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POTATO GENETICALLY MODIFIED BY 14-3-3 PROTEIN REPRESSION IN GROWING RAT DIETS. PART II: HEALTH STATUS OF EXPERIMENTAL ANIMALS

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For 4 weeks, male outbred IF_2Jaz rats, divided into 4 groups (n=10), were administered *ad libitum* diets with 30% addition of dried tubers of transgenic potatoes with repression of isoform a, c as well as a and c of 14-3-3 protein (experimental groups) or with the addition of non-transgenic potatoes of Desiree cultivar (control group). The administration of transgenic potatoes did not affect the growth rate of model animals nor most of the analysed parameters of their health status. Neither anti-nutritional, nor immunostimulatory or toxic effects of the experimental diets were demonstrated. Yet, in liver tissue of rats fed a diet with transgenic potatoes J4 and G1 the concentration of 8-oxo-2'deoxyguanosine – a biomarker of oxidative damage to DNA – was higher than in animals administered a diet with non-transgenic potatoes. Results obtained in the study indicate a threat to the health status of animals fed diets with a high content of genetically-modified potatoes with repression of 14-3-3 protein.

INTRODUCTION

Potatoes are cultivated in *ca*. 125 countries and constitute a basis of an everyday diet to over a billion of people. They are a rich source of starch, but also of vitamin C and potassium. The improvement of agrotechnical characteristics and quality attributes of potatoes, feasible due to advance in techniques of genetic modification of plants, may affect their increased attractiveness in the economic aspect. Still, metabolism of plants is significantly affected by transgenesis which leads to their increased resistance to biotic stresses [Catchpole *et al*., 2005] and is likely to affect dietetic or anti-nutritional properties [Matthews *et al*., 2005] of an edible part of crop. The content of biologically-active substances, secondary metabolites, changed upon transgenesis may contribute not only to differentiated degree of nutrients utilization, but may also yield health-promoting or detrimental as well as immunostimulatory and anti-inflammatory effects or may induce disturbances in redox balance – a change in the oxidative status of body cells, degradation of DNA *etc*. Thus, administration of a transgenic plant in a diet may modify metabolism of an animal and, indirectly, affect the functioning of the whole organisms, *i.e.* its homeostasis.

Due to anxieties of consumers, demands of opponents of contemporary biotechnology implementations as well as recommendations of EFSA [2004] and OECD [2003], safety analyses of the use of genetically-modified organisms (GMO) in diets of animals and humans require, among others, the evaluation of their potential toxicity, carcinogenicity, and allergenicity that might by unintentional effects of transgenesis.

Assays of the chemical composition of tubers of genetically-modified lines of potato with repression of a and c isoforms of 14-3-3 protein obtained by means of antisense transformations indicated small differentiation in the content of basic nutrients. Our previous feeding experiment carried out on rats demonstrated also similar digestibility of a diet with 30% addition of dry tubers of transgenic and non-transgenic plants [Kosieradzka *et al*., 2008]. However, according to results of experiments conducted by Żuk *et al*. [2003], the repression of 14-3-3 protein isoforms changed also the activity of enzymes responsible for the mechanisms of binding nitrogen and carbon as well as enhanced antioxidative properties of potato plants. The increased antioxidative activity, being probably an effect of modification of polyphenols synthesis in a plant, may trigger changes in dietetic properties of tubers, whereas their administration with diet may modulate metabolism of a consumer.

The objective of a subsequent stage of investigations was, therefore, to evaluate the effect of changes in tissue composition of an edible part of a genetically-modified plant on metabolism and health status of experimental animals.

MATERIAL AND METHODS

To determine the effect of GMO administration in a diet on selected parameters of health status of model animals, a 4-week growth experiment was carried out on male outbred IF_2Jaz rats with the initial body weight of *ca*. 52 g. The animals were divided into 4 experimental groups (n=10) and kept in individual growth cages in a room with a constant temperature of 21°C, humidity of 60% and 12 h light/dark cycle.

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Rats from the experimental groups were administered *ad libitum* a semi-synthetic diet with 30% addition of dry tubers of the examined transgenic potatoes *Solanum tuberosum* L. cv. *Desiree* with repression of 14-3-3 protein isoforms: a (line J4), c (line J5) or a and c (line G1). An iso-protein diet for control rats contained an analogous addition of non-transgenic potatoes of Desiree cvr. Apart from dried potatoes, the diets contained: casein, cellulose, soybean oil, mineral mix, vitamin mix, choline chloride, dl-methionine and maize starch [Kosieradzka *et al*., 2008]. Body weight of rats and feed intake were monitored over the experimental period.

On termination of the feeding experiment and after 12-h fasting, the rats were weighed and anaesthetized by ketamine overdose (50 mg/kg b.w.). Blood to be analysed was sampled from heart whereas selected internal organs (brain, liver, small intestine, kidneys and pancreas) were prepared and weighed. Liver tissue was frozen and kept at a temperature of –70°C until subjected to chemical analyses.

Potential disorders in the functional status of organs and balance of metabolic processes were evaluated by means of multiple parameters whose values might indicate organism's response determined by the presence of a substance with toxic or allergenic activity.

Blood samples collected on an coagulant (EDTA) were assayed for the following morphological parameters: number of red blood cells (RBC), haematocrit (HCT), mean cell volume (MCV), concentration of haemoglobin (HGB), number of white blood cells (WBC) and number of platelets (PLT), with standard laboratory methods using an ABACUS hematological analyser (Diatron).

Blood plasma was determined for the following biochemical parameters: total protein, albumin, glucose, urea N, cholesterol, HDL, triglycerides, asparagine transferase (AST) and alanine transferase (ALT), Fe and Ca – with the spectrometric method, using a Vitros analyzer and Ektachem DT-60- II system (with DT, DTE, DTSC module) and a set of film slides by Johnson & Johnson Clinical Diagnostics.

The activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) was analysed spectrophotometrically by means of Randox Laboratories Ltd. kits. The concentration of immunoglobulins IgE, IgA, IgM, IgG in blood plasma was determined with the chemiluminescene method, using an Immulete 2000 analyzer. The concentration of interleukin IL-4 in blood plasma was assayed with the ELISA method using a kit by R&D System and following producer's instructions. Measurements of absorbance were performed at a wavelength of 405 nm with an Anthos 2010 reader after plate incubation for 30 min. In the analysis of TNF-α concentration with the ELISA method use was made of a commercial kit by Peprotech and 96-well microplates by Bethyl Lab. The assay was carried out according to the producer's instructions. A colour reaction was developed using an ABTS dye (Sigma). The reading was performed at a wavelength of 405 nm with an Anthos 2010 reader. After coating, the plate was incubated for 24 h.

The concentration of products of oxidative degradation of protein, lipids and DNA was determined in liver tissue. Concentrations of NO_3^- and NO_2^- were assayed with the HPLC method (Waters), whereas the sum of thiobarbituric acid reacting substances (TBA-RS) – with the colorimetric method (with 1.2.3.3.-Tetraethoxypropane – TEP used as a standard). After isolation and DNA hydrolysis of liver tissue, the concentration of 8-oxo-2'-deoxyguanosine DNA hepatocytes was analysed with HPLC Dionex, an electrochemical detector (at a wavelength of 350 mV), a UV/VIS detector (at a wavelength of 245 nm), and Supelcosil LC-18-S column (250 mm x 4.6 mm x 5 μm). The concentration of 8-oxo-2'-deoxyguanosine (8-oxo-2'dG) in DNA was expressed as the number of 8-oxo-2'dG molecules per 10^6 of unmodified molecules of 2'-deoxyguanosine (2'dG) [Foksiński *et al*., 2000].

Results obtained were elaborated statistically using a one-way analysis of variance ANOVA with Duncan's range test using Statgraphics 6.0 Plus software.

RESULTS AND DISCUSSION

Administration of a diet with 30% addition of transgenic potatoes with repression of 14-3-3 protein did not affect differentiation of the growth rate nor body weight gains of the rats. Utilization of diet and dietary protein per body weight gain unit, likewise the coefficient of dietary protein utilization per body weight gain, indirectly determining the biological value of protein, were not statistically significant between control and experimental groups (Table 1).

The intake of transgenic potatoes of J4, J5 and G1 lines by experimental rats had no statistically significant effect on differentiation in the relative mass of liver, small intestine or brain. In turn, the mass of kidneys of rats receiving diets with their addition was significantly higher (Table 2), which could indicate that the diets contained a substance demonstrating

TABLE 1. Growth parameters of rats fed diets with dried transgenic and non-transgenic potatoes and nutritional value of dietary protein.

Parameter	Group - diet				SEM	p
	control – Desiree cv.	transgenic				
		J4	J5	G1		
Initial body weight (g)	51.88	52.19	52.13	52.25	0.6837	0.9814
Final body weight – 4 weeks (g)	221.44	221.25	219.69	226.38	2.8805	0.4015
Daily body gain (g)	5.71	5.89	5.44	6.04	0.1859	0.1432
Total body gain (g)	169.56	169.06	167.56	174.13	2.8828	0.4240
Dietary protein utilization per g of body gain (g/g)	0.379	0.386	0.359	0.376	0.0095	0.2433
Body weight gain per g of dietary protein (g/g)	2.659	2.598	2.798	2.667	0.0670	0.2208
Diet utilization/body gain (g/g)	2.34	2.28	2.24	2.26	0.0300	0.2098

TABLE 2. Relative mass of selected internal organs (g/100 g b.w.).

Organ	Group - diet				SEM	p
	control – Desiree cv.	transgenic				
		J4	J5	G1		
Brain	0.67	0.65	0.63	0.62	0.0164	0.1809
Liver	4.96	5.02	5.13	5.00	0.2022	0.7096
Small intestine	2.98	3.02	2.96	2.84	0.0757	0.4846
Kidneys	0.64^{A}	0.72^{B}	0.73^{B}	0.75^{B}	0.0041	0.0041
Pancreas	0.46	0.43	0.43	0.44	0.0201	0.6889

A, B – means in rows with different letters difer significantly at $p<0.01$.

relative toxicity (*e.g.* glycoalkaloids) or result from disturbances in mineral metabolism evoked by the diet.

The diet containing transgenic potatoes did not affect values of most of the analysed biochemical parameters of blood whose differentiation might have indicated disturbances in the metabolism of proteins, lipids or carbohydrates (Table 3), disorders in the functioning of organs elicited by *e.g.* considerable concentration of an anti-nutritional substance in a diet. Blood of rats receiving diets with transgenic potatoes contained more Fe (by *ca.* 60%), however the statistical analysis did not demonstrate any significance of those differences.

Diets with transgenic potatoes were also demonstrated not to affect the analysed hematological parameters of rats (Table 4).

Administration of an allergenic factor with a diet is likely to stimulate the body for the production of specific IgE antibodies. In the case of a lack of allergic reaction, IgE occurs in blood plasma in extremely low concentrations. In individuals without atopic predisposition, the immune response is linked, in turn, with the production of IgG antibodies specific to extrinsic proteins and neutralizing protein antigens in serum; whereas their increased concentration manifests a defensive response of a body [Romański, 1998]. In investigations of the effect of GMO on the immune system it is advisable to identify food proteins that induce sensitization of consumer body, *i.e.* IgG stimulation, despite a lack of typical allergenic properties [Kimber & Dearman, 2003].

In the reported experiment, the concentration of IgE produced as a response to the presence of an allergenic factor did not change upon the administration of a diet containing transgenic potatoes. In addition, the diet was found not to affect concentrations of other antibodies, *i.e.* IgA, IgG, IgM (Table 5).

The long-term (in a 4-week experiment) exposure to dietary protein with allergenic activity or a substance modifying the response of the immune system may stimulate the synthesis of interleukin–4 that serves a significant function in the course of proliferation of T lymphocytes responsible for, among others, production of IgE antibodies. However, the concentration of IL-4 in blood serum of rats fed diets with GM potatoes appeared to be very low. Likewise, the concentration of TNF-α – a cytokine controlling the secretion of IL-4 from basophiles, did not exceed the detection limit with the selected analytical method in spite of the fact that potato glycoalkaloids are capable of stimulating proteins of the TNF family [Thorburn., 2004]. In the reported experi-

TABLE 3. Biochemical blood parameters of rats fed diets with transgenic and non-transgenic potatoes.

Parameter	Group - diet				SEM	p
	control – Desiree cv.	transgenic				
		J4	J5	G1		
Total protein (g/dL)	5.90	6.43	5.78	5.65	0.2733	0.2517
Albumins (g/dL)	3.17	3.38	3.15	3.15	0.1454	0.6505
Globulins (g/dL)	2.73	3.05	2.63	2.50	0.1433	0.0925
Urea N (mg/dL)	11.00	11.00	9.25	8.75	0.8354	0.1723
AST (U/L)	138.0	203.0	106.5	141.5	49.7778	0.5937
ALT (U/L)	44.50	48.75	38.75	36.75	5.7396	0.4657
Glucose (mg/dL)	180.5	190.5	163.0	166.3	10.4988	0.2683
HDL (mg/dL)	37.25	43.50	42.50	40.50	2.7453	0.4228
Total cholesterol (mg/dL)	69.25	76.75	72.75	71.00	3.9005	0.5824
Triglycerides (mg/dL)	161.25	134.25	138.25	130.25	25.9169	0.8338
Fe (μg/dL)	160.75^{A}	256.0^{B}	248.5^{AB}	259.0^{B}	30.2220	0.1160
Ca (mg/dL)	11.13	10.38	10.48	10.33	0.2155	0.7410

A,B – means in rows with different letters difer significantly at $p<0.01$.

TABLE 4. Haematological blood parameters of rats fed diets with transgenic and non-transgenic potatoes.

Parameter	Group - diet				SEM	p
	control – Desiree cv.	transgenic				
		J4	J5	G1		
Erythrocytes (T/L)	6.16	6.07	6.06	5.86	0.1595	0.6099
Leukocytes (G/L)	4.15	3.20	3.04	3.47	0.3536	0.1813
Haemoglobin (g/L)	12.17	12.32	12.00	11.75	0.2273	0.3616
Haematocrit (L/L)	37.3	38.1	37.7	36.81	0.8607	0.7318
MCV (f/L)	60.55	62.87	62.38	62.80	0.6427	0.0807
Red blood cells (G/L)	860.0	671.2	574.5	838.8	84.6789	0.0994
Lymphocytes ($10^3/\mu$L)	3.37	2.78	2.54	2.75	0.3364	0.3776
Monocytes ($10^3/\mu$L)	0.029	0.103	0.151	0.154	0.0416	0.1732
Neutrophils ($10^3/\mu$L)	0.656^B	0.161^A	0.154^A	0.500^B	0.0834	0.0021

A, B - means in rows with different letters difer significantly at $p<0.01$.

TABLE 5. Potential allergenicity of protein – the level of IgE immunoglobulin in blood serum of rats fed diets with transgenic and non-transgenic potatoes.

Parameter	Group - diet				SEM	P
	control – Desiree cv.	transgenic				
		J4	J5	G1		
IgE (IU/mL)	< 1	< 1	< 1	< 1	-	-
IgA (IU/mL)	< 0.05	< 0.05	< 0.05	< 0.05	-	-
IgG (IU/mL)	0.51	0.61	0.54	0.61	0.0296	0.0789
IgM (IU/mL)	0.10	0.11	0.11	0.11	0.0099	0.8026
IL-4 (pg/mL)	< 5	< 5	< 5	< 5	-	-
TNF- α (pg/mL)	< 5	< 5	< 5	< 5	-	-

ment, a lack of significant differences in the concentrations of IL-4 and TNF-α points to a lack of the allergic response of the body to the change in the composition of tubers upon transgenesis (Table 5).

The presence of biologically-active substances with toxic activity (*e.g.* alkaloids), whose slightly increased concentration was observed in dry transgenic potatoes [Kosieradzka *et al*., 2008], as well as substances antioxidative in character (*e.g.* polyphenols) in a diet may evoke disturbances in the balance of oxidation and reduction processes in cells [Azevedo *et al*., 2003]. A research by Shahjahan *et al*. [2005] demonstrated that glycoalkaloids of plants of the family *Solanum* (solanine, solasonine and solamarine) might inhibit lipid peroxidation and that their long-lasting effect might be an increase in the activity of endogenous antioxidants and reduction of TBA-RS. Simultaneously, the presence of flavonoids is likely to enhance the activity of glycoalkaloids that model the enzymatic and non-enzymatic defense system against oxidative stress [Shahjahan *et al.*, 2005]. Diets with 30% addition of dry potatoes, despite differentiated contents of compounds with antioxidative (flavonoids) and toxic properties (glycoalkaloids), did not affect the intensity of lipid degradation processes, whereas the concentration of compounds reacting with thiobarbituric acid (TBA-RS) was similar in rats from all feeding groups (Table 6). An insignificant effect of mixtures with GM potatoes on TBA-RS value may, therefore, point to a lack of the toxic influence of the diet (and a lack of exposure of experimental animals to oxidative stress) or

TABLE 6. Selected parameters of degradation of the following compounds: lipids (TBA-RS), DNA (8-oxo-2'deoxyguanosine), and proteins (NO_3^-, NO_2^-).

Parameter	Group - diet				SEM	p
	control – Desiree cv.	transgenic				
		J4	J5	G1		
TBA-RS (μg/mL)	1.570	1.988	1.585	1.719	0.1178	0.0924
8-oxo-2'deoxyguanosine (8odG/$10^6$2dG)	6.164^A	7.996^B	5.686^A	7.635^B	0.1839	0.0000
NO_2^- NO_3^- (mg/kg)	2.38	2.34	2.54	2.18	0.1248	0.2817

A,B means in rows with different letters difer significantly at $p<0.01$.

TABLE 7. Activity of antioxidant enzymes in blood of rats fed diets with transgenic and non-transgenic potatoes.

Parameter	Group - diet				SEM	P
	control – Desiree cv.	transgenic				
		J4	J5	G1		
GSH-Px (U/g Hb)	262.6	256.7	260.0	257.2	2.1873	0.2336
SOD (U/g Hb)	2.33	1.97	2.04	2.23	0.1012	0.0937

to a protective or "repair" effects of other dietary constituents, *e.g.* polyphenolic compounds.

Modification of diet composition (*e.g.* contents of anti- or prooxidants) is also one of the exogenous factors affecting the concentration of 8-oxo-2-deoxyguanosine (8-oxo-dG) [Chen *et al*., 1999] in the tissue of liver – an organ playing a key role in detoxification processes, protecting the organism against toxic activity of dietary xenobiotics and reactive metabolites [Valentova *et al*., 2007]. 8-oxo-2'deoxyguanosine is acknowledged as a biomarker of oxidative damages to DNA [Shigenaga & Ames, 1991]. In hepatic tissue of rats receiving transgenic potatoes J4 and G1 in diets the process of oxidative degradation of DNA proceeded with a higher intensity and the concentration of an adduct – 8-oxo-2'deoxyguanosine was higher in the rats fed a diet with non-transgenic potatoes (Table 6). It seems that the elevated antioxidative potential of transgenic potatoes, resulting from the repression of 14-3-3 protein [Żuk *et al*., 2003], could diminish the generation of reactive oxygen species such as hydroxyl radical and peroxides, thus protecting molecules of DNA, lipids and proteins against the negative effects of Reactive Oxygen Species (ROS) and reducing the risk of cancer. Still, bioavailability of potentially-antioxidative substances from diets containing transgenic potatoes is, presumably, too low to exert and immediate effect on ROS activity. Contemporary research [Frei & Higdon, 2003] suggest rather the intermediate effect of dietary antioxidants that consists in the inhibition of transcription factors, *e.g.* NF-κB – a key regulator of the action of the cellular antioxidant system (activated by ROS generated *e.g.* upon the action of a toxic substance), inhibition of prooxidative enzymes, *e.g*. nitrogen oxide synthase (iNOS), lipoxygenase, cycloxygenase (COX) or induction of antioxidant enzymes involved in biotransformation of *e.g*. glutathione transferase (GST) or superoxide dismutase (SOD). In the conducted experiment, no significant differences were observed in the concentration of nitrites and nitrates (NO_2^-, NO_3^-) in liver tissue determined as markers of oxidative degradation of proteins produced upon the oxidizing activity of peroxynitrite. Also blood concentration of antioxidant enzymes – superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) (Table 7), likewise the concentration of thiobarbituric acid reactive substances (TBA-RS), were not subject to changes as a result of administration of transgenic potatoes (Table 6).

The effect of the analysed potatoes on the evaluated parameters may result from an interaction occurring between multiple components of a semi-synthetic diet containing the whole tissue of an edible part of a GM plant, as well as between biologically-active substances whose concentrations changed upon transgenesis.

CONCLUSIONS

The application of transgenic potato tubers with the repression of isoform a (line j4), isoform c (line J5) and isoforms a and c (line G1) of 14-3-3 protein in diets for rats did not deteriorate growth parameters nor diet utilization; was not implicated with the occurrence of an allergic reaction or food intolerance in the experimental animals; and did not induce definitely toxic effect of the diet, though GMO components were observed to affect the extent of oxidative degradation of DNA in liver tissues.

The feeding experiment conducted with the application of a considerable addition (30%) of transgenic potatoes with 14-3-3 protein repression in diet enabled concluding that their consumption does not pose any severe health risk. The absolute values of the assayed parameters determining health status did not exceed values typical of healthy animals (reported in available literature or reference values).

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