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Repression of the 14-3-3 Gene Affects the Amino Acid and Mineral Composition of Potato Tubers

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Recently, transgenic potato plants were created showing underexpression of the 20R isoform of the 14-3-3 protein. The transgenic plants grown in tissue culture showed a significant increase in nitrate reductase activity and a decrease in nitrate level. The transgenic line with the lowest 14-3-3 quantity was field-trialed (1997−2000) and analyzed. The reduction in the 14-3-3 protein level consistently resulted in a starch content increase and in an increase in the ratio of soluble sugars to starch in the tubers, although the latter was only barely visible. The determination of amino acid composition in the tubers showed a significant increase in methionine, proline, and arginine content and a slight but consistent increase in hydrophobic amino acid and lysine content in the cells of the transgenic potato plants. We also observed an increase in the crude protein content, from 19 to 22.1% of the control value in consecutive years. It is proposed that all of these changes might have resulted from the downregulation of nitrate reductase and sucrose phosphate synthase activities by 14-3-3, although other potential mechanisms cannot be excluded (e.g., an increase in enzyme protein level). 14-3-3-repressed transgenic plants showed a significant increase in calcium content in their tubers. It is thus proposed that a function of the isolated 14-3-3 isoform is in the control of amino acid synthesis and calcium metabolism. However, the mechanism of this control is as yet unknown.

KEYWORDS: 14-3-3 protein; transgenic plant; amino acids; minerals; *Solanum tuberosum*

INTRODUCTION

One of the aspects of modern biotechnology research is the creation of new plant species by means of genetic transformation. The expectation is to obtain plants with modified metabolic pathways and less sensitivity to biotic and abiotic stresses. The genetic engineering of plants also appears to be a good method for gene function studies. Most of the modifications in the plant genome accomplished to date lead to changes in the activity of enzymes involved in specific metabolic pathways. In recent years, manipulating the expression of the regulatory genes and thus coordinating whole metabolic pathways has come under extensive scrutiny. The results of in vitro and in vivo studies on the 14-3-3 protein family strongly suggest that they fulfill the role of metabolism coordinators in plants (*1*−*3*). These proteins are widely distributed in nature. As well as being found in plants, they are present in mammals, insects, and fungi (*2*, *4*). In plant tissues, the 14-3-3 protein has regulatory functions in the process of nitrogen fixation and carbohydrate metabolism via direct interaction with nitrate reductase (NR) and sucrose phosphate synthase (SPS), respectively (*5*, *6*). According to the proposed model, 14-3-3 interacts with the phosphorylated forms of NR and SPS enzymes, thus downregulating their activity (for review, see *7*) . Moreover, cultivation experiments conducted on model *Arabidopsis thaliana* plants and on tobacco plants with modified levels of 14-3-3 protein synthesis showed its involvement in the adaptation of the plant to environmental stresses such as cold and salinity (*8*, *9*).

Recently, six isoforms of the 14-3-3 protein were identified and isolated from potato plants—*Solanum tuberosum*, cv. Desiree. The homology between these sequences ranged from 74 to 87%. Western blot analysis using the antibodies directed against the 14-3-3 recombinant protein revealed five 14-3-3 isoforms in potato leaves with molecular masses from 26.4 to 32.5 kD. The pattern of the isoform expression was dependent upon leaf maturity (*3*).

Potato plants were genetically transformed using an antisense transformation, and transgenic plants underexpressing of the 20R 14-3-3 isoform were created. Studies on the cultivated lines of transgenic plants showed that 14-3-3 protein repression leads to an acceleration of the vegetative cycle and an increase in tuber size and the fresh weight of tubers per plant. We also observed a decrease in the catecholamine and soluble sugar contents in the leaves of the transgenic plants. It was proposed that 14-3-3 affects carbohydrate metabolism in potato plant cells

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via regulation of catecholamine synthesis, presumably by affecting tyrosine hydroxylase activity (*10*, *11*). However, to date, there is no convincing data on the specificity of 14-3-3 protein action on plant metabolism.

The inhibitory function of 14-3-3 on NR activity may suggest that this protein plays a role in amino acid synthesis. Thus, the objective of this study is to measure amino acid content in potato tubers from field experiment plants with a decreased level of the 14-3-3 protein. Because it was shown that the interaction of 14-3-3 and NR is strongly magnesium ion-dependent (*12*), we also measured the metal ion content in 14-3-3 antisense plants. The presented results are part of a research project that includes the evaluation of the nutritional value of transgenic potato plant tubers with various levels of 14-3-3 protein synthesis and their usefulness in food processing.

MATERIALS AND METHODS

Plant Material. Potato plants (*S. tuberosum* L. cv. Desiree) were obtained from "Saatzucht Fritz Lange KG" (Bad Schwartau, FRG). In the tissue culture, the plants were grown under a 16 h light/8 h dark regime on an MS medium containing 0.8% sucrose. In the greenhouse, the plants were cultivated in soil under a 16 h light (22 °C)/8 h dark (15 °C) regime. The plants were grown in individual pots and were watered daily. Tubers were harvested 3 months after the transfer of the tissue culture plants to the greenhouse. Field trials were performed in the vicinity of Wrocław, Poland. Tubers were harvested after 20 weeks of growth, between April and September 1997, 1998, 1999, and 2000.

Construction of Transgenic Plants. In this study, a selected single transgenic line, J4.18, underexpressing the potato 14-3-3 isoform cDNA 20R (EMBL/GenBank database account no. X87370), was used. For leaf explant transformation, a binary vector containing cDNA 20R in reverse orientation under the control of a 35S promoter and Nos terminator was used (*13*). The selection marker was neomycin phosphotransferase. The transgenic plants were preselected by the polymerase chain reaction (PCR) with primers specific for the selecting enzyme (neomycin phosphotransferase) and then selected by means of northern and western blot analysis as described previously (*14*, *15*). The 20R 14-3-3 isoform was almost completely repressed—the corresponding band was only faintly visible on the western blot.

Enzyme Extraction from Tissue. For each of six plants of each type (transgenic and wild type), three tissue samples (mainly from tubers, from leaves where indicated) were harvested, weighed, and immediately frozen in liquid N_2. A 0.5 g amount of tissue was homogenized in a chilled mortar in 0.25 g of PVP and 2 mL of an appropriate extraction buffer (30 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES)−NaOH, pH 6.9, 10 mM dithiothreitol, 1 mM $MgSO_4$, 0.5 mM ethylenediaminetetraacetic acid, 0.5% (w/v) bovine serum albumin, and 0.5% (w/v) PVP) at 4 °C. The homogenate was centrifuged at 16 000*g* for 15 min. The supernatant was desalted using Sephadex G-25 columns. The final volume of desalted extract was 1 mL.

Determination of Enzyme Activities. Extracts were kept at 4 °C until assayed. The enzyme assays were carried out at 25 °C. All of the assays, except for SPS, were carried out in a reaction volume of 250 μL using an AnthosIII plate reader (Anthos Laboratories). The changes in absorption were monitored over time at A_{340}, other than for SPS, nitrite reductase (NiR), and NR, the activities of which were measured in a stop assay.

NR, NiR, and Glutamine Synthase (GS). The activities of these enzymes were measured in leaves using the methods described in (*16*−*19*).

SPS. The activity of this enzyme (V_{max} and V_{sel}) was measured by the anthrone method described by Huber and Huber (*19*).

The data obtained were expressed per gram of fresh weight of tissue.

Determination of Starch and Soluble Sugar Content. For each of six plants of each type (transgenic and wild type), three tissue samples (tuber slices and leaf disks) were collected. The tissue samples 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 (*20*). For starch measurement, the 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 level was determined enzymatically.

Determination of Nitrate Content. For each of six plants of each type (transgenic and wild type), three tissue samples were dried at 80 °C for 96 h and then NO_3^- was extracted with doubly distilled water (1:100, w/v) for 60 min. The extracts were filtered through Millex-HV_{13} (0.45 μm) filters, and the NO_3^- content was determined on high-perfomance liquid chromatography (Waters) with the use of an IC-Pak Anion HR column filled with polymetacryl resin containing quaternary amine groups.

Determination of Crude Protein Content in Potato Tubers from the Field Trial. The crude protein content in tuber extracts was determined by the standard Kjeldahl procedure (*21*) with a Kjeldahl apparatus type K-424/K-314 (Büchi, Germany). Each season, the yield of tubers from 75 plants (both transgenic and wild type) was collected, and at least three medium-sized tubers (3 kg weight each) were taken as samples for analyses. The tubers were peeled and cut into 1 cm thick slices, then freeze-dried, and powdered. At least three measurements were performed for each sample.

Determination of Amino Acid Contents in Potato Tubers from Field Trial. The amino acid content was determined with the use of a T 339 amino acid analyzer (Mikrotechna, Czech Republic). At least three medium-sized tubers (3 kg weight each) were taken as samples for analyses. Freeze-dried and powdered tuber samples were acid-hydrolyzed, other than for the tryptophan analysis, where alkaline hydrolysis was conducted (*21*). The sulfur amino acid contents were determined after performic acid oxidation of the sample followed by acid hydrolysis (*21*). Standard amino acid solutions (Sigma-Aldrich, St. Louis) were used to calibrate the analyzer.

Determination of Mineral Contents in Potato Tubers from the Field Trial. The Mg, Fe, Zn, and Cu ion contents in tubers were determined by means of atomic absorption spectroscopy (*17*). The calcium content was measured by means of atomic emission spectroscopy using an AAS 1N apparatus (Carl Zeiss Jena, Germany). Each season, the yield of tubers from 75 plants (both transgenic and wild type) was collected, and at least three medium-sized tubers (3 kg weight each) were taken as samples for analyses. The tuber samples were peeled and homogenized and then dry-mineralized at 450 °C. The ash was dissolved in 0.64 M HNO_3 for analysis. Spectrally pure reagents and standards from Merck (Germany) were used. The phosphate content in the potato tuber samples, dry-mineralized at 500 °C, was determined using the spectrophotometric molybdovanadate method (*17*).

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

RESULTS AND DISCUSSION

Transgenic Potato with 14-3-3 Gene Repression. Transgenic plants with the repressed 20R isoform were recently created. The gene construction was prepared in the same way as described previously and contains 20R cDNA under the control of the 35S promoter and Nos terminator in the pBinAR vector. The vector carried the kanamycin resistance gene. The leaf explants were transformed with *Agrobacterium tumefaciens* as described previously. Because of the high homology between the isoforms and the very similar size of their mRNA, the transgenic plants were preselected by PCR and then selected by means of western analysis as recently described (*14*). Of the four transgenic lines analyzed with the lowest 14-3-3 quantity (J4.11, J4.18, J4.19, and J4.54), 4 years of field trial data were obtained for J4.18 and these are presented. The data from 1−2 years of field trials on other transgenic potato lines (J4.11, J4.19, and J4.54) were very similar to those obtained for J4.18 (not shown).

Phenotype of the Transgenic Plants. 14-3-3 antisense plants maintained in tissue culture were visually indistinguishable from

Table 1. Effect of 14-3-3 Manipulation on Tuber Development[a]

	tuber fresh weight (g) per plant				tuber number per plant				mean fresh weight (g) per tuber			
	year of experiment											
plant	1997	1998	1999	2000	1997	1998	1999	2000	1997	1998	1999	2000
D	539 ± 30	646 ± 35	420 ± 40	532 ± 35	10.1 ± 0.4	8.3 ± 0.2	7.0 ± 0.3	7.9 ± 0.4	53.2	78.0	60.0	67.0
J4	567 ± 25	699 ± 30	298 ± 25	541 ± 30	11.3 ± 0.3	7.7 ± 0.3	5.2 ± 0.2	8.0 ± 0.3	50.2	91.0	57.5	69.2

[a] Plants of a transgenic line with repression of the 20R (J4) 14-3-3 protein isoform were analyzed and compared to the control (D, Desiree). Seventy-five control and transgene plants were grown.

Table 2. Carbohydrate Content in Percent of Dry Weight (DW) for Control Tubers (D, Desiree) and Tubers of Transgenic Potato Plants with Repression of the 20R (J4) 14-3-3 Protein Isoform[a]

	starch %DW			sucrose %DW			hexose %DW			soluble sugar/ starch × 10^{-2}		
	year of experiment											
	1998	1999	2000	1998	1999	2000	1998	1999	2000	1998	1999	2000
D	66.0 ± 6.4	68.2 ± 5.0	75.8 ± 4.5	2.5 ± 0.1	0.6 ± 0.08	1.6 ± 0.10	1.9 ± 0.1	0.6 ± 0.06	0.6 ± 0.05	6.6	1.8	2.9
J4	69.1 ± 7.0	72.7 ± 4.5	77.0 ± 6.4	2.2 ± 0.2	0.5 ± 0.04	1.3 ± 0.08	1.4 ± 0.2	0.8 ± 0.04	0.6 ± 0.02	5.2	1.7	2.4

[a] Seventy-five control and transgene plants were grown.

Table 3. Effect of 14-3-3 Manipulation on Nitrate Content and the Activity of Enzymes Involved in Nitrogen Fixation[a]

	nitrate (mg/g FW)	NR (μmol/ h/g FW)	NiR (μmol/ h/g FW)	GS (nmol/ h/g FW)
D	7.6 ± 0.8	9.7 ± 1.1	59.3 ± 4.3	1.3 ± 0.2
J4	2.5 ± 0.3	13.8 ± 2.1	53.1 ± 5.8	1.2 ± 0.1

[a] Transgene leaves (the first six from the top of the plant) with repression of the 20R (J4) protein isoform were analyzed and compared to the control (D).

nontransformed control plants. When grown in the greenhouse, there was also no dramatic change in the phenotype of the aerial parts of the transgenic plants. However, a significant difference in the phenotype of the tubers formed was observed. The data from the field experiments are presented in **Table 1**. Over 4 years of experiments (1997−2000), significant but not consistent differences in several measured parameters were found.

14-3-3 Protein and Carbohydrate Synthesis. Because 14-3-3 interacts and regulates in vitro SPS, changes in enzyme activity concomitant with sugar content were expected. A reduction in the 14-3-3 protein level consistently resulted in a very significant increase in enzyme activity for J4 tubers (V_{max}: 1554.9 ± 123.2 μmol/g FW; V_{sel}: 681.5 ± 70.0 μmol/g FW) as compared to that for the wild type tubers (V_{max}: 650.3 ± 59.8 μmol/g FW; V_{sel}: 129.6 ± 43.1). A concomitant increase in starch content and a decrease in the soluble sugars/starch ratio, both slight and not statistically significant, were detected in the transgenic tubers (**Table 2**).

14-3-3 Affects NR Activity. It has been reported several times that 14-3-3 proteins might be involved in nitrogen fixation by modulating NR activity. This suggestion comes from two lines of evidence. First, 14-3-3 is immunoprecipitated with NR, and second, recombinant 14-3-3 inhibits enzyme activity. To date, there is no evidence that the same may occur in vivo. Very recently, we analyzed our transgenic plants grown in tissue culture for enzyme activity and nitrate contents. All of the plants showed a significant increase in NR activity in their leaves suggesting that the regulation of NR occurs in vivo (**Table 3**). The increase in NR activity resulted in a significant decrease in the nitrate level. Two other enzymes involved in nitrogen fixation, NR and GS, were also analyzed, but they were only very slightly changed in the transgenic plants.

As a consequence of the NR increase and decrease in nitrate content in the aerial part of the transgenic plants, an increase in amino acid and protein level was expected. Thus, we analyzed the amino acid and protein content in tubers from the J4 plants.

Protein Content and Amino Acid Composition in the Potato Tubers from the Field Trial. Potatoes serve as a major, inexpensive food source for energy, vitamin C, and good quality protein. Mature potato tubers contain about 2% of protein per g/100 fresh weight, and potato protein, along with rapeseed, soybean, and bean proteins, is considered to be the best amino acid-balanced protein among plant proteins (*22*). Potato protein quality is possibly higher than indicated by amino acid composition, as suggested by the results of human feeding trials (*23*). For the estimation of the protein content in the transgenic potato tubers from field trial, the standard Kjeldahl method was applied. This method gives a value called crude protein (total nitrogen × 6.25). This method is officially endorsed in the E.U. and U.S.A. for food labeling. The results obtained from the 3 year experiment are presented in **Table 4**. The data show a significant increase in the crude protein value for tubers from the transgenic line J4.18 in comparison to that for the tubers of the wild type plants. The increase ranged from about 19% in the two first years to 22.1% in the third year. It is argued that the crude protein parameter obtained by multiplying the total nitrogen content by a general factor 6.25 does not deviate much from the protein content in potato tubers (*24*). It is proposed that the sum of the amino acids involved in protein synthesis (net protein, NP) is a more suitable parameter for defining the nutritional value of food. The NP contents in the examined potato tubers from the field trial (1999 and 2000) were only 8.8 and 5.6% lower (respectively) than the crude protein value in the control tubers and 13.9 and 24.2% lower (respectively) in the tubers of transgenic plants. The significant differences between the crude parameters and the NP parameters in the transgenic tubers in comparison with the control tubers suggest the presence of larger amounts of nonprotein amino acids and other nitrogen compounds in the tubers of the transgenically modified plants.

The sum of the essential amino acids in the transgenic potato plant samples from the 1999 experiment was almost the same as in the control plant and 10% higher in the consecutive year

Table 4. Crude Protein Contents in Percent of DW in Potato Tubers of Wild Type (D) and Transgenic (J4) Potato Plants from Field Trials Performed in 1998–2000

	year of experiment					
	1998		1999		2000	
plant	DW (%)	crude protein (%DW)	DW (%)	crude protein (%DW)	DW (%)	crude protein (%DW)
D	17.7 ± 0.9	9.52 ± 0.26	19.6 ± 1.4	12.13 ± 1.31	20.7 ± 0.9	8.55 ± 0.71
J4	18.1 ± 0.25	11.36 ± 0.44	19.4 ± 0.6	14.46 ± 0.65	21.5 ± 0.9	10.44 ± 0.61

Table 5. Amino Acid Contents in g/kg DW in Wild Type (D) and Transgenic (J4) Potato Tubers from Field Trials

	year of experiment			
	1999		2000	
plant	D	J4	D	J4
Essential amino acids				
Thr	3.65	3.70	2.09	2.25
Cys	1.64	1.60	1.00	1.10
Met	1.75	2.54	1.21	1.79
Ile	3.73	4.68	1.97	2.13
Leu	5.19	6.08	2.54	2.96
Tyr	3.96	4.70	1.26	1.78
Phe	13.89	9.41	6.90	7.12
Lys	5.66	6.32	4.88	5.71
Val	5.24	5.99	3.83	3.60
Trp	1.20	1.42	0.90	0.96
sum content of essential amino acids	45.91	46.44	26.58	29.40
Endogenous amino acids				
Asp	29.75	34.04	21.24	20.74
Ser	3.36	4.27	2.10	2.08
Glu	13.81	18.49	15.60	13.16
Pro	4.47	6.35	7.83	9.86
Gly	2.61	2.70	1.76	1.67
Ala	2.95	2.82	2.00	1.58
His	3.37	3.52	1.32	1.23
Arg	4.40	5.83	2.57	3.61
sum content of endogenous amino acids	64.72	78.02	50.53	49.09
total amino acid content (NP)	110.63	124.46	81.00	83.33

(**Table 5**). The comparison of the absolute content of the essential amino acids in the control and transgenic potato tubers showed that in both years of the experiment, the content of methionine (45 and 48%, respectively), tyrosine (19 and 41%, respectively), and lysine (12 and 17%, respectively) was higher than in the control plants. The content of tryptophan and hydrophobic amino acids was also slightly higher in the transgenic tubers than in the control, except in the case of valine in the last year's harvest—a slight decrease was detected.

In the transgenic tubers, among the endogenous amino acids, proline and arginine show the highest increase in content as compared to the control. The proline content is increased by 42 and 26% in the J4 plants harvested in 1999 and 2000, respectively. A significant increase in arginine content (33 and 40%, respectively) in the transgenic potato plants was also detected. The other amino acid contents only show very slight increases or decreases in the transgenic plants when compared to the wild type potato tubers.

Contents of Minerals in Potato Tubers from the Field Trial. The control and transgenic potato tubers from the field trial were examined for their content of minerals of great nutritional importance—among them calcium, magnesium, iron, zinc, and copper ions. The phosphorus content was also determined. Besides potassium, phosphorus is the main element present in the potato tubers. The phosphorus content in the control tubers was at the expected level (*21*, *25*) and was not significantly different in the tubers from transgenic plants. The transgenic tubers harvested in 1999 showed a 2-fold lower quantity of phosphorus than those from 1998. It is known that the phosphorus level in potatoes varies greatly in response to environmental conditions (*26*).

Table 6. Phosphorus, Magnesium, Calcium, Iron, Zinc, and Copper Content in mg% of DW in Wild Type (D) and Transgenic (J4) Potato Tubers from Field Trials

	year of experiment			
	1998		1999	
plant	D	J4	D	J4
phosphorus	343.7 ± 16.3	362.1 ± 21.2	220.7 ± 41.7	178.5 ± 33.5
magnesium	81.4 ± 3.96	83.6 ± 6.12	75.9 ± 7.00	84.0 ± 4.54
calcium	14.0 ± 1.05	24.1 ± 3.01	34.8 ± 1.35	60.8 ± 5.39
iron	2.38 ± 0.23	2.23 ± 0.78	2.53 ± 0.38	2.46 ± 0.27
zinc	2.12 ± 0.11	1.84 ± 0.64	2.30 ± 0.10	2.65 ± 0.03
copper	0.79 ± 0.08	1.39 ± 0.10	0.45 ± 0.08	0.41 ± 0.06

The data presented in **Table 6** show a consistent and significant increase in the calcium content in the transgenic potato tubers. An only slight increase in magnesium content was detected. The content of the other measured metal ions in the tubers was rather unaffected by 14-3-3 protein repression.

CONCLUSION

While substantial progress has been made in the identification of the diverse partners of 14-3-3 in recent years, at least one important question needs to be answered: does this binding affect plant metabolism or physiology in vivo? Because the in vitro findings show nitrogen reductase and SPS activity reduction by 14-3-3, an important question for biotechnology is whether 14-3-3 gene regulation is useful for protein metabolism manipulation. To answer this question, a transgenic potato plant with repressed 14-3-3 protein was created and investigated. We analyzed tubers from plants grown in the field. The protein content, amino acid quantity, and metal ion level were measured. The phenotype of transgenic potato plants grown in the field was also analyzed. In the 4 years of experiments, significant but not consistent changes in several measured parameters were found. The reduction in the 14-3-3 protein level consistently resulted in a starch content increase and in an increase of the ratio of soluble sugars to starch, although the latter was only slight and not statistically significant. Repression of 14-3-3 induced a significant increase in the crude protein value and also in the methionine, proline, and arginine contents in the field trial tubers. A slight but consistent increase in hydrophobic amino acid and lysine content was detected in the transgenic tubers. It should be pointed out that 14-3-3-repressed transgenic plants showed a significant increase in calcium content. It is thus proposed that a function of the isolated 14-3-3 isoform is in the control of amino acid synthesis and calcium metabolism; however, the mechanism of this control is as yet unknown.

Recently analyzed transgenic potato plants with overexpression of the 14-3-3 protein from *Cucurbita pepo* showed no change in protein content and instead a significant change in lipid content (*27*). We present here tubers from 14-3-3-repressed plants showing a significant increase in protein content and no change in lipid level (not shown). This may suggest that 14-3-3 isoforms show specificity in their action on plant metabolism.

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