JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

14-3-3 Protein Down-regulates Key Enzyme Activities of Nitrate and Carbohydrate Metabolism in Potato Plants

MAGDALENA ZUK,† RYSZARD WEBER,§ AND JAN SZOPA*,†

Institute of Biochemistry and Molecular Biology, University of Wrocław, Przybyszewskiego 63/77, 51-148 Wrocław, Poland, and Institute of Soil Science and Plant Cultivation, Lakowa 2, 55-230 Jelcz-Laskowice, Poland

The 14-3-3 protein is one of the best candidates for coordinating all plant metabolic pathways. To verify this suggestion transgenic potato plants with repression of one (J4 and J5 plants), two (G1 plants), and six (G3 plants) constitutive 14-3-3 protein isoforms as well as plants overexpressing the 14-3-3 protein were studied. Reduction in the 14-3-3 protein level in the J4 and J5 transformants, the G1 transformants, and the G3 transformants was close to 29, 41.5, 38, and 55%, respectively. In the case of the 14-3-3 overexpressing plants (J2), a 30% increase in protein content was detected. Changes in nitrate reductase (NR), sucrose phosphate synthase (SPS), and starch synthase (SS) activities in the transgenic plants perfectly reflect the overall 14-3-3 protein level. The highest increase in enzyme activities was observed for the G3 plants and the lowest for the J4 transformants. The same was detected for the measured metabolites. The highest increase in the protein, starch, and sucrose levels was detected in the tubers from the G3 transgenic plants. Because there was almost no change in the isoform ratio in the transgenic plants when compared to the control, it is suggested that it is the overall content of the 14-3-3 protein, rather than the content of particular isoforms, which plays a crucial role in the regulation of enzyme activities and thus in metabolite synthesis. The properties of the 14-3-3 overexpressing plants are very similar to those of the control ones, suggesting that the protein is in excess in the nontransformants and a further increase in its content is not recognized by cell metabolism. A considerable influence of the 14-3-3 protein level on potato plant metabolism was demonstrated. This effect was observed in key metabolic enzyme activities and metabolite content as well. A high variability between mean values, representing individual transgenes, with respect to nitrate reductase, sucrose phosphate synthase, and starch synthase activities in the examined genotypes was noted. These changes were closely correlated with metabolite levels, among them protein, starch, reducing sugars, and sucrose. The results obtained for the five types of transgenic potato plants in comparison with the control were statistically assessed using discriminate function and cluster analyses.

KEYWORDS: Transgenic plants; 14-3-3 proteins; nitrate reductase; sucrose phosphate synthase; starch synthase

INTRODUCTION

The 14-3-3 proteins constitute a family of highly homologous proteins. They exist as acidic 30 kDa molecules that form homo- and heterodimers and within a cell have been found in cytosol, chloroplasts, nuclei, and nuclear matrix (*1*, *2*). Members of this protein family are considered to be present in all eucaryotic cells (*3*, *4*). The best-evidenced feature of the 14-3-3 proteins is their interaction with other proteins, which finally results in the activation or inactivation of the interacting proteins. Results of in vitro experiments suggest that the members of this protein family affect nitrate fixation by regulating nitrate reductase, carbohydrate metabolism by binding to sucrose phosphate synthase, and regulation of activity of starch synthase (*5*). They might also influence cell communication by direct interaction with plasma membrane H^+-ATPase, the cell cycle by binding to cdc25 phosphatase and exporting it to the nucleus in response to DNA damage (*6*), and antioxidant capacity by affecting phenolic compounds biosynthesis (*7*).

Up to now many other proteins known to be a target for 14-3-3 have been identified, and the list of partner proteins is still growing. Until quite recently it was considered that all interactions of the 14-3-3 proteins with partners were through phosphopeptide motifs in target proteins (*8*), but in recent years the same interactions, however without phosphorylation, have been observed (*9*).

* Author to whom correspondence should be addressed (telephone +48 071 375 62 02; fax +48 071 325 29 30; e-mail szopa@ibmb.uni.wroc.pl).
† University of Wrocław.
§ Institute of Soil Science and Plant Cultivation.

10.1021/jf0485584 CCC: $30.25
Published on Web 03/29/2005

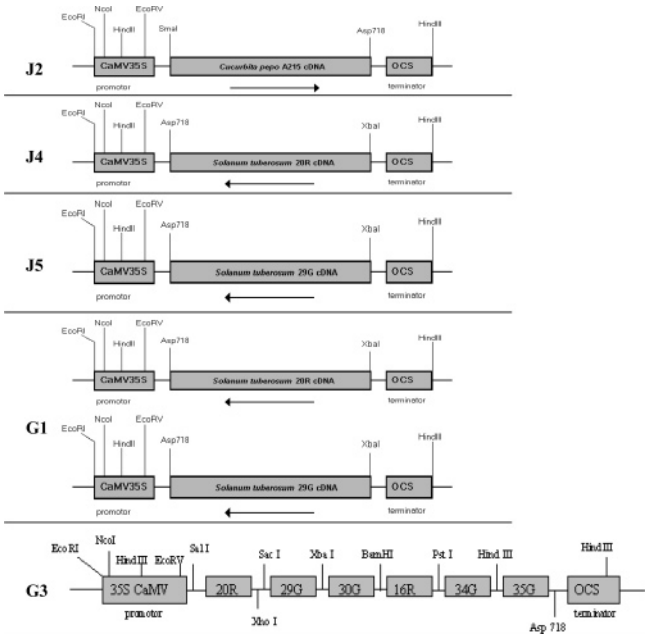

Figure 1. Characteristics of the analyzed genetic types of potatoes with modified 14-3-3 protein synthesis.

By screening of cDNA libraries obtained from leaves, roots, and epidermal fragments, six different cDNAs encoding isoforms of 14-3-3 had been previously found in potato plants. All of them were present in each analyzed tissue. However, the level of expression of a given isoform strongly varied depending on plant age and the tissue investigated (*10*, *11*).

To discover the significance of the 14-3-3 gene for plant metabolism, transgenic potato plants overexpressing this protein and plants with simultaneous repression of one, two, and six 14-3-3 mRNAs were created. Using this model we tried to answer the following questions:

(i) Does binding of ligand proteins affect plant metabolism in vivo?

(ii) Is there any specificity among the 14-3-3 isoforms in the binding of diverse partners?

(iii) What is significant for the affected enzyme activities: the total content of the 14-3-3 protein or a certain individual isoform level?

The data presented in this paper clearly demonstrate the in vivo regulatory function of 14-3-3 on nitrate reductase (NR), sucrose phosphate synthase (SPS), and starch synthase (SS) activities. It is also demonstrated that it is the total level of the 14-3-3 protein rather than a particular isoform content that affects the targeted enzyme activities.

MATERIALS AND METHODS

Plant Material. Potato plants (*Solanum tuberosum* L. cv. Desiree) were obtained from Saatzucht Fritz Lange KG (Bad Schwartau, Germany). Wild-type plants were modified by leaf explants transformation mediated by *Agrobacterium* infection (*11*, *12*), and potato lines of six transgenic types with various rates of 14-3-3 protein isoform synthesis were obtained. Genetic constructs used for generation of transgenic plant types are presented in **Figure 1**. The transgenic plants were preselected by PCR using primers specific to the respective phosphotransferase (Kan) gene and then selected by means of the Northern and Western blot analyses as described previously (*11*, *12*).

Three transgenic lines of each transgenic type were selected for further cultivation. The plants were grown in a greenhouse in soil under a 16 h light (22 °C)/8 h dark (15 °C) regimen. Tubers from the greenhouse were cultivated in the field. The field experiment was performed from April to September 2002 in the vicinity of Wrocław, Poland. After harvesting of mature tubers from 20 plants of each transgenic line, mean samples, representing the size distribution of the whole batch, were collected for analysis. For analyses of the control line Desiree, three mean samples from the whole batch were collected. The tubers were washed, wiped dry, peeled (the thickness of the peel was 1−2 mm), and then cut into 0.5 mm disks. The tissue was homogenized in a chilled mortar.

Western Blot Analysis. The assessment of the expression of the 14-3-3 genes by means of the Western blot analysis using the rabbit IgG antirecombinant 14-3-3 protein was conducted as described previously (*11*).

Briefly, solubilized protein was run on 12% SDS−polyacrylamide gels and blotted electrophoretically onto nitrocellulose membranes (Schleicher and Schuell). Following transfer, the membrane was sequentially incubated with blocking buffer (5% dry milk) and then with antibodies directed against the 14-3-3 recombinant protein (1:3000 dilution). Formation and detection of immune complexes were performed as described previously (*13*). Alkaline phosphatase-conjugated goat anti-rabbit IgG served as a second antibody and was used at a dilution of 1:1500.

SPS Assay. N_2 tissue (0.5 g) frozen in liquid was homogenized in a chilled mortar in 0.5 mL of an extraction buffer containing 30 mM Hepes−NaOH, pH 6.9, 10 mM DTT, 1 mM $MgSO_4$, 0.5 mM EDTA, 0.5% (w/v) BSA, and 0.5% (w/v) PVP at 4 °C (*14*). The homogenate was centrifuged at 16000*g* for 10 min.

The activity of the enzyme was measured according to the anthrone method (*15*). Forty-five microliters of desalted extract was assayed in 50 mM Mops−KOH, pH 7.4, 12 mM $MgCl_2$, 1 mM DTT, 12 mM Fru-6-P, 36 mM Glc-6-P, and 6 mM UDPglc, in a total volume of 70 μL. Anthrone reagent (1 mL of 0.14% w/v anthrone in 85% w/v H_2SO_4) was added to the reaction mixture, which was then incubated at 40 °C for 20 min. Then absorbance was measured at 620 nm and compared to that of a standard curve containing 0−100 μM glucose.

NR Assay. The tissue was harvested, weighed, and immediately frozen in liquid N_2. Two hundred and fifty milligrams of tissue was homogenized in a chilled mortar in 750 μL of an extraction buffer containing 100 mM Hepes−NaOH, pH 7.5, 5 mM $Mg(OAc)_2$, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 4 mM DTT, 0.1% Triton X-100, 1 mM PMSF, 1 mM benzamidine, 1 mM 6-aminocaproic acid, 20 μM FAD, 5 μM Na_2MoO_4, 0.1 μM ocadaic acid, 20 mM NaF, and 2% (w/v) PVP at 4 °C.

The activity of the enzyme was assayed by measuring NO_2^- formation, which was then determined colorimetrically at 540 nm (*16*). The assay mixture contained 250 μM NADH, 5 mM KNO_3, 5 mM EDTA, 100 mM Hepes buffer, pH 7.0, and 35 μL of the enzyme extract, in a final volume of 240 μL.

SS Assay. Starch synthase was assayed by measuring the incorporation of ^{14}C from ADP−glucose into a methanol−KCl insoluble product (*17*). Enzyme extract (10 μL) was incubated in 50 mM Tricine−NaOH, pH 8.5, 25 mM potassium acetate, 2 mM EDTA, 2 mM DTT, 1 mg of potato amylopectin, and 1 mM ADP−[U-^{14}C]glucose (at a specific activity of 0.2 mCi/mmol) in a final volume of 100 μL at 25 °C for 30 min. The reaction was stopped by the addition of 1 mL of methanol−KCl [75% (v/v):1% (w/v)]. The precipitate was collected by centrifugation and washed with methanol−KCl. Three milliliters of a Ready Safe scintillation mix dissolved in 400 μL of 0.1 M NaOH was added, and radioactivity was measured in a liquid scintillation counter.

Determination of Starch and Soluble Sugar Contents. The potato tuber slices and leaf disks were extracted with 80% ethanol−50 mM Hepes−KOH, pH 7.4, at 80 °C. The supernatant was used for enzymatic analysis of glucose, fructose, and sucrose (*14*). For starch measurement, the extracted plant material was homogenized in 0.2 M KOH, and following an incubation at 95 °C it was adjusted to pH 5.5 with 1 M acetic acid. Starch was hydrolyzed with amyloglucosidase, and the released glucose was determined enzymatically.

Determination of Crude Protein Content. The crude protein content in the tuber extracts was determined according to the standard Kjeldahl procedure (*18*) with a K-424/K-314 Kjeldahl apparatus (Büchi, Germany). At least three middle-sized tuber samples obtained from three to five plants, representing each transgenic line, as well as at least three control plants were taken for the analysis. The tubers were

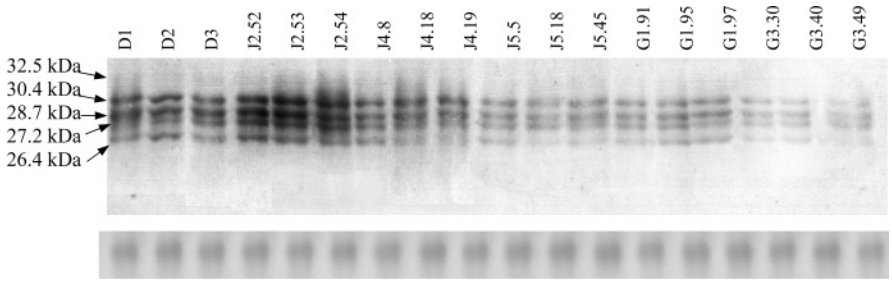

Figure 2. Western analysis of proteins isolated from leaves of control plants (D) and different lines of transgenic potato plants (numbered). Eighty micrograms of protein was applied onto each slot of SDS–polyacrylamide gel electrophoresis, and the blot was probed with antibody antirecombinant 14-3-3 protein. On the left, the molecular mass is marked. Loading control (lower panel), BuBisCo large subunit.

peeled and cut into 1 cm thick slices, then freeze-dried and powdered. At least three measurements were performed for each sample.

RESULTS

Generation of Five Types of Transgenic Plants. In this study five types of transgenic plants were investigated: (i) J2, overexpressing the 14-3-3 protein from *Cucurbita pepo* var. *patissonina* with high homology to the potato 14-3-3 protein (20R isoform); (ii) J4, underexpressing the potato 20R cDNA isoform (*11*); (iii) J5, underexpressing the potato 29G cDNA isoform; (iv) G1, underexpressing both 20R and 29G of the 14-3-3 isoforms; and (v) G3, repressing six (16R, 20R, 29G, 30G, 34G, 35G) 14-3-3 isoforms (*12*).

The leaf explants were transformed using the binary vector (BinAR) containing corresponding cDNAs in sense (J2) or reverse orientation under the control of a 35S CaMV promoter and a Nos terminator (for details see **Figure 1**). The neomycin phosphotransferase gene was used as a selection marker, except for the G1 plants, which were simultaneously transformed with two vectors: one containing a kanamycin and the other a hygromycin resistance gene. The transgenic plants were preselected using PCR and selected by means of Northern and Western blot analyses as described previously (*11*, *12*). The following transgenic lines were used for further investigation: J2.52, J2.53, and J2.54 among the J2 transgenes; J4.8, J4.19, J4.54, J5.5, J5.18, and J5.45 among the J4 and J5 transgenes, respectively; G1.91, G1.95, and G1.97 among the G1 transgenes; and G3.30, G3.40, and G3.49 in the case of the G3 plants.

14-3-3 Protein Isoform Content. We have previously reported that the 14-3-3 gene expression is developmentally regulated (*11*). An analysis of leaves along the plant stem revealed that only the 35G isoform is constitutively expressed along the plant stem; the other two isoforms, 29G and 20R, are synthesized predominantly in the youngest (1−3 plastochrons) leaves. When the tissue was analyzed by immunoblotting, the same expression pattern was detected. Thus, for comparison of the 14-3-3 protein levels in different transgenes, leaves from the same stem sector (7−10 plastochrons) were harvested and analyzed by immunoblotting (**Figure 2**). As previously indicated (*11*), the uppermost protein band (32.5 kDa) corresponds to the 29G isoform (faintly seen in the analyzed plant stem sector); the following band (30.4 kDa) refers to the 34G isoform. The most intense, third band (28.7 kDa) is the sum of two isoforms: 16R and 30G. The lowest two bands (27.2 and 26.4 kDa) correspond to the 20R and 35G potato 14-3-3 isoforms, respectively.

In the case of the plants with overexpression of the 14-3-3 protein, the third band is visually intensified by a protein from *Cucurbita pepo* 14-3-3 cDNA translation (*19*).

It was expected that a very high homology in the amino acid sequence between isoforms would result in an overall decrease

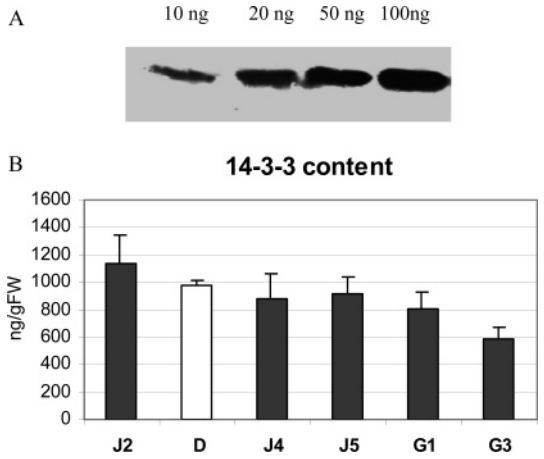

Figure 3. Content of the total 14-3-3 protein (all measured isoforms): (**A**) different quantities of the recombinant 20R isoform of the 14-3-3 protein probed with anti-14-3-3 antibody (bands after densitometry used as standard curve for calculation of 14-3-3 content in plant sample); (**B**) quantity of 14-3-3 expressed in nanograms per gram of fresh weight of leaves.

in the level of all isoforms upon single isoform repression, and this was the case. The content of total and individual 14-3-3 isoforms was determined by densitometry of Western blot analysis.

On the basis of densitometry of Western blot standardized with a recombinant 14-3-3 protein (20R), we calculated the quantity of the total 14-3-3 protein per gram of fresh weight tissue (leaves) (data presented in **Figure 3**). As expected, the highest content of 14-3-3 was detected in the plants with overexpression of the 14-3-3 protein (1254 ng/g of FW). The content of protein in the control plants was 30% lower (975 ng). For the plants with the lowest 14-3-3 level (G3 plants), the content was 589 ng. In the J4 and J5 plants, with repression of a single 14-3-3 isoform, the contents of 14-3-3 per gram of leaves were 878 and 911 ng, respectively, and for the G1 plants it was 800 ng/g of FW.

It was found that the plants overexpressing the 14-3-3 protein (J2) showed a 30% increase in the total level of 14-3-3 when compared to the nontransformants. The opposite changes were expected for the plants with repression of the 14-3-3 protein. Underexpression of the potato 20R cDNA isoform resulted in complete repression of respective mRNA [based on Northern and Western analyses (*11*, *20*)] and additionally in a decrease in the total 14-3-3 protein level to 71% of the control plant level. In the plants with repression of the 29G isoform, except for a specific mRNA level decrease, the total level of the 14-3-3 protein attained 58.5% of the wild-type plants. Underexpression of both 20R and 29G of the 14-3-3 isoforms caused both a reduction in mRNA synthesis and a decrease in the total

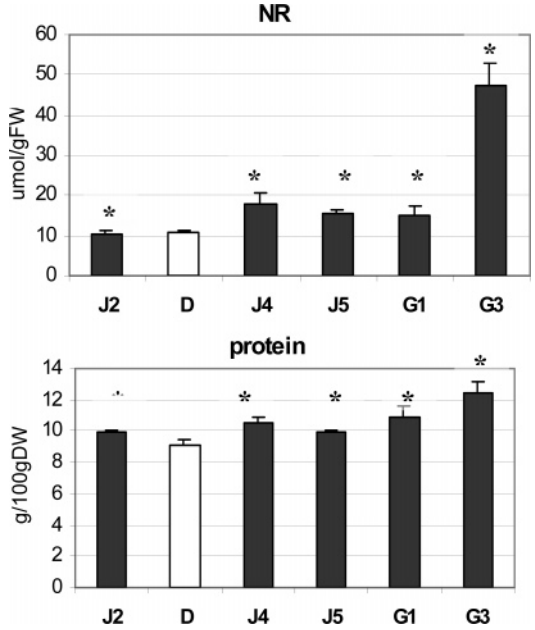

Figure 4. Nitrate reductase (NR) activity and protein content in the tubers of the control (D) and transgenic (J2, J4, J5, G1, and G3) potatoes. *, Significant differences $\alpha = 0.05$.

14-3-3 level by 38%, which is near the level for a single transformant. However, the biggest change in the 14-3-3 quantity was a characteristic feature of the plants with repression of all known 14-3-3 protein isoforms. For them (G3), the measured content of the 14-3-3 protein ranged from 43 to 46% of the control level, depending on the examined transgenic line.

Thus, the expression of different 14-3-3 cDNAs in reverse orientation resulted in a series of transgenes with different isoform inhibition but with a similar overall quantity of 14-3-3, with the exception for the G3 plants, and these were further studied for the effect of protein content on plant metabolism.

NR Activity and Protein Content. Nitrate reductase is a key enzyme in amino acid synthesis and thus potentially also affects protein content in plant cells. The interaction of 14-3-3 with NR is one of the earliest findings in 14-3-3 studies. First, it was found that 14-3-3 is immunoprecipitated with nitrate reductase, and, second, that recombinant 14-3-3 inhibits enzyme activity. To verify this effect in vivo, we analyzed transgenic potato plants with different levels of repression of the 14-3-3 protein (*10*). The obtained transgenic lines showed an NR activity 1.6 (J5 and G1) to 1.9 (J4) times higher than that of the control plants (mean values for each transgene are presented in **Figure 4**). It should be also pointed out that in three types of the transgenic plants (J4, J5, and G1) only a slight difference in NR activation was detected. This may suggest that the lower the total 14-3-3 protein content, the higher NR activity. Thus, we repressed all known potato 14-3-3 proteins and expected an even higher NR activity increase than this obtained for single- and double-isoform repression (*12*).

All transgenic lines of the G3 type showed a significant increase (5-fold on average) in NR activity. We suggest that NR activity is not 14-3-3 isoform dependent and that the lowest total 14-3-3 quantity results in the highest NR activity level. This conclusion is further confirmed by an in vitro experiment in which extract of transgenic plants was supplemented with recombinant protein from either potato or *C. pepo* and resulted in a decrease in NR activity to the control value (*12*). Plants overproducing the 14-3-3 protein do not show significant differences in NR activity in comparison to control plants.

It was expected that modification of NR activity in transgenic plants would result in an increase in the amino acid content and thus in the protein level. For the estimation of the protein content in the transgenic potato tubers from the field trial, the standard Kjeldahl method was applied. The method gives a value called crude protein (total nitrogen $\times$ 6.25). This method is officially endorsed in the European Union and the United States for food labeling. The data showed an increase in the crude protein value for tubers from all of the transgene types in comparison to that for the tubers of the wild-type plants (**Figure 4**). The highest level of total protein (137% of the control plant protein content) was observed in the plants with repression of all the 14-3-3 protein isoforms. For the other types of transgenes, the observed changes in the total protein level were rather slight. This suggests that a strong correlation between NR activity and the 14-3-3 protein level is not the case when the protein level is considered. However, the calculated correlation factor between NR activity and the protein content showed a reasonable value (0.617), which suggests a rather strong relationship between both parameters.

Carbohydrate Metabolism. Carbohydrates are one of the most important primary metabolic compounds in plants. They serve as a source of energy and provide a carbon skeleton for many other compounds synthesized in plant cells. The enzyme that plays a key role in carbohydrate metabolism is sucrose phosphate synthase (SPS). Because 14-3-3 interacts and regulates in vitro SPS (*21*, *22*), changes in enzyme activity, concomitant with sugar content, in plants with a modified 14-3-3 protein level were expected (**Figure 5**).

We observed a significant increase in SPS activity in the 14-3-3 repressed plants. In the transgenic plants with repression of either the 29G or 20R, or both, isoforms, the increase ranged from 1.4- to 1.9- fold of the control level. For the plants with repression of all the 14-3-3 isoforms (the G3 plants), an outstanding, >8-fold increase in SPS V_{sel} activity was detected.

The data reveal that the repression of six 14-3-3 isoforms is more effective in increasing SPS activity than the repression of one or two isoforms. The data also suggest that there is no specificity in controlling the enzyme by a particular 14-3-3 isoform. The latter was confirmed by complementation of the SPS activity in an extract from transgenic plants by adding recombinant 14-3-3 protein isoforms from either *C. pepo* or potato. There was no difference in the complementation reaction among potato 14-3-3 isoforms (*12*).

Unexpectedly, the plants overexpressing the 14-3-3 protein showed only a very slight decrease in SPS V_{max} activity and no changes in SPS V_{sel} activity in comparison to the nontransformants. It is postulated that an increase in 14-3-3 protein content is negligible for enzyme activity.

Recently, a direct interaction of the 14-3-3 protein with starch synthase (SS) in *Arabidopsis thaliana* was detected, which affects starch synthesis in this plant (*5*). The measured activity of SS in the transgenic tubers confirmed this finding. All of the transgenic plants with a decreased content of the 14-3-3 showed an increase in enzyme activity ranging from 20% for the J5 to >60% for the G3 tubers, and the plants overexpressing 14-3-3 indicated an ~20% decrease in the examined activity (**Figure 5**).

A significant alteration in the carbohydrate content of the tubers from the plants with different levels of the 14-3-3 protein was noted. In consequence of the SPS activity enhancement, an increase in sucrose and subsequently in the starch level was expected. All of the transgene types with repression of the 14-3-3 protein showed an increase in sucrose quantity when

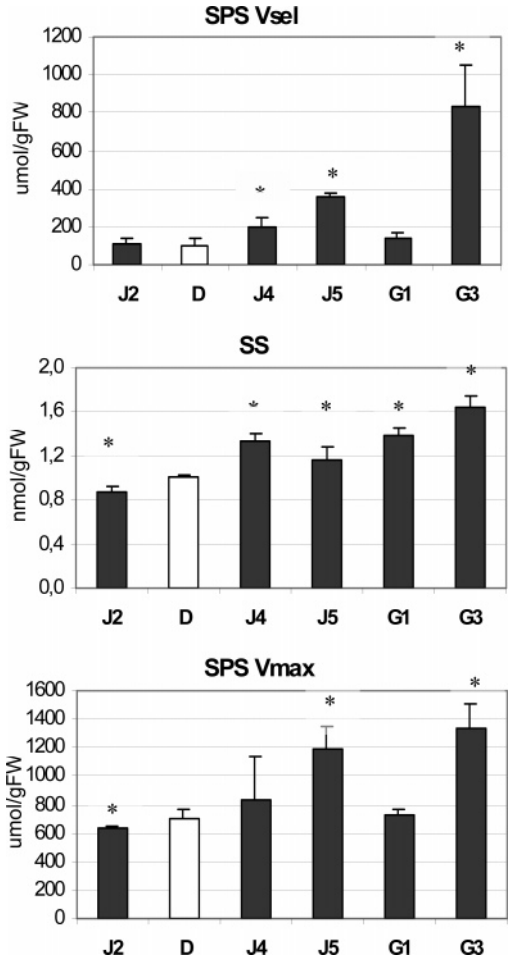

Figure 5. Key carbohydrate metabolism enzyme activities—sucrose phosphate synthase (SPS V_{max} and V_{sel}) and starch synthase (SS) in the transgenic (J2, J4, J5, G1, and G3) and control (D) potatoes. *, Significant differences $\alpha = 0.05$.

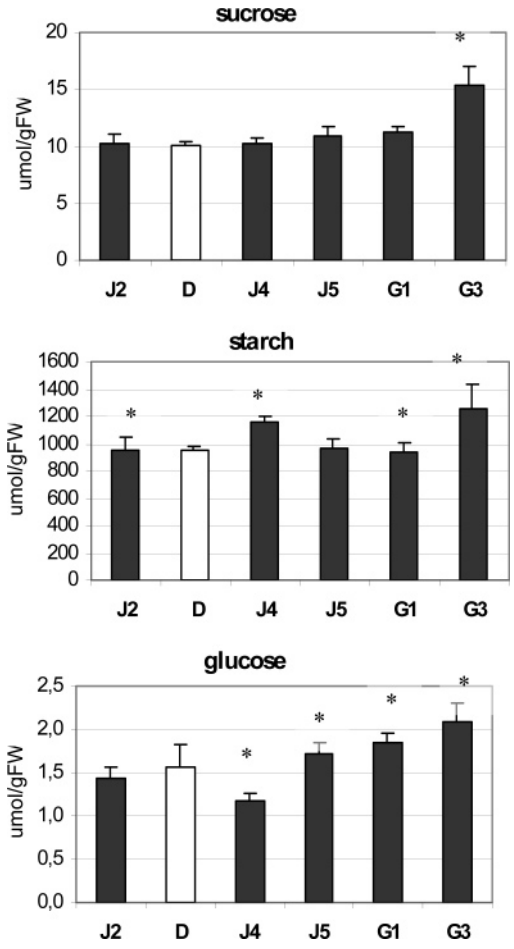

Figure 6. Carbohydrate content in the transgenic (J2, J4, J5, G1, and G3) and control (D) potatoes. *, Significant differences $\alpha = 0.05$.

compared to the control plants (**Figure 6**); however, the increase varied depending on the transgene type. Again, the highest increase was detected for the G3 plants (*12*); in the case of the others only a slight increase was noted. It is interesting to note that the correlation coefficient for SPS activity and sucrose content in the transgenic plants showed a very high (0.7) positive value.

Most of the transgene types showed changes in tuber starch accumulation. However, only those repressing all of the 14-3-3 isoforms indicated a significant increase; in the others the changes in starch content were not so clear. This may suggest that transgenic plants with increased SPS activity produce more sucrose, which is immediately transformed to a polymerized form. It may also be speculated that this effect derives directly from SS activation by a reduction of the 14-3-3 protein content.

The correlation factor for SS activity and the level of starch for the plants with repression of all the 14-3-3 isoforms (G3) was very high (0.67); a reasonably lower value of the coefficient (0.22) was observed for the other transgenes.

The content of glucose in the transgenic plants was also measured. Most of the plants with the level of the 14-3-3 protein lower than that of the control plants showed an increase in glucose content in tubers, and, as in the other cases, the biggest changes were observed in the G3 plants (**Figure 6**); the reason for this, however, is at the moment unknown.

Statistical Analysis of Variability. To describe multiple diversification of the investigated transgenic types with regard to 14-3-3 regulated enzymatic activities (NR, SPS, and SS) and their potential metabolic products (protein, sucrose, and starch), we used the discrimination function analysis and the cluster analysis method of "furthest neighbors" (*23*−*25*).

A multidimensional analysis of variance (MANOVA) showed a significant differentiation of the transgenic types and the control plants.

Table 1. Values of Standardized Coefficients for the Canonical Variables

variable	root 1	root 2	root 3	root 4	root 5
NR	0.898	–0.609	0.386	0.283	–0.396
SPS V_{max}	2.230	–2.202	0.593	–0.513	–0.130
SPS V_{sel}	–2.470	2.891	0.310	0.586	0.444
SS	–0.008	–0.848	–0.166	–0.777	0.198
glucose	0.107	–0.249	–0.455	0.213	0.534
sucrose	0.639	–0.150	–0.087	0.165	–0.078
starch	–0.257	–0.257	–0.425	0.149	–0.155
protein	0.523	–1.212	–0.023	0.816	0.182
eigen value	14.97	3.74	0.95	0.45	0.27
cumulative %	0.73	0.92	0.96	0.98	1.00

Table 1 shows the multiple differentiation between the groups of homogeneous objects in space of canonical variables. We use standardized canonical coefficients (roots 1−5) and correlation coefficients between the mean values of activities or metabolite content for the objects and canonical variables to interpret the meaning of the canonical variables. The high absolute values of canonical coefficients (**Table 1**) and the significant correlation between the examined variables and

Table 2. Factor Matrix Correlation: Variables–Canonical Roots

variable	root 1	root 2	root 3	root 4	root 5
NR	0.539	–0.194	0.265	0.298	–0.234
SPS V_{max}	0.142	0.006	0.655	–0.036	0.566
SPS V_{sel}	0.006	–0.093	0.691	0.131	0.579
SS	–0.117	–0.617	0.225	–0.427	0.479
glucose	0.439	0.158	–0.294	0.007	0.477
sucrose	0.585	0.124	–0.196	–0.120	0.088
starch	–0.157	–0.303	–0.192	0.277	–0.426
protein	–0.492	–0.488	0.245	0.523	0.254

Table 3. Squared Mahalanobis Distances between the Analyzed Genotypes[a]

genotype	Desi	J2	J4	J5	G1	G3
Desi	0	12.88**	35.37**	17.24**	38.70**	106.05**
J2	12.88**	0	17.12**	7.48*	19.97**	122.42**
J4	35.37**	17.12**	0	9.26**	6.93*	103.87**
J5	17.24**	7.48*	9.26**	0	16.98**	99.84**
G1	38.70**	19.97**	6.93*	16.98**	0	105.01**
G3	106.05**	122.42**	103.87**	99.84**	105.01**	0

[a] *, significant differences, $\alpha = 0.05$; **, significant differences, $\alpha = 0.01$.

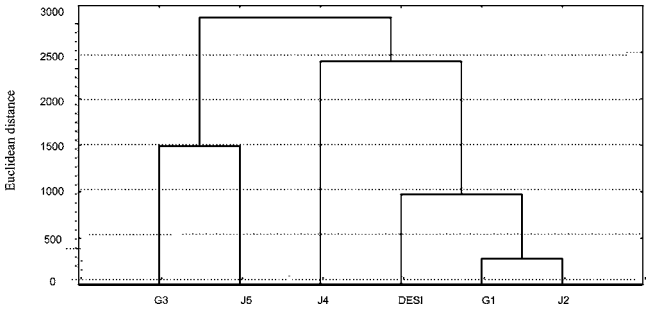

Figure 7. Dendrogram of the analysis of the clusters—similarity of the examined genotypes with respect to the investigated traits.

canonical roots (**Table 2**) indicate a significant participation of single enzyme activity and metabolite content in the discrimination of the tested genotypes.

The variability of the investigated genotypes was also estimated by means of the Mahalanobis distance, which is the distance between two objects expressed as a sum of squared differences of mean values of the investigated traits for the object (**Table 3**). The overall suggestion is that as all of the analyzed genotypes vary from one another in the 14-3-3 protein level, significant differences with respect to the analyzed enzyme activities and metabolite content are indicated. A special position could be ascribed to the transgenes with repression of all the 14-3-3 protein isoforms (the G3 type), which are characterized by a considerably longer distance from the other analyzed transgenes and the control as well.

To assess the multifeature similarity of the investigated objects, we employed a dendrogram tree by means of the furthest neighbors method (*24*). Such an analysis determines the Euclidean distances in space defined by three described enzyme activities and five metabolite contents (**Figure 7**). The nearer the position of each transgene in comparison to the control or other analyzed plants, the greater is similarity between the groups of the investigated genotypes. It is remarkable that in this analysis again the G3 plant type displays a significant distance from the other analyzed plant types. This observation establishes the outstanding characteristic of this transgene when compared to the other transgene types and the control with respect to the analyzed enzyme activities and metabolite content.

DISCUSSION

In potato six different 14-3-3 isoforms were identified. They showed high amino acid sequence homology, ranging from 65 to 95% identity in the coding region. Recently we showed that the regulatory fragments of the 14-3-3 genes differed substantially. It was proposed that each isoform function is replaceable by the others and that their promoters precisely regulate the spatiotemporal 14-3-3 isoform gene expression.

It was noted several times in in vitro experiments that the 14-3-3 protein plays a modulatory function in different kinds of enzyme activities (*6*, *21*, *26*). To analyze the in vivo function of 14-3-3 the sense−antisense technology was used. Five different transgenic types with gradually decreasing 14-3-3 protein levels were obtained. Selected transgenic plants known to interact in vitro with 14-3-3 proteins and metabolites, which are the products of the interacting enzymes, were analyzed for enzyme activities, and the data were statistically treated.

There is a distinct correlation between the 14-3-3 protein level and the measured enzyme activities and the metabolite content exhibited by the genetically modified plants. The potato overexpressing *C. pepo* 14-3-3 (the J2 type) indicates in several cases characteristics opposite those of the 14-3-3 repressing plants; however, in other features it does not differ from the control plants. More distinct changes in enzyme activities and metabolite levels are observed for all of the plants with a reduced 14-3-3 protein level. In several analyzed enzymes a significant increase in NR, SPS, and SS activities was observed. It is interesting to note that the level of all the measured activities strongly depends on the 14-3-3 protein content in the generated transgenic plants, thus suggesting that it is the quantity of the 14-3-3 protein, rather than particular isoforms, that controls interacting enzymes.

Statistical analysis of variability demonstrates a significant differentiation of transgenic genotypes with regard to the analyzed enzyme activities and the content of nutritive components in potato tubers. Analysis of the Mahalanobis distance and the dendrogram tree by means of the furthest neighbors method suggests that the distance between the transgenic and the nontransformant plants clearly correlates with the 14-3-3 level. The longest distance was found for the G3 transgene, which showed the lowest 14-3-3 level. The results of canonical analysis demonstrate that, of the analyzed plant properties, the best parameters for transgene differentiation are NR and SS activities. Of the metabolites, the sucrose content reasonably differentiates transgene types. These results strongly suggest a coordinating role of 14-3-3 protein in primary metabolite synthesis.

LITERATURE CITED

(1) Bihn, E. A.; Paul, A.-L.; Wang, S. W.; Erdos, G. W.; Ferl, R. J. Localisation of 14-3-3 proteins in the nuclei of arabidopsis and maize. *Plant J.* **1997**, *12*, 1439−1445.

(2) Sehnke, P. C.; Henry, R.; Cline, K.; Ferl, R. J. Interaction of plant 14-3-3 protein with the signal peptide of a thylakoid-targeted chloroplast precursor protein and the presence of 14-3-3 isoform in chloroplast stroma. *Plant Physiol.* **2000**, *122*, 235−241.

(3) Aitken, A.; Collinge, D. B.; van Hausden, G. P. H.; Isobe, T.; Roseboom, P. H.; Rosenfeld, G.; Soll, J. 14-3-3 proteins: a highly conserved, widespread family of eucariotic proteins. *Trends Biochem. Sci.* **1992**, *17*, 498−501.

(4) Ferl, R. J. 14-3-3 proteins and signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1996**, *47*, 49−73.

(5) Sehnke, P. C.; Chung, H.-J.; Wu, K.; Ferl, R. J. Regulation of starch accumulation by granule-associated plant 14-3-3 proteins. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 765−770.
(6) Finnie, C.; Borch, J.; Collinge, D. B. 14-3-3 proteins: eucariotic regulatory proteins with many functions. *Plant Mol. Biol.* **1999**, *40*, 545−554.
(7) Łukaszewicz, M.; Matysiak-Kata, I.; Aksamit, A.; Oszmiański, J.; Szopa, J. 14-3-3 protein regulation of antioxidant capacity of transgenic potato tubers. *Plant Sci.* **2002**, *163*, 125−130.
(8) Moorhead, G.; Douglas, P.; Cottelle, V.; Harthill, J.; Morrice, N.; Meek, S.; Deiting, U.; Stitt, M.; Scarabel, M.; Aitken, A.; MacKintosh, C. Phosphorylation-dependent interactions between enzymes of plant metabolism and 14-3-3 proteins. *Plant J.* **1999**, *18*, 1−12.
(9) Aitken, A.; Baxter, H.; Dubois, T.; Clokie, S.; Mackie, K.; Mitchell, K.; Peden, A.; Zemlickova, E. Specificity of 14-3-3 isoform dimer interactions and phosphorylation. *Biochem. Soc. Trans.* **2002**, *30*. 351−360.
(10) Szopa, J.; Wróbel, M.; Matysiak-Kata, I.; Świędrych, A. The metabolic profile of the 14-3-3 repressed transgenic potato tubers. *Plant Sci.* **2001**, *161*, 1075−1082.
(11) Wilczyński, G.; Kulma, A.; Szopa, J. The expression of 14-3-3 isoforms in potato is developmentaly regulated. *J. Plant Physiol.* **1997**, *153*, 118−126.
(12) Żuk, M.; Skała, J.; Szopa, J. Repression of six 14-3-3 protein isoforms resulting in activation of nitrate and carbon fixation key enzymes from transgenic potato plants. *Plant Sci.* **2003**, *165*, 731−741.
(13) Szopa, J.; Rose, K. M. Cleavage of the 190 kDa subunit of DNA-dependent RNA polymerase I yields small polypeptides capable of degrading DNA. *J. Biol. Chem.* **1986**, *261*, 9022−9028.
(14) Stitt, M.; Lilley, R. M. C.; Gerhart, R.; Heldt, H. W. Metabolite levels in specific cells and subcellular compartments of plant leves. *Methods Enzymol.* **1994**, *174*, 518−552.
(15) Huber, J. L. A.; Huber, S, C. Protein phosphorylation as a mechanism for regulation of spinach leaf sucrose-phosphatase activity. *Arch. Biochem. Biophys.* **1989**, *270*, 681−690.
(16) Nejidat, A.; Zhang, G.; Grinberg, M.; Heimer, Y. M. Increased protein content in transgenic *Arabidopsis thaliana* over-expressing nitrate reductase activity. *Plant Sci.* **1997**, *130*, 41−49.
(17) Abel, G. J.; Springer, F.; Willmitzer, L.; Kossmann, J. Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (*Solanum tuberosum* L.). *Plant J.* **1996**, *10*, 981−91.
(18) *Official Methods of Analysis of the AOAC*, 15th ed.; Association Official Analytical Chemicts: Arlington, VA, 1990.
(19) Markiewicz, E.; Wilczyński, G.; Rzepecki, R.; Kulma, A.; Szopa, J. The 14-3-3 binds to nuclear matrix endonuclease and has a possible function in the control of plant senescence. *Cell. Mol. Biol. Lett.* **1996**, *1*, 319−417.
(20) Szopa, J. Transgenic 14-3-3 isoforms in plants: the metabolite profiling of repressed 14-3-3 protein synthesis in transgenic potato plants. *Biochem. Soc. Trans.* **2002**, *30*, 405−410.
(21) Chung, H.-J.; Sehenke, P. C.; Ferl, R. J. The 14-3-3 proteins: cellular regulators of plant metabolism. *Trends Plant Sci.* **1999**, *4*, 367−371.
(22) Toroser, D.; Athwal, S. G.; Huber, S. C. Site specific regulatory interaction between spinach leaf sucrose-phosphate synthase modulation. *Plant J.* **1998**, *17*, 407−413.
(23) Caliński, T.; Dyczkowski, A.; Sitek, M. Procedury testów jednoczesnych w wielozmiennej analizie wariancji. *Mat. Stosowana* **1979**, *14*, 5−31.
(24) Mądry, W. Studia statystyczne nad wielowymiarową oceną zróżnicowania cech ilościowych w kolekcjach zasobów genowych zbóż, Warszawa, 1993.
(25) Morrison, D. F. Wielocechowa analiza statystyczna, Warszawa, 1990.
(26) Sehnke, P. C.; Ferl, R. J. Plant metabolism—enzyme regulation by 14-3-3 proteins. *Curr. Biol.* **1996**, *6*, 1403−1405.

Received for review August 31, 2004. Revised manuscript received February 14, 2005. Accepted February 24, 2005. This work was financially supported by Grant OPTI 2 from the EU and Grant PBZ-KBN-089/PO6/2003 from the Polish State Committee for Scientific Research.

JF0485584