. The tubers were peeled and cut into 1 cm thick slices, freeze-dried, and powdered. At least three measurements for each sample were conducted.

Determination of Amino Acid Contents in Potato Tubers from Field Trial. The amino acid content was determined exactly as recommended by the Official Methods of AOAC with the use of amino acid analyzer T 339 (Mikrotechna, Czech Republic). The 3 kg tuber sample was freeze-dried, powdered, and acid-hydrolyzed, except for the tryptophan analysis in case of which alkaline hydrolysis was conducted. The sulfur amino acid contents were determined after performic acid oxidation of the sample followed by acid hydrolysis. Standard amino acid solutions (Sigma-Aldrich, St. Louis, MO) were used to calibrate the analyzer.

Lipid Content and Composition. The total fat (raw) in the peeled potato tubers was determined using the gravimetrical method. Extraction was performed using the Bligh and Dyer method (*14*). The chloroform extract was evaporated under nitrogen, and the solid remains were weighed after drying at 105 °C. The extracted fat was fractionated into nonpolar (neutral) and polar fractions using the chromatography on a silica gel column. The nonpolar lipids were eluted with chloroform, and the polar fraction was eluted with methanol as described (*15*). The obtained fractions were quantitatively evaluated using the gravimetrical method, which includes solvent evaporation under nitrogen atmosphere, drying the remaining part at 105 °C and finally weighing the solid matter.

Analysis of Fatty Acids. The fatty acid composition of the total fat from tubers and its nonpolar fraction was examined using the gas chromatography method. Methyl esters of the fatty acids were obtained by means of esterification of fat samples (*16*). Then the methyl esters mixture was separated in a capillary column CP-Sil 88 Chromopack (50 m × 0.25 mm). Helium was used as a carrier, and the separation was carried out at a programmed temperature from 150 °C (for 6 min) to 235 °C; the temperature was increased at a rate of 6 °C/min. The identification of particular fatty acids was accomplished by comparing the retention time with external standards.

Determination of Starch and Soluble Sugars Contents. Potato tuber slices were extracted with 80% ethanol−50mM HEPES−KOH, pH 7.4, at 80 °C. The supernatant was used for enzymatic analysis of glucose, fructose, and sucrose (*17*). 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 determined enzymatically.

Determination of Flavonoids Content. A 10 mg amount of lyophilized tuber peels was extracted with 1 mL of methanol −1% HCl (1:1, v/v) solution. The predominant fractions of anthocyanins and phenolic acids were resolved on LiChroCART125-3Purospher RP-18 (Merck Labs) column and detected with the use of a Hitachi diode array detector L-7455 coupled to D-7000 HSM multisolvent delivery system. The compounds were identified and determined on the basis of standard analysis (as described in ref *18*). The total anthocyanin content was determined spectrophotometrically in acid methanol extracts at 536 and 600 nm. The difference in absorbance at 536 and 600 nm was calculated in order to correct the anthocyanin quantification. The most abundant potato anthocyanin, petunidine (Fluka), was used as a standard.

Vitamin C Determination. The vitamin C content in peeled potato tubers was determined according to the 2,6-dichloroindophenol trimetric method (*13*).

Determination of Antioxidant Activity of Tuber Extracts. The acid methanol extract (the preparation procedure was the same as in the case of anthocyanins) of potato tuber epiderms was diluted in the range between 1:1000 and 1:15000 with water and directly analyzed. The chemiluminescence method (*19*) was used to determine 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 N Tris-HCl buffer, pH 9.0, and 4 mM AAPH (2,2″-azobis(2-amidinopropane) dihydrochloride), freshly prepared. Thermolysis of AAPH at room temperature generated free radicals, which oxidized the luminol and resulted in photon production. The luminol solution (100 μM) was automatically injected. The photons produced in the reaction were counted on an EG&G Berthold LB96p microplate luminometer at 30 °C. Subsequently, after the reaction had proceeded for 60 s, the diluted extract was automatically injected and the reduction of photon production was measured. The antioxidant potential (IC 50) was defined as the amount of tuber dry weight (μg) extracted which quenches luminol chemiluminescence by 50%.

Sample Preparation and Glycoalkaloids Determination. The mean sample (3 kg) of tubers of a single transgenic line, representing the size distribution of the whole batch, was collected for analyzes. The sample was freeze-dried and analyzed for α-solanine and α-chaconine content. The 1 g of freeze-dried sample was extracted with 50 mL of the water−methanol mixture (8:92, v/v) (*20*) for 15 min using the ultrasonic bath. Following the filtration (Whatman No.1), 5 mL of methanolic extract containing glycoalkaloids was purified using an SPE C_{18} microcolumn (3 mL, 500 mg; Baker Bond). The identification and determination of α-chaconine and α-solanine were performed on HPLC with the use of respective external standards (Sigma).

Statistical Analysis. Statistical calculations were done using the *t*-test. The term significant is used when $P > 0.05$ with the *t*-test.

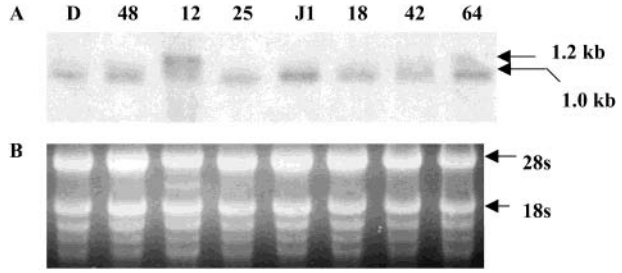

Figure 1. Northern analysis of RNA isolated from leaves of control and transgenic potato plants: D, control plant; J1, ARF-antisense potato plant; G2, different double antisense (ARF and 14-3-3) transgenic plants—numbered. The blot was probed with ^{32}P labeled 29G cDNA. On the right side the length of RNA is marked. (A) A 40 μg amount of total RNA from each sample was loaded in each lane. (B) The size of ribosomal RNA is marked.

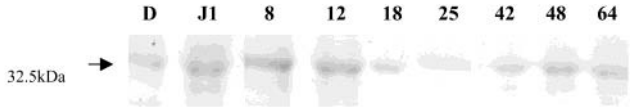

Figure 2. Immunodetection of 14-3-3 protein in the tuber extract from the control plant (D), the ARF-antisense plant (J1), and the double transformant (G2)—numbered. A 50 μg amount of protein was applied onto each lane, and following the SDS PAGE the blot was probed with polyclonal immunoglobulins anti-recombinant 14-3-3 protein. On the left side the molecular mass is marked.

RESULTS AND DISCUSSION

Results. The analysis of ARF antisense potato plants grown in a field was the primary goal of this study. Since these plants revealed increased 14-3-3 protein synthesis, the study included double mutants, in which the 14-3-3 was brought to the control plant level.

Selection of Double Transformed Plants. Previously it was evidenced that repression of ADP-ribosylation factor synthesis in potato plants resulted in substantial phenotype and carbohydrate metabolism changes and also in significant increase in 14-3-3 (29G isoform) protein content. To complement increased quantity of 14-3-3 protein, the double transformants (called G2) were obtained, analysed, and compared to the control and single transformed plants (J1.11). Leaf explants of previously obtained ARF-antisense potato plants (*7*) were transformed using *Agrobacterium tumefaciens* containing the plasmid construct with 29G isoform of 14-3-3 in reverse orientation. Leaf samples (first, second, and third leaf from the top) were taken from 2 months old double transgenic, control, and J1.11 (further called J1) plants and analyzed for the content of 29G isoform of 14-3-3. Figure 1 shows a northern blot experiment with 29G cDNA as a probe. In the case of control and J1, the signal for endogenous fragment (1kb) was observed. The G2 transgenic plant may have an additional mRNA band with the length of 1.2kb representing introduced gene product. Transgenic plants with the 14-3-3 mRNA level similar to the control plant were taken for further selection, which was performed using the western blot analysis (Figure 2). Plants with the 14-3-3 (32.5 kDa) content similar to the control plant level (G2.18, G2.25, G2.42) were selected. Finally, the transgenic lines G2.18 and G2.25 with clear correlation between northern and western analysis results with respect to 14-3-3 protein repression were chosen for field growth and further analysis. The repression of ARF gene in double transformants was confirmed by the western blot analysis, and its result is presented in Figure 3. Using antibodies against ARF protein, it was indicated that the repression of ARF protein

Figure 3. Western blot analysis of ARF protein in a tuber extract from the control plant (D), the ARF-antisense plant (J1), and the double transformant (G2)—numbered. A 50 μg amount of protein was applied onto each lane, and following the SDS PAGE the blot was probed with polyclonal immunoglobulins anti-recombinant ARF.

Table 1. Height (μM) of Palisade Parenchyma Cells in Leaves of the 3 Months Old Control Plants (D), Transgenic Plants with Repressed ARF-Protein (J1), and Transgenic Plants (G2) with Repression of Both ARF and 14-3-3 Proteins

	for given leaf no. starting from the bottom of the plant				
plant	7	8	9	11	13
D	110 ± 8	100 ± 9	80 ± 6	35 ± 3	22 ± 3
J1	140 ± 11	133 ± 11	118 ± 8	87 ± 6	58 ± 4
G2.18	135 ± 10	126 ± 10	112 ± 8	80 ± 5	50 ± 4
G2.25	128 ± 8	120 ± 8	105 ± 6	82 ± 6	50 ± 5
G2.42	125 ± 9	118 ± 8	108 ± 6	76 ± 5	52 ± 4

synthesis in G2 plants was comparable to repression in single transformant J1 (initial line used for transformation), which suggests that this protein level is not affected by a second round of transformation with the 14-3-3 gene construct.

Phenotype Analysis. It was previously reported that an ARF-antisense potato plant differed from a wild type with regard to the shape and size of leaves. The more detailed analysis showed that a transgenic plant contains two or three leaves more than the wild type, and these additional leaves are located in an upper sector of the plant; thus, the plant transgene is enriched with very young still proliferating leaves. This feature is complemented in ARF-antisense plants transformed with 14-3-3 cDNA in reverse orientation, the number of leaves along the plant stem in all three G2 transgenic lines is the same as in the control plants (not shown). Thus, the number of leaves on a stem is not related to the amount of ARF protein. Also stolon reduction in ARF-antisense potato is completely restored upon repression of 14-3-3 protein synthesis in double transformants G2 (not shown). There are, however, two other features associated with ARF content, and these are the number of cell layers in the youngest leaves and the height of palisade parenchyma cells. It was found that leaves of the wild type contain six cell layers, while seven layers were noticed in ARF-antisense and double antisense potato. The cells of palisade parenchyma from a transgenic potato are significantly higher than those of the wild type (Table 1).

Previously we reported a decrease in tuber number per plant and an increase in the size of individual tubers from ARF-antisense plants when grown in a greenhouse, which indicates a possible increase in sink strength in these plants. The data presented in Table 2 suggest that this feature of J1 plants cannot be changed by 14-3-3 gene repression, and it is interesting to note the higher sensitivity of all transgenic plants to low temperature. The tuber fresh weight of a transgenic plant grown at 4 °C is about half the weight of tubers obtained from plants grown at 20 °C. This suggests that ARF is involved in plant resistance to low temperature and the 14-3-3 protein does not affect this feature. Since the data of phenotype analysis of all three G2 transgenic lines are very similar, the transgenic lines G2.18 and G2.25 were chosen for further analysis of double transformed potato grown in the field.

Table 2. Greenhouse Trial of Wild Type Potato (D) and Transgenes Grown at 20 °C (Bold Numbers) and at 4 °C (Nonbold Numbers): Transgenic Plant with Repressed ADP–Ribosylation Factor (J1) and Double Transformant with Repressed ARF and 14-3-3 29G Isoform[a]

	tuber fresh wt (g) per plant	tuber quantity per plant	mean fresh wt (g) per tuber
D	**652 ± 45**	**12.0 ± 3**	**54.0 ± 4**
	270 ± 30	11.0 ± 3	24.5 ± 5
J1	**601 ± 30**	**5.6 ± 2**	**100 ± 8**
	143 ± 40	6.0 ± 2	24.0 ± 4
G2.25	**524 ± 35**	**4.9 ± 2**	**107 ± 11**
	147 ± 40	6.6 ± 2	22.2 ± 5
G2.18	**540 ± 40**	**5.1 ± 2**	**106 ± 10**
	135 ± 30	6.1 ± 2	22.1 ± 4

[a] In the case of the control plant and each transgenic line, the results are means calculated for at least 25 plants.

Table 3. Field Trial of Wild Type Potato (D), Transgenic Plant with Repressed ADP–Ribosylation Factor (J1) and Double Transformant with Repressed ARF and 14-3-3 29G Isoform[a]

	tuber fresh wt (g) per plant	tuber quantity per plant	mean fresh wt per tuber
D	734 ± 45	8.3 ± 0.3	78.0
J1	522 ± 30	5.2 ± 0.2	100.0
G2.18	535 ± 32	5.3 ± 0.4	98.9
G2.25	531 ± 30	5.3 ± 0.2	100.0

[a] In the case of the control plant and each transgenic line, the results are means calculated for at least 75 plants.

Field Trials of Potato Transgenic Plants. To prove the results obtained for transgenic plants grown in standard greenhouse conditions, the field trial was performed and the relevant data are presented in Table 3. Two double transformed transgenic lines G2.18 and G2.25 were used for field trial, and the obtained data were compared to data obtained for single transformant J1 and for control plants. Significant changes in several measured parameters of field grown J1 transgenic plants were found. The reduction in ARF protein level resulted in the decrease of tuber fresh weight per plant and in the significant increase in mean fresh weight per tuber and in the decrease of tuber number per plant. The repression of 14-3-3 gene expression in these plants does not affect tuber phenotype. Thus, the field trial confirmed the greenhouse data with respect to tuber yield, tuber number per plant, and mean fresh weight of individual tubers (Table 3), which strongly suggests that all these parameters are controlled by ARF gene expression.

Carbohydrate Level. Potato plants grown in the field were analyzed for the quantity of the major soluble and insoluble carbohydrates, and the obtained data are presented in Figure 4.

The most visible differences between examined plant lines were observed in the case of soluble sugars. Both transgenic plants showed the significant increase in the level of sucrose, and in the case of double transformed G2 plants the substantial increase in glucose content may also be noticed. This resulted in the increase of the soluble sugar-to-starch ratio parameter, when compared to un-transformed plants.

The increased sucrose quantity did not, however, result in the substantial increase of the starch level in a plant transformant. It is supposed that the glucose and sucrose may be consumed in the synthesis of other compounds such as lipids and amino acids, where carbohydrates serve as a donor of carbon skeleton.

Amino Acids and Protein Content. The level of amino acids essential for human and animal diets was examined. The data

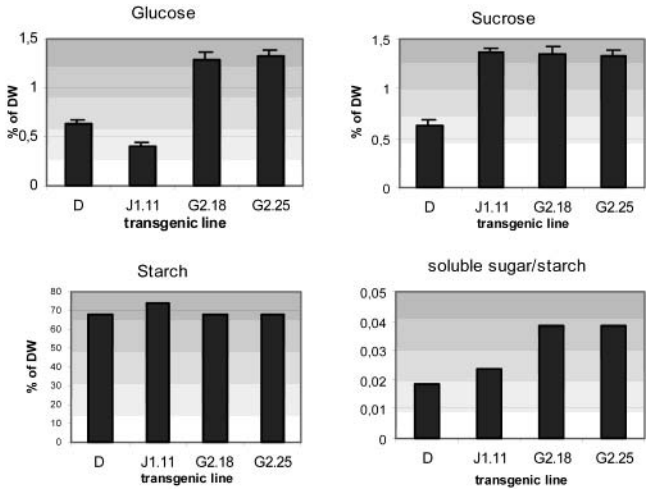

Figure 4. Soluble carbohydrates and starch contents in tubers from the control plant (D) and transgenic plants (J1, G2). The ratio of soluble sugars to starch is also indicated. In the case of both control and transgenic lines the results were obtained for at least 3 kg samples. The data are expressed in percent of dry weight ± standard deviation.

Table 4. Amino Acids Contents (g/(kg DW)) in Wild Type and Transgenic (J1, G2) Potato Tubers from Field Trials

	D	J1	G2.18	G2.25
Essential Amino Acids				
Thr	3.65	3.04	3.89	3.92
Cys	1.64	1.74	1.53	1.52
Met	1.75	1.91	1.58	1.58
Ile	3.73	3.76	3.05	3.03
Leu	5.19	4.33	3.50	3.46
Tyr	3.96	4.56	4.47	4.49
Phe	13.89	10.72	13.02	12.88
Lys	5.66	5.35	5.79	5.81
Val	5.24	2.22	5.43	5.46
Trp	1.20	1.31	1.44	1.50
total content of essential amino acids	45.91	38.94	43.70	43.65
Endogenous Amino Acids				
Asp	29.27	31.35	35.78	36.45
Ser	3.36	3.63	3.86	3.93
Glu	13.81	16.80	14.71	14.61
Pro	4.47	5.16	4.13	4.10
Gly	2.61	2.17	2.51	2.56
Ala	2.95	2.22	3.34	3.35
His	3.37	3.88	2.35	2.73
Arg	4.40	4.09	4.38	4.51
total content of endogenous amino acids	64.72	69.30	73.92	72.24
total content of amino acids	110.63	108.24	117.62	115.89

indicate (Table 4) a slight decrease in the quantity of essential amino acids in ARF-antisense plants, while their sum calculated for G2 plants is close to control plants. This suggests that the reduction of 14-3-3 protein may have affected the level of the essential amino acids.

In a group of endogenous amino acids slight changes in the level of aspartic acid were detected. The smallest quantity of this amino acid was observed in control plants (29.27 mg/(g of dry weight (g DW))). In the J1 transgenic line the level of this amino acid is 7% higher (31.35 mg/(g DW)), in G2.25 plants 25% higher (36.45 mg/(g DW)), and in G2.18 plants 22% higher (35.78 mg/(g DW)) than in the wild type plants. The opposite effect may be noticed in the case of glutamic acid. The level of glutamic acid in ARF-antisense plants (16.80 mg/(g DW)) is found to be 22% higher than in the controls (13.81 mg/(g DW)),

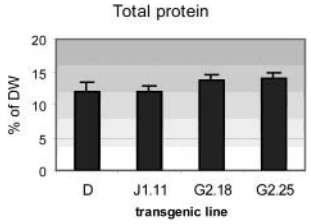

Figure 5. Total protein content in tubers from the control plant (D), the ARF-repressed plant (J1), and both ARF and 14-3-3 protein repressed plants (G2) grown in a field. In the case of both control and transgenic lines the results were obtained for at least 3 kg samples. The protein content is expressed as percent of dry weight ± standard deviation.

and in G2.18 and G2.25 plants it is nearly the same as in the controls (14.71 and 14.61 mg/(g DW), respectively).

The data describing the total protein content in investigated plants (Figure 5) also showed only slight and insignificant differences. In the case of G2 plants a slight increase in total protein content when compared to the control was observed. The level of protein in ARF-antisense plants remains nearly the same as in the wild plants.

Lipid Content and Fatty Acids Analysis. The quantities of total lipids, phospholipids, and triglycerides measured in transgenic and control tubers are presented in Figure 6. When compared to the control, there were only very slight changes in the quantities of various groups of lipids in the transgenic plants. However, a significant difference in fatty acids composition was found. The percentage share of linoleic acid, the main unsaturated fatty acid of potato tubers, in total lipids is significantly higher in transgenic plants when compared to the control. In the case of ARF-antisense plant the level of linoleic acid is 30% higher, in G2.18 plant 21% higher, and in G2.25 plant 25% higher than in the wild type plants (Table 5).

Flavonoids Content and Antioxidant Potential. Flavonoids serve as an important oxidant and pathogen protectant. The level of this compound is presented in Figure 7. In the case of the anthocyanins content, the difference between J1 and G2 plants was found. The only significant reduction in anthocyanins level was observed in the case of G2 plants. The change in flavonoids content in 14-3-3 gene repressed potato plants was recently reported (*22*). The data for chlorogenic acid and total phenolic acids reveal that their contents are downregulated in all analyzed transgenes. The significant decrease in total phenolic compounds may lead to the decrease of antioxidant capacity in analyzed transgenic tubers. To evaluate the antioxidant properties of transgenic tuber extract, the method based on the measurement of chemiluminescence (CL), originating from treatment of luminol with free radicals, was used. The scavenging of free radicals with antioxidants may be observed as a luminescence quenching. The amount of extract, which results in luminescence quenching by 50% (IC50), is presented in Figure 8. To our surprise, both transgenes showed the significant increase in antioxidant capacity (IC50 decrease). This may result from the high level of vitamin C detected in transgenic tubers (Figure 9). Thus, the level of antioxidant potential is a result of both phenolic compound content and vitamin C content in potato tubers.

Table 5. Relative Composition of Fatty Acids (Percent of Total Fatty Acids) in the Nonpolar Fraction of Tuber Lipids from Control and Transgenic Potato Lines

	D	J1	G2.18	G2.25
palmitic acid C16:0	40.62 ± 0.89	30.50 ± 1.05	34.89 ± 0.85	35.12 ± 0.97
stearic acid C18:0	6.19 ± 0.39	6.81 ± 0.24	10.24 ± 0.36	10.11 ± 0.33
oleic acid C18:1	7.63 ± 0.62	8.41 ± 0.29	8.89 ± 0.71	8.81 ± 0.51
linoleic acid C18:2	19.84 ± 0.36	26.18 ± 0.94	23.97 ± 0.69	24.58 ± 0.83
linolenic acid C18:3	14.42 ± 0.55	16.53 ± 0.25	10.97 ± 0.58	10.81 ± 0.44
other acids	11.30 ± 0.26	11.57 ± 0.85	10.81 ± 0.95	10.57 ± 0.99

Glycoalkaloids Level. Steroidal glycoalkaloids are the compounds, which determine the flavor of tubers and protect potato plants against pests. In potato tubers α-solanine and α-chaconine are most represented. Both of these glycoalkaloids are toxic for humans at concentrations greater than 200 mg/(kg FW) and the most dangerous is α-chaconine. Thus the decrease in contents of these compounds is advantageous for animal and human diet [for review see ref *23*]. Significant reduction of the level of glycoalkaloids was detected in transgenic plants (Figure 10). The highest difference concerns the α-chaconine quantity. In peeled tubers obtained from a transgenic plant, the level of α-chaconine was about one-third of the level observed in the control plant. Steroidal glycoalkaloids are mainly present in the potato peels, and their level is much higher in the control than in transgenes.

Discussion. To prove the physiological significance of the ADP-ribosylation factor in plants, the transgenic potatoes with repressed ARF synthesis were recently constructed and analysed (*7*, *24*). The transgene grown in controlled greenhouse conditions showed large changes in plant phenotype and significant changes in carbohydrate synthesis. The dramatic reduction of stolons, the appearance of knobby tubers, and the significant changes in leaf shape and size were characteristic features of these plants. The ARF-antisense plants showed a small increase in starch accumulation in tubers and a high increase in glucose synthesis in sink organs. The exceptional feature of the transgenes was the increase in 14-3-3 protein synthesis. The 14-3-3 proteins affect many enzyme activities in vitro, including nitrate reductase and sucrose phosphate synthase (8). Thus, to verify the in vivo significance of ARF protein for phenotype and carbohydrate metabolism, the double transgenic plants with repression of ARF and 14-3-3 protein were generated. It cannot be ruled out that repression of the 29G isoform of the potato 14-3-3 protein may have also resulted in partial repression of other isoforms. Since there is no specificity in 14-3-3 isoform action on plant metabolism, the partial repression of other isoforms does not seem to affect analyzed plant parameters.

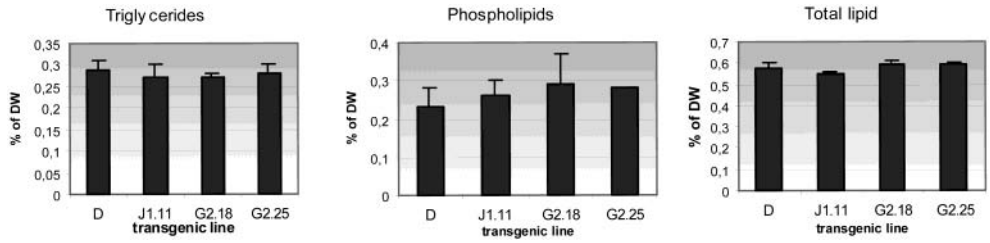

Figure 6. Level of lipids measured in tubers from the control plant (D), the ARF-antisense plant (J1), and the double transformed plants (G2), expressed as a percent of dry weight. At least 3 kg samples were analyzed.

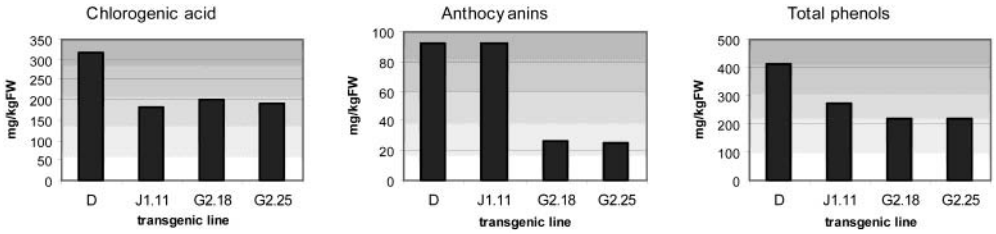

Figure 7. Level of chlorogenic acid, anthocyanins, and total phenolics measured for at least 3 kg tuber samples from the control plant (D), the ARF-antisense plant (J1), and the double transformed plants (G2). The data are expressed in [mg/(kg FW)] ± SD.

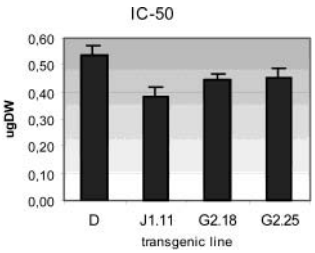

Figure 8. Antioxidant activity (IC50) measured for control and transgenic tubers. The data are expressed in μg of dry weight tissue (μg DW) extracted and added to the reaction mixture.

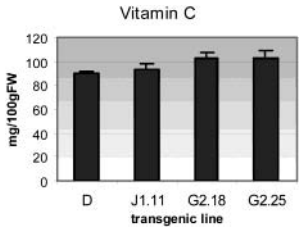

Figure 9. Content of vitamin C in the control plant, the ARF-antisense plant (J1), and the double transformed plants (G2). The data are expressed in [mg/(100 g DW)] ± SD.

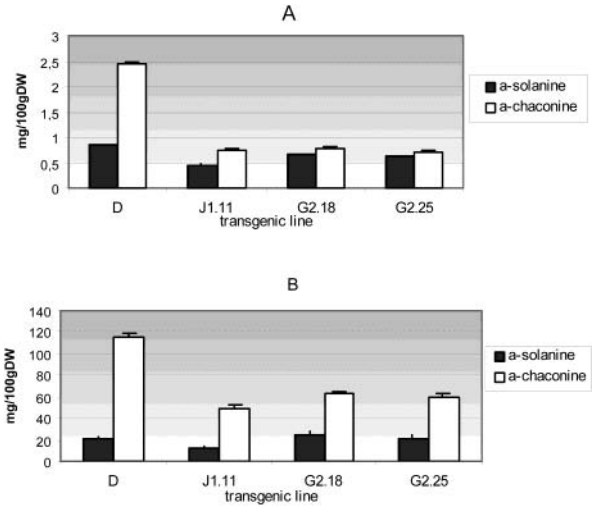

Figure 10. Content of glycoalkaloids in peeled tubers (panel A) and in peels (panel B) from the control plant (D), the ARF-antisense plant (J1), and the double transformants (G2). In the case of both control and transgenic lines the results were obtained for at least 3 kg samples. The data are expressed in [mg/(100 g DW)] ± SD.

The double transgenes were analyzed with respect to phenotype and carbohydrate synthesis as well. The analyzed plants were field grown, and primarily their phenotype was observed. The phenotype of double transgene plants (G2) was the same as that of the control plant. This suggests that the ARF-antisense (J1) plant morphological features can be complemented with repression of 14-3-3 gene expression.

The carbohydrate analysis of transgenic plants grown in the field reveals changes in glucose, sucrose, and starch syntheses. However, these changes are inconsistent with ARF and 14-3-3 protein level. The significant increase in the sucrose level and the slight increase in the starch level are accompanied by a slightly lower glucose content in J1 as compared to the wild type plant. In a double transformant, the significant increase in glucose and sucrose levels can be observed. The starch content, however, is the same as in the control plant. The data thus suggest that the sucrose synthesis is specifically affected by the ARF repression. The substantial increase in the sucrose level in all transgenes may suggest its use as a carbon skeleton for synthesis of other organic compounds in these plants. Thus, we determined the levels of proteins, lipids, amino acids, flavonoids, and glycoalkaloids, which are known to contain a sugar backbone. Compared with the control plant, the increase in tyrosine and the decrease in leucine contents for both J1 and G2 transgenic tubers were found. Consequently there were only very slight changes in protein content. The analysis of lipids also showed only a very slight increase in phospholipids and a decrease in triglyceride levels in all transgenic plants. However, it is interesting to note the substantial increase in linoleic acid content in tubers of G2 plants. The significant reduction in phenolic acids and glycoalkaloids contents in all transgenes was detected. It appears advantageous that the chlorogenic acid, responsible for tuber darkening, and the toxic glycoalkaloids contents are substantially diminished in transgene tubers. The obtained results suggest that in all transgenes the accumulated sucrose does not affect cellular metabolic pathways. In case of anthocyanins, the much lower level of these compounds in transgenic G2 plants is difficult to explain in terms of ARF and 14-3-3 gene repression, and therefore further experiments will have to be undertaken to resolve this problem.

From among the analyzed metabolites, the sucrose accumulation and the level of steroidal glycoalkaloids as well as phenolic acids and vitamin C levels in tubers are affected by the ARF level. The latter may result in an advantageous increase in the antioxidant capacity of transgenic tubers.

CONCLUSIONS

The sucrose content increases, and changes in glycoalkaloids content in all analyzed transgenic plants clearly indicate the ARF regulatory function in potato metabolism. The changes in phenolic compound content resulted in the increase of the antioxidant capacity of transgenic tubers. The valuable increase in the linoleic acid level was also detected. Thus, the ARF appears to be a protein, which when manipulated may improve qualities of potato tubers important for the human diet.

Abbreviations: ARF, ADP ribosylation factor; FW, fresh weight; DW, dry weight.

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Received for review July 15, 2002. Revised manuscript received September 30, 2002. Accepted October 2, 2002. This paper was supported by Grant No 5P06A 02319 from the National Research Committee.

JF020779R