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Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

The effect of diet containing genetically modified triticale on growth and transgenic DNA fate in selected tissues of mice*

Antoni Baranowski[1], Stanisław Rosochacki[2,4], Rafał Parada[2], Kazimierz Jaszczak[2], Janusz Zimny[3], Jarosław Połoszynowicz[2]

[1] Department of Animal Improvement,
Polish Academy of Sciences Institute of Genetics and Animal Breeding,
Jastrzębiec, 05-552 Wólka Kosowska, Poland

[2] Department of Molecular Cytogenetics,
Polish Academy of Sciences Institute of Genetics and Animal Breeding,
Jatrzębiec, 05-552 Wólka Kosowska, Poland

[3] Department of Plant Biotechnology and Cytogenetics,
Plant Breeding and Acclimatization Institute,
Radzików, 05-870 Błonie, Poland

[4] Chair of Sanitary Biology and Biotechnology, Białystok Technical University,
Wiejska 45a, 15-351 Białystok. Poland

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C57Bl/6J mice were fed with pellets containing 20% of conventional >Bogo< triticale grain *vs* pellets containing 20% transgenic triticale grain obtained from the same cultivar, but resistant to BASTA herbicide with phosphonithricin as an active substance (control and experimental group, respectively). Feeding regimen (with or without transgenic triticale) was maintained continuously in both groups over five generations, and body weight of animals was recorded on day 21, 42, 63 and 91 of life. No significant inter-group differences in body weight were found. During the experiment, mice of both groups showed no pathological manifestations, were found healthy and did not produce any visible skin changes. The PCR method used to analyse blood, kidneys, liver, spleen and thigh muscle of experimental animals did not show the presence of transgenic DNA (PCR product of 637

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bp) in any of the tissues. It is concluded that transgenic triticale used for experimental group had no effect on either growth of mice, or on the appearance of transgene in their tissues.

KEY WORDS: genetically modified organism / growth / mice / PCR /transgenic feed / triticale

Genetic modification has become the domain of molecular biology and genetic engineering and is widely applied in agriculture, thus creating GM crops which are distributed all over the world [Zduńczyk 2001, Twardowski *et al.* 2003]. Just 80 per cent of all transgenic plants produced are used as components for animal feeds [Kosieradzka 2002]. Substantial evidence has been established on the basis of European [Aumaitre *et al*. 2002] and American [Faust 2002] data, leading to the conclusion that pest- or herbicide-resistant transgenic plants used as components of animal diet (GM feed) are equivalent in basic chemical characteristics and nurtitive value to their isogenic analogue crops. There is no direct evidence that transgenic (GM) feed may potentially be dangerous to animals or can yield the transgenic protein in animal products used as food for humans [Einspanier *et al*. 2001, Phipps *et al*. 2001, Weber and Richert 2001, Aumaitre *et al*. 2002, Faust 2002, Sanden *et al*. 2004]. On the other hand, however, the investigation conducted with viral DNA [Schubbert *et al*. 1994, 1997] showed, that foreign DNA of bacteriophage M13mp18 was not completely degraded in the gastrointestinal tract of mice and was identified in their blood leukocytes, spleen and liver. Additionally, some foreign DNA fragments of bacteriophage M13 delivered to pregnant mice by Schubbert *et al*. [1998] were found out in their liver, spleen and kidney as well as in the foetal organs (brain, eyeball) or in the next generation of mice (in brain, eyeball, liver and spleen). Pusztai [1998] in rats fed transgenic potatoes that were modified by the insertion of the snowdrop lectin gene (insect-resistant) observed retarded growth, under-development of some organs, as well as damaged intestine and disturbed immunological system. Also, some changes in the liver cells of mice fed with GM soybean resistant to glyphosate (active substance of the herbicide ROUNDUP READY) were shown by Malatesta *et al*. [2002]. Ermakova [2005] revealed, that ROUNDUP READY soybean could have a negative effect on the offspring of Wistar rats, causing high mortality and decreasing weight gain in some of the pups. The present state of knowledge (literature on interactions between GM feed and living organism is still quite poor) does not exlude the potential effect of prolonged feeding with transgenic feeds on animals and their prosterity. This is especially dangerous when alergenic or even mutagenic activities of transgene are concerned. Including transgenic plants into the nutritional chain of animals promotes further intensive research aiming at the detection of foreign DNA metabolic pathways as well as supplying irrefutable evidence confirming or excluding the negative effect of GM feeds on farm animals health (safety feeds) and safety of animal products (safety foods).

This experiment aimed at finding out whether transgenic triticale grain used as a component of feed affects the health and growth intensity of mice and leads to appearance of transgenic DNA in their tissues.

Material and methods

Conventional and transgenic triticale

Both conventional and transgenic triticale used in this experiment were produced in Plant Breeding and Acclimatization Institute, Radzików, Poland. To obtain transgenic triticale crop, the immature embryos of cultivar >Bogo< were used as described by Zimny *et al*. [1995]. Plant tissue material was bombarded with the plasmid pDB1 [Becker *et al*. 1994] containing the β-glucuronidase gene *(uidA)* under the control of rice actin-1 promoter (Act 1) – Mc Elroy *et al*. [1990] – and a selectable gene marker *bar* (phosphinothricin acetyl transferase) under the control of the CaMV 35S promoter. From bombarded scutella plants were regenerated. These regenerants were screened for enzyme activity by the histological glucuronidase (GUS) assay, and tested by spraying the plants with BASTA herbicide solution. Twenty four regenerants showed GUS-activity and survived the BASTA spraying. PCR tests as well as Southern blot analysis showed the presence of both marker genes integrated in the genome of transgenic plants. This way it has been shown that *in vitro* cultures of triticale were genetically transformed and regenerated to phenotypically normal, fertile plants. Those plants were grown to maturity and set seeds. Then, transgenic line obtained in that way has been reproduced under closed conditions to get grain subsequently used as feed component in experiment described below.

Feeds

Pelleted diet for control group, containing 20% (by weight) of conventional triticale grain and another one for experimental group, containing 20% (by weight) of transgenic triticale grain were both prepared in the feed-producing plant specialized in feeds for laboratory rodents (certified by Polish Standard PN ISO 9001). Pellets for both groups (Tab. 1) contained neither additives of animal origin, pharmateutics, and probiotics nor GM products (other than transgenic triticale in pellets for experimental group), and were completely balanced as far as protein, energy and mineral-vitamin additives are concerned. From a pool of conventional and transgenic triticale grain as well as from respective pellets one kg samples were withdrawn (n=4) to perform proximate analyses as well as to confirm the presence of transgene (DNA analysis by PCR).

Animals

91 days-old breeding mice (strain C57BL/6J) obtained from the Centre of Experimental and Clinical Medicine of the Polish Academy of Sciences were mated brother × sister to create parental generation (F0). From mating, during pregnancy and litter rearing the F0 animals were offered exclusively the control diet (pellets containing 20% conventional triticale – Tab. 1). At the age of 91 days the F0 animals were mated (brother × sister) to create (i) the first control generation, further fed

Table 1. Composition (% weight) of conventional (control) and experimental pelleted diet

Component	Control pelleted diet (containing conventional triticale)	Experimental pelleted diet (containing transgenic triticale)
Maize grain	20.0	20.0
Conventional triticale	20.0	–
Transgenic triticale	–	20.0
Oat grain (defattened)	10.0	10.0
Soybean oilmeal	27.1	27.1
Wheat bran	15.0	15.0
Yeast	4.0	4.0
Ca_2-phosphate	1.0	1.0
Chalk	1.5	1.5
NaCl	0.4	0.4
Premix LRM	1.0	1.0

with control diet – Tab. 1) and (ii) first experimental generation, further fed with experimental diet – Tab. 1). These animals are further referred to as generation F1-con and generation F1-exp, respectively. From F1-con and F1-exp, further generations, *i.e*. F2-con, F3-con, F4-con, F5-con, as well as F2-exp, F3-exp, F4-exp F5-exp were derived which, over all periods of life (including gestation and lactation), were fed with only control and experimental pellets, respectively. During the experiment the animals were maintained at the Institute mice farm where all the regulations of rodent keeping and breeding have been observed. The mice were haused in plastic cages with wire tops, and were supplied *ad libitum* with their respective control or experimental pellets, with free access to water.

At weaning (day 21 of age) and then on day 43, 63 and 91 of life (the latter at the time of mating to get the new generation) the offspring born in 10 litters by individual dams from each of five generations and each of two feeding groups was weighed. Moreover, every day the mice were visually examined for well-being, and conceivable skin changes. At the age of 91 days five females and five males were removed randomly from each group and each generation and sacrificed by cervical dislocation. Their blood, kidney, liver, spleen and thigh muscle were collected to check for the presence (or absence) of transgenic DNA.

PCR analyses of transgenic triticale based on the CaMV 35S promoter and *bar* gene primers

DNA was isolated from 50 mg of triticale grain using the GeneMATRIX Plant & Fungi DNA Purification Kit (EURx). Grain was firstly ground in the mortar. The plasmid pDB1 used for triticale transformation contains the *uideA* gene under

the control of the actin-1 promoter and the *bar* gene under control of CaMV 35S promoter.

PCR based on primers of the CaMV 35S promoter. Two primers were used:
cf3: 5'- CCACGTCTTCAAAGCAAGTGG - 3' (length 21 bp) and
cr4: 5'- TCCTCTCCAAATGAAATGAACTTCC - 3' (length 25 bp).

The complete PCR contained: 5 µl 10 × Taq amplification buffer, 2.5 µl 4 mM nucleotide mixture, 5 µl 25 mM $MgCl_2$, 1.25 µl of both primers, 0.025 U Taq-polymerase in a final volume of 48 µl. PCR product was 123 bp long. The PCR of 50 cycles were: 25 s at 95°C, 30 s at 62°C and 45 s at 72°C. Final extension lasted 7 min at 72°C .

PCR based on primers of the *bar* gene (*bar1* and *bar2*). Two primers were used:
bar1: 5'- GAGACCAGTTGAGATTAGGCC - 3' (length 21 bp) and
bar2: 5'- ATCTGGGTAACTGGCCTAACT - 3' (length 21 bp).

The complete PCR mixture contained: 5 µl 10 × Taq-amplification buffer, 2.5 µl mM nucleotide mixture, 5 µl 25 mM $MgCl_2$, 1.25 µl of both primers and 0.025 U Taq polymerase in a final volume of 48 µl. PCR product was 500 bp long. The PCR of 30 cycles were: 30 s at 94°C, 90 s at 62°C and 2 min 30 s at 72°C. Final extension lasted 5 min at 72°C.

PCR analyses of tissues of mice and supplementary of triticale and pellets, all based on *uideA* gene primers

Total genomic DNA was isolated from tissues (100 µl blood or 20-40 mg tissue) based on the protocol GeneMATRIX Blood DNA Purification kit or GeneMATRIX Tissue DNA Purification Kit ((EURx). PCR analyses of all tissues of mice as well as supplementary PCR of triticale and pellets were performed based on primers of the gene *uideA* (*uideA1* and *uideA2*). The following primers were used:
uideA1: 5'- CAGGAAGTGATGGAGCATCAG - 3'
(position on *uideA* 603→ 623, length 21 bp) and
uideA2: 5'-TCGTGCACCATCAGCACGTTA -3'
(position on *uideA* 1220← 1240, length 21 bp).

The complete PCR mixture contained: 10 ng of DNA in 1.0 µl and 2.5 µl 10 × Taq-amplification buffer, 0.25 µl 2.5 mM nucleotide mixture (EURx, 0.025 mM final concentration), 0.10 µl of 50 µM of both primers (0.2 µM final concentration of *uideA1* and *uideA2*, supplied by the Polish Academy of Science Institute of Biochemistry and Biophysics), 0.25 µl 5 U Taq-polimerase (0.05 U/µl) in a final volume (with 20.80 µl water) of 25 µl. The PCR of 30 cycles were: 35 s at 94°C, 30 s at 62°C and 45 s at 72°C. PCR product was 637 bp long. Final extension lasted 5 min at 72°C.

Statistical

The results were subjected to analysis of variance using the least squares method [Harvey 1990]. The following model was used:

$$Y_{ijkl} = \mu + a_i + b_j + c_{ik} + e_{ijkl}$$

where:

Y_{ijkl} – the analysed trait;

μ – overall mean;

a_i – effect of generations (i = 1..4);

b_j – effect of sex (j = male 1, female 2);

c_{ik} – effect of diet (k = 1 – control, 2 – eperimental) in i-th generation;

e_{ijkl} – error.

Results and discussion

The presence of the incorporated gene in experimental pellets containing transgenic triticale was detected using three different pairs of primers. It was possible to amplify the inserted gene construct in all transgenic triticale samples as well as in pellets containing 20% of transgenic triticale. With primers cf3 and cr4 originating from CaMV 35S promoter, we detected a 123 bp fragment, with bar1 and bar2 primers of a *bar* gene – a 500 bp fragment, and with *uideA1* and *uideA2* primers of the *uideA* gene – a 637 bp-long band. All measurements showed positive results (Photo 1) in the transgenic triticale grains and experimental pellets.

In accordance with published review-data on substantial equivalence of genetically modified and conventional forage plants [Zduńczyk 2001, Aumaitre *et al*. 2002, Faust 2002, Baranowski 2004], the conventional triticale used in our experiment appeared

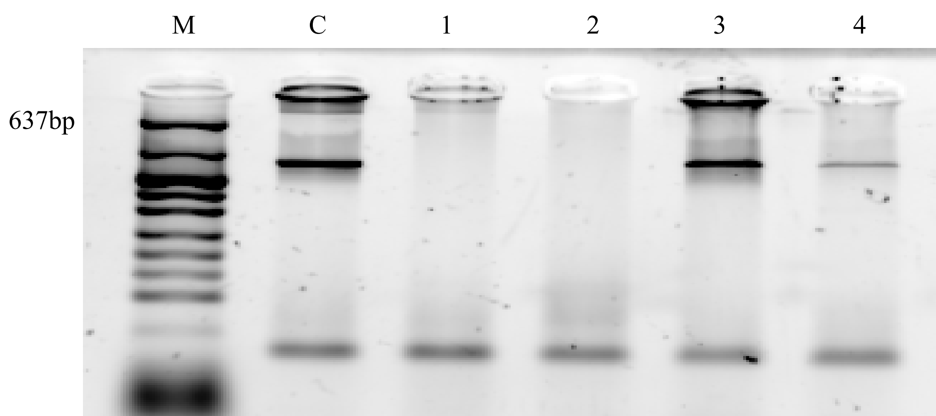

Photo 1. Electrophorogramme of pellets DNA. M-marker; C – control (*uideA* transgen); 1– conventional triticale; 2 – pellets containing conventional triticale; 3 – transgenic triticale; 4 – pellets containing transgenic triticale.

similar to transgenic one as far as the results of proximate analysis are concerned (Tab. 2). No significant differences in proximate composition were identified between the pellets made with conventional or transgenic triticale (Tab. 3). This allows to assume that control and experimental diet used in our feeding programme represented similar nutritive value.

Table 2. Proximate composition of conventional and transgenic triticale

Item	Conventional triticale (n=4)	Transgenic triticale (n=4)
Dry matter (%)	88.92 (±2.41)	87.90 (±1.34)
In dry matter (%)		
protein (N×6.25)	14.57 (±1.21)	14.55 (±2.66)
fibre	3.12 (±0.54)	3.13 (±0.47)
ether extract	1.38 (±0.18)	1.24 (±0.04)
ash	2.09 (±0.15)	2.19 (±0.34)
N-free extractives	78.84 (±1.54)	78.90 (±3.02)

n – number of samples.

Table 3. Proximate composition of control and experimental pelleted diet

Item	Control pellets (containing conventional triticale, n=4)	Experimental pellets (containing transgenic triticale, n=4)
Dry matter (%)	87.45 (±1.76)	86.94 (±0.52)
In dry matter (%)		
protein (N×6.25)	25.15 (±0.31)	25.00 (±0.18)
fibre	4.91 (±1.11)	4.77 (±1.34)
ether extract	3.13 (±0.41)	3.10 (±0.58)
ash	7.08 (±0.40)	7.47 (±0.44)
N-free extractives	59.73 (±0.97)	59.66 (±1.41)

n – number of samples.

The growth of mice was not affected by the diet (Tab. 4). In each examined generation the mice fed pellets containing transgenic triticale (F1-exp, F2-exp, F3-exp, F4-exp and F5-exp) did not differ significantly in body weight on 21, 42, 63 and 91 day of life from conventional triticale-fed mice (F1-con, F2-con, F3-con, F4-con and F5-con). In last two generations, but principally in F5, at each age,

Table 4. Body weight of mice fed with control (con) and experimental (exp) pelleted diet, across five generations (F1...F5)

Generation	Age (days)							
	21		42		63		91	
	N	LSM (SE)	N	LSM (SE)	N	LSM (SE)	N	LSM (SE)
F1-con	56	7.7 (0.15)	52	19.2 (0.23)	51	22.3 (0.22)	49	23.8 (0.22)
F1-exp	55	8.0 (0.15)	54	19.4 (0.22)	54	22.0 (0.21)	54	24.0 (0.20)
		ns		*ns*		*ns*		*ns*
F2-con	55	7.9 (0.15)	52	17.9. (0.22)	50	21.2 (0.21)	52	23.3 (0.20)
F2-exp	53	7.8 (0.15)	50	18.2 (0.23)	50	21.7 (0.22)	50	23.1 (0.21)
		ns		*ns*		*ns*		*ns*
F3-con	55	8.0 (0.15)	54	18.9 (0.22)	54	21.7 (0.21)	54	23.7 (0.22)
F3-exp	57	8.2 (0.15)	55	18.8 (0.22)	55	21.9 (0.21)	55	23.6 (0.20)
		ns		*ns*		*ns*		*ns*
F4-con	60	7.5 (0.15)	58	17.7 (0.21)	58	21.2 (0.20)	58	23.1 (0.20)
F4-exp	55	7.4 (0.15)	52	17.1 (0.22)	54	20.7 (0.21)	52	22.6 (0.21)
		ns		*ns*		*ns*		*ns*
F5-con	54	6.9 (0.15)	48	14.3 (0.27)	46	18.7 (0.23)	46	21.3 (0.22)
F5-exp	50	6.5 (0.15)	42	12.6 (0.25)	41	18.4 (0.24)	41	21.0 (0.23)
		ns		*ns*		*ns*		*ns*

ns – differences between diets were not found significant.
N – number of animals.

the inbred depression occurred shown by decreased body weight ($P \leq 0.01$, Tab. 5). Simultaneously, no stillbirths occurred. In all generations of mice, no visual changes on the skin were noticed indicating the alergy. According to Twardowski *et al*. [2001] transgenic diet with 40% of glyphosynate-tolerant (LIBERTY LINK) maize did not affect the gain of body weight and of certain vital organs and also morphological parametres of blood in the first two generations of adult rats. In a study by Brake and Evenson [2004] body weight of mice fed the diet cotaining 21.4% of glyphosate-tolerant ROUNDUP READY soybean from 8th to 87th day of life did not differ from that of controls. Simultaneously, the transgenic feed had no effect on macromolecular synthesis or cell growth and differentiation as evidenced by no differences in the percentages of testicular cell populations (haploid, diploid and tetraploid).

Contrary to our results, in the work of Ermakova [2005] genetically modified ROUNDUP READY soybean (glyphosate-tolerant) introduced to the diet was possibly

Table 5. Effect of generations and sex on body weight of mice (control and experimental diets pooled)

Generation	Age (days)							
	21		42		63		91	
	N	LSM (SE)	N	LSM (SE)	N	LSM (SE)	N	LSM (SE)
Parental	53	7.8 (0.15)	53	19.0 (0.34)	53	not measured	53	22.8 (0.48)
F1	111	7.8 (0.11)^{A}	106	19.3 (0.16)^{A}	105	22.1 (0.15)^{A}	103	23.9 (0.15)^{A}
F2	108	7.9 (0.11)^{A}	102	18.0 (0.16)^{B}	100	21.4 (0.15)^{aB}	104	23.2 (0.15)^{aB}
F3	112	8.1 (0.11)^{A}	109	18.8 (0.15)^{C}	109	21.8(0.15)^{AB}	109	23.6 (0.14)^{bA}
F4	115	7.5 (0.11)^{B}	110	17.4 (0.15)^{D}	112	21.0 (0.14)^{bC}	111	22.8 (0.14)^{B}
F5	104	6.7 (0.06)^{C}	90	13.4 (0.19)^{E}	87	18.6 (0.17)^{D}	87	21.2 (0.16)^{C}
Males	277	7.7 (0.06)^{A}	259	18.8 (0.11)^{A}	257	23.9 (0.10)^{A}	210	25.5 (0.09)^{A}
Females	273	7.4 (0.06)^{B}	258	16.0 (0.11)^{B}	257	18.6 (0.10)^{B}	217	20.4 (0.09)^{B}

$^{aA...}$Means in columns bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.
N – number of animals.

the cause of 55.6% mortality of rat pups and their lower – by 36% – body weight. She suggested, that this could be the result of transformation and insertion of foreign genes, which could penetrate into sexual/stem cells, or/and into cells of the foetus, as it was observed by Schubbert *et al.* [1998]. Also Malatesta *et al.* [2002] suggest, that glyphosate-tolerant transgenic ROUNDUP READY soybean (14% of the diet) might be the reason of significant changes in hepatocyte nuclear features in young and adult mice.

In this study while analysing the tissues of mice fed experimental (transgenic) feed we used a set of primers of the *uideA* gene for identifying the amplifiable plant DNA fragments. The 637 bp band was only present in positive control (plasmid pDB1). Blood, kidney, liver, spleen and thigh muscle were the tissues withdrawn from mice of all five generations and of both sexes. No signal for any of the investigated tissues occurred positive (for illustration shown are exemplary electrophorogrammes of DNA isolated from tissues of generation 5 only – Photos 2, 3, 4 and 5). The negative and positive controls confirmed that each PCR reaction proceeded properly. These results are similar to those published by Einspanier *et al*. [2001], who in cattle and poultry muscle, liver, spleen and kidney did not find any plant DNA. Furthermore, *Bt* gene fragments, possibly representing the uptake of recombinant maize, were not detected in any sample from cattle and poultry. Investigations on atlantic salmon fed with 17.2% GM soybean meal indicated, that certain transgenic sequences of that feed may survive passage through the gastrointestinal tract (only 120 bp fragment could be amplified from the content of the stomach, pyloric region, mid intestine and distal

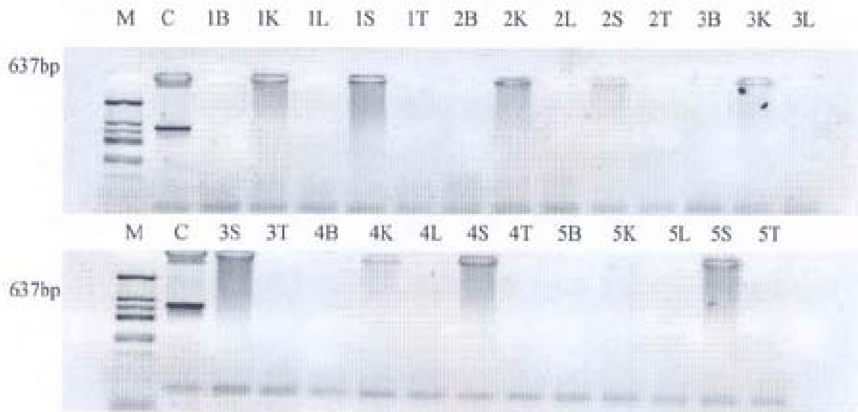

Photo 2. Exemplary electrophorogrammes of DNA of tissues of female mice fed with control diet (generation 5, female 1,2,3,4,5). M – marker; C-control (*uideA* transgen); B – blood; K – kidney; L – liver; S – spleen; T – thigh muscle.

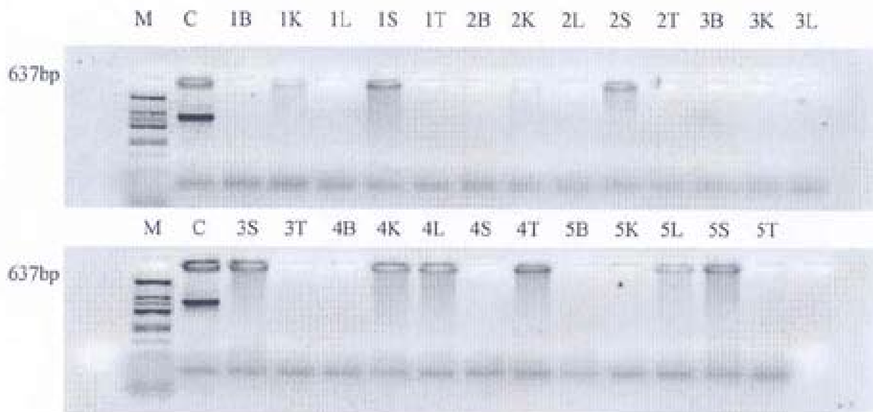

Photo 3. Exemplary electrophorogrammes of DNA of tissues of male mice fed with control diet (generation 5, male 1,2,3,4,5). M – marker; C – control (*uideA* transgen); B – blood; K – kidney; L – liver; S – spleen; T – thigh muscle.

intestine), but could not be traced in the fish tissues (liver, muscle or brain) – Sanden *et al*. [2004]. There are also reports upon the presence of foreign DNA in some tissues of mice. Schubbert *et al*. [1994] delivered the plasmid pEFGFP containing the green fluorescent protein gene or the bacteriophage M13 DNA to pregnant mice. Foreign DNA was then detected in fragmented form in the cells of gastrointestinal tract, which

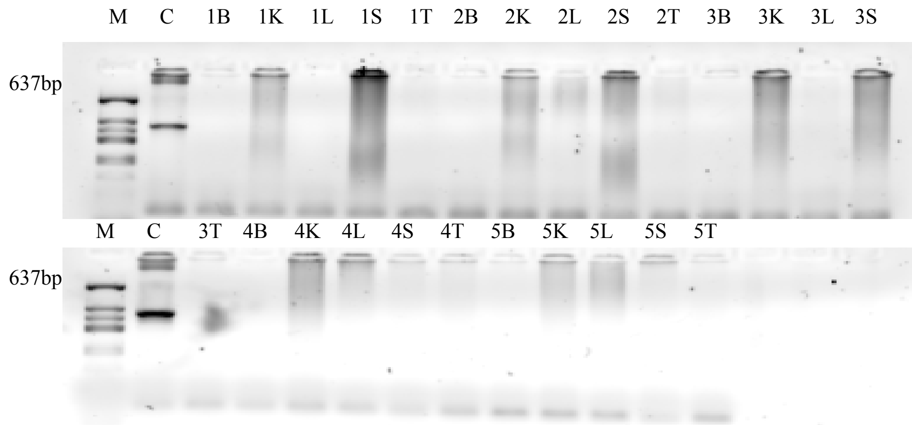

Photo 4. Exemplary electrophorogrammes of DNA of tissues of female mice fed with experimental diet (generation 5, female 1, 2, 3, 4, 5). M – marker; C – control (*uideA* transgen); B – blood; K – kidney; L – liver; S – spleen; T – thigh muscle.

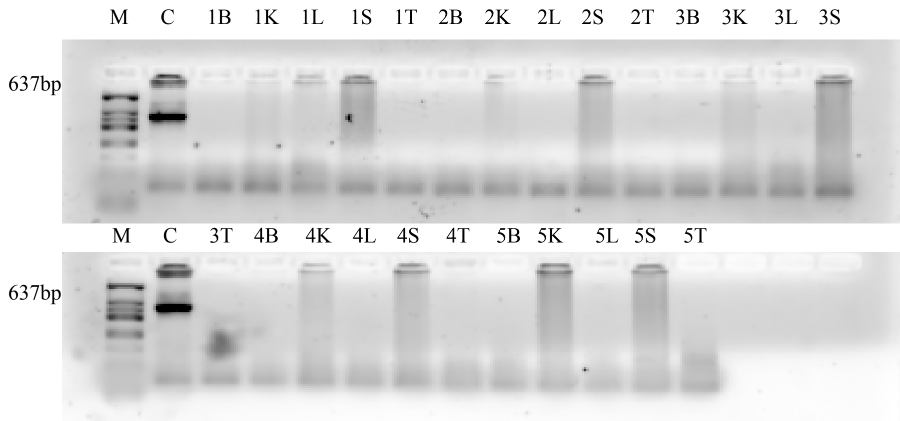

Photo 5. Exemplary electrophorogrammes of DNA of tissues of male mice fed with experimental diet (generation 5, male 1, 2, 3, 4, 5). M – marker; C – control (*uideA* transgen); B – blood; K – kidney; L – liver; S – spleen; T – thigh muscle.

could penetrate the intestinal wall and reach the nuclei of leukocytes, spleen and liver cells. Later Schubbert *et al.* [1998] have never found all cells of the foetus to be transgenic for the foreign DNA. They considered, that this distribution pattern argues for a transplacental pathway rather, than for germline transmission which might be expected only after long-term feeding. Ermakova [2005] claims, that negative effect of transgenic soybean on mice can also be connected with the accumulation of herbicide

residues and the instability of gene constructs in GM soybean [Windels *et al.* 2001] and rice [Yang *et al*. 2005]. Ermakova [2005] does not exclude the interaction of a number of antinutrients in soybean (such as the lectins, tripsin inhibitors *etc*.) and also female hormone-like substances as earlier indicated by Pusztai [1998].

Conventional triticale used in our experiment was equivalent to transgenic (phosphinothricin-tolerant) triticale in basic chemical composition. Pelleted feed containing 20% of transgenic triticale grains was used to feed mice over five generations and did not lead to any visible changes on the skin that could be ascribed to allergy. The diet with transgenic triticale did not affect the postnatal growth of mice. In blood, kidney, liver, spleen and muscle of mice fed with the diet containing transgenic triticale, the transgene introduced was not found out. In light of the results of other experiments indicating negative effect of transgenic feeds on animals (even when transgenic and conventional feed were nutritionally equivalent), there is a need for further complex investigations to confirm the safety of transgenic feeds for animals and further safety of their products for humans (food safety).

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Antoni Baranowski, Stanisław Rosochacki, Rafał Parada,
Kazimierz Jaszczak, Janusz Zimny, Jarosław Połoszynowicz

Wpływ diety z udziałem genetycznie zmodyfikowanego pszenżyta na wzrost myszy i obecność w ich tkankach transgenicznego DNA

S t r e s z c z e n i e

Myszy szczepu C57BL/6J żywiono przez pięć kolejnych pokoleń wyłącznie granulatem zawierającym 20% pszenżyta transgenicznego, odpornego na fosfinotrycynę (substancja czynna herbicydu Basta) lub granulatem zawierającym 20% pszenżyta konwencjonalnego tej samej odmiany (odpowiednio grupa doświadczalna i kontrolna). Dieta z udziałem pszenżyta transgenicznego nie wpływała ujemnie na wzrost myszy (w 21, 42, 63 i 91 dniu życia i we wszystkich pokoleniach zwierzęta doświadczalne nie odbiegały pod względem masy ciała od kontrolnych). We krwi, nerkach, wątrobie, śledzionie i mięśniu uda myszy żywionych dietą z udziałem pszenżyta genetycznie zmodyfikowanego nie stwierdzono (analiza PCR, 637 p.z.) obecności transgenicznego DNA.