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Evaluation of the possibility of horizontal gene transfer and accumulation of transgenic DNA from the diet in the bodies of rats*

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ABSTRACT

The procedures for GMO safety tests include traceability of transgenic protein and transgenic DNA if the plant constitutes a component in the diet for an animal. This is due to the possibility of horizontal transfer of genes, accumulation of transgenic DNA in consumer's organs, or induction of antibiotic resistance in gastrointestinal tract microflora. The last possibility is related to the use of marker genes in the process of transformation. In an *in vivo* experiment conducted on laboratory rats with the use of transgenic cucumbers expressing the pre-prothaumatin gene, the presence of transgenic DNA in the tissue of kidneys and liver was not detected. Resistance to neomycin of gastrointestinal tract microflora of the rats fed the GMO diet was not found, despite the use of marker genes (npt II) in the process of transformation of the investigated plants.

KEY WORDS: GM cucumber, gene transfer, antibiotic resistance, *in vivo* experiment, rats

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INTRODUCTION

Evaluation of the potential risk connected with consumption of transgenic protein and transgenic DNA is an element of studies on the safety of including genetically modified plants in human and animal diets. Safety is mainly considered in the context of the effect of transgenic protein on the consumer's organism but also in the context of the possible transfer and accumulation of recombined DNA in organs. The horizontal transfer of antibiotic-resistance marker genes employed in the process of obtaining a transgenic plant creates the threat of inducing antibiotic resistance in gastrointestinal tract microflora of the GMO consumer. The procedures of the studies on safety include, therefore, investigations of the fates of transgenic protein and transgenic DNA of the plant included in the diet. The European Parliament Directive 2001/18/WE (EC, 2001) recommends paying special attention to GMOs that contain genes expressing resistance to antibiotics and assessment of the potential risk resulting from the possibility of transferring the genes from GMOs onto other organisms. Pursuant to the procedure of approving GMOs in the EU, if the application for authorization to place a GMO product on the market concerns GMOs containing genes of resistance to antibiotics employed in treatment of people or animals and which may cause a risk for humans and the environment, the said authorization is not granted (Regulation of the European Parliament and of the Council No1829/2003/EC; EC, 2003).

The aim of the conducted studies was to verify the hypothesis according to which the consequences of feeding animals a diet containing GM plants may include horizontal transfer of genes - accumulation of transgenic protein and DNA in organs or acquiring antibiotic resistance by gastrointestinal tract microflora.

MATERIAL AND METHODS

Experimental material

The experimental material consisted of the fruits of transgenic plants of *Cucumus sativus* L. cucumber, of Borszczagowski variety, obtained by the method of vector transformation with the application of sweet protein gene-thaumatin. Transgenic plants (generation T2 of the independent transgenic line 224 09), the fruits of which were used, possessed insert T-DNA with 2 chimerical genes: 35S-cDNA of the pre-prothaumatin gene (gene of sweet protein derived from *Thaumatococccus daniellii* Benth.) and nos-nptII, expressing resistance to antibiotics from the neomycin group (expression of neomycin phosphotransferase) (Szwacka et al., 2002). Transformation of cucumber using *Agrobacterium tumefaciens* was conducted at the

Department of Plant Genetics, Breeding and Biotechnology of Warsaw University of Life Sciences (Szwacka et al., 1996). The cucumber plants were cultivated under greenhouse conditions. Fruits 8-10 cm long were successively harvested, frozen and freeze-dried (lyophilized). The ground lyophilizate was analysed for chemical composition and used in the diets for rats.

Animals and management

Twenty rats from the outbred IF_2Jaz herd with an initial body weight of about 65-70 g were allocated to two groups (10 animals per group). The rats were fed *ad libitum* with iso-protein semi-synthetic diets containing 10% of lyophilizates of transgenic cucumbers (experimental group) or lyophilizates of isogenic line fruits (control group). The mentioned diets were balanced based on the analysis of the composition of ingredients (according to AOAC, 1996, in accordance with the requirements according to NRC, 1996). The composition of the mixture included casein (12.75 vs 12.84%), cellulose (3.35 vs 3.15%), soya oil (4.0 vs 4.0%), minerals (3 vs 3%), vitamins (1 vs 1) and maize starch (65.6 vs 65.71). The animals were kept in individual balance cages, in a room with constant, controlled environmental conditions: light/darkness 12 h/12 h, temperature 21°C and humidity 60%. After completion of the experiment, 12 h of starvation, the animals were killed by administration of ketamine (50 mg/kg of body weight), which was overdosed afterwards.

Faeces were collected from the rats throughout the experiment, and when the experiment was finished, livers, kidneys and small intestine digesta were collected and frozen (-70°C).

Detection of thaumatin II protein in plant and animal material

Protein extraction from cucumber fruit probes and animal material as well as immuno-analysis were performed as described by Szwacka et al. (2002). Protein extracts were fractionated on SDS-PAGE and subjected to immunoblot analysis using specific anti-thaumatin rabbit polyclonal antibodies (Kucharczyk) and anti-rabbit IgG goat antibodies conjugated with alkaline phosphatase (Roche). Immunoblot was developed using the NBT/BCIP alkaline phosphatase substrate kit (Roche).

Detection of the thaumatin II gene in animal tissues

DNA isolated from animal tissues according to the procedure developed by Zimowska et al. (1997) was used for PCR amplification. The sequence of

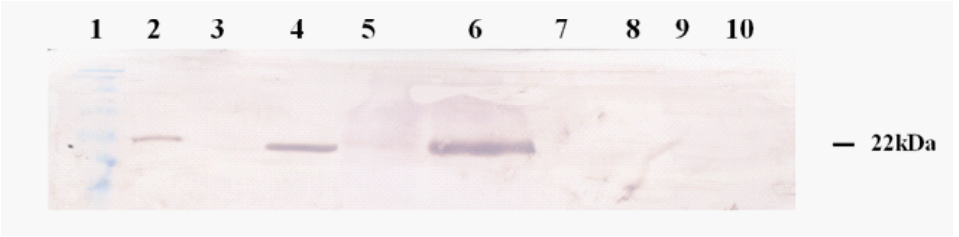
the primers for the thaumatin II gene was 5'catcgacatctccaacatc3' forward, 5'cggggcaggtgacggtggttgg3' reverse, yielding a fragment of 267 bp. The PCR cycle programme consisted of initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min. The PCR product was resolved by 1.0 % agarose gel electrophoresis in 1× Tris-acetate-EDTA (TAE) buffer.

Microfora resistance to antibiotic

The intestinal contents of each of the rats from the control and experimental groups were inoculated on 7 Petri dishes with agar medium containing the addition of neomycin (*neomycini sulphas*) at the following concentrations: 0, 0.005, 0.01, 0.1, 0.2, 0.3 and 0.5%. The dishes were incubated under aerobic conditions, at a temperature of 37°C. After 48 h of incubation, the presence and number of colonies on the plate was determined. The result of the microbiological test was analysed using the SAS 8.2. programme with the Kruskal-Walles test (SAS, 2003).

RESULTS

Digestion and accumulation of transgenic protein thaumatin in organ tissues. The presence of recombinant thaumatin II in the extract of protein of fresh cucumber of the transgenic line used in the experiment (Figure 1, lane 4) and in the lyophylizate of the cucumbers added to the diet (Figure 1, lane 6) was

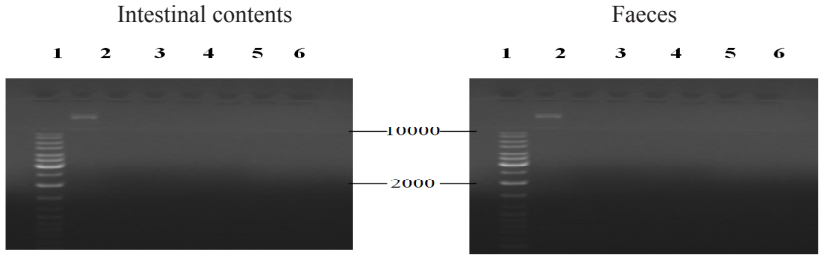


From the left: 1. Marker of proteins' size; 2. Protein - thaumatin (0.25 µg); 3. Borszczagowski cucumber - negative control; 4. Transgenic cucumber 224 09 - fresh - positive control; 5. Transgenic cucumber of line 210 06 - lyophilizate (bring not utilized in the nutritional experiments); 6. Transgenic cucumber of line 224 09 - lyophilizate (administrated to rats in the diets). Material collected from the rats, receiving GM cucumbers 224 09 in the diet; 7. Liver; 8. Kidneys; 9. Jejunum contents; 10. Faeces

Figure 1. Analysis of the presence of recombined thaumatin (22kDa) in the selected samples by Western blotting method

confirmed by Western blotting. Detection of the transgenic protein in faeces and small intestine digesta collected from the animals after the end of the experiment was unsuccessful. The presence of thaumatin in the material was not found (Figure 1, lanes 9 and 10). Recombinant thaumatin II was not accumulated in the tissues of kidneys or livers of the experimental rats fed the diets containing transgenic cucumbers (Figure 1, lanes 7 and 8).

Digestion and accumulation of transgenic DNA. No dietary transgenic DNA was detected in the intestinal digest or faeces of any experimental rats fed diets containing the GMO (Figure 2, lanes 3, 4, 5 and 6).

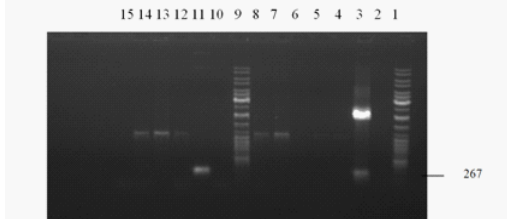


From the left: 1. DNA marker (1.5 μg); 2. plasmid pRUR 528 (1 ng); 3,4,5,6 intestinal contents of the rats, fed the GM cucumbers

From the left: 1. DNA marker (1.5 μg); 2. plasmid pRUR 528 (1 ng); 3,4,5,6 faeces of the rats, fed the GM cucumbers

Figure 2. Analysis of the presence of transgenic DNA in material, collected from the experimental animals

Similar results of the PCR analysis were obtained when the genomic DNA isolated from organ tissues (livers and kidneys) collected after the end of the experiment was analysed. The presence of the thaumatin gene in the studied tissues was not detected (Figure 3).



From the right: 1. marker; 2. sample without DNA; 3. plasmid DNA; 4 – 8. DNA of rat liver; 9. marker 10. sample without DNA, 11. plasmid DNA; 12,13,14,15. DNA of rat kidneys

Figure 3. Analysis of the presence of transgenic DNA in kidneys and liver tissue, as conducted by PCR method (61°C – temperature of connecting the starters; 30 cycles)

Microflora resistance to antibiotics - the possibility of marker gene transfer. In the process of transformation of experimental plants, marker genes nptII, encoding neomycin transferase, active in relation to aminoglycoside antibiotics (e.g., kanamycin, neomycin, gentamycin), were used. No differences in the neomycin sensitivity of microorganisms from the gastrointestinal tract of the rats fed the GM cucumbers or their isogenic equivalents were found. Statistically significant differences in growth of intestinal microflora of rats fed the GMO and control diets on plates with agar containing neomycin would indicate the possibility of marker gene (nptII) transfer from transgenic plants to organisms living in the gastrointestinal tract. The number of plates with medium containing neomycin in concentrations from 0 to 0.5%, on which the presence of microbial colonies was detected after incubation in the control and experimental groups was, however, similar. Studies on microflora DNA (met PCR) were not conducted because resistance to antibiotics, which might be induced by built-in nptII genes, was not found.

DISCUSSION

The absence of recombined thaumatin in the contents of intestines and faeces of the rats receiving GMO in their diet is evidence of the complete digestion in the upper gastrointestinal tract of the sweet protein produced by the GM plant. According to the results of the studies of Hsu et al. (1977), Edwards (1981), and Gibbs et al. (1996), pure thaumatin, i.e. protein isolated from plants and administered to animals during *in vivo* experiments, is easily digested in the gastrointestinal tract and decomposed to amino acids. In the experiment conducted within the framework of the present study, considerable effectiveness of digesting GMO-derived thaumatin constituting a component of transgenic plant fruit was observed.

The inability to detect transgenic DNA in the contents of intestines and faeces from any of the experimental animals, similarly as the failure to find the presence of transgenic DNA, thaumatin gene, in liver and kidney tissues of the rats fed the diet containing the transgenic cucumbers, may be proof of a considerable degree of its digestion in the gastrointestinal tract. When interpreting these results, however, it should be taken into consideration that the sensitivity of the diagnostic method used may be too low for detection of a small concentration of transgenic DNA in the examined material. Such a result of the experiment supports the possibility of safe utilization of cucumbers with taumatin in the diet, found in previous experiments showing no influence of GMO on growth and selected health parameters of model animals (Kosieradzka et al., 2001, 2004).

Vegetal transgenic DNA is decomposed in the gastrointestinal tract of animals similarly as non-transgenic DNA, but its survivability may be dependent on many exo- and endogenous factors (Aeschbacher et al., 2005). Recently published studies have not confirmed the thesis that assumes the possibility of transfer of genes from transgenic plants included in a diet to the consumer's organism; the presence of transgenic DNA in animal organs has not been recorded. Transgenic DNA in blood, kidneys, liver and muscles of mice receiving genetically modified herbicide-resistant triticale in their diets was not detected by Baranowski et al. (2006). Vegetal (non-transgenic) DNA was found in the content of rumen and duodenum, faeces, trace amounts - in milk, eggs, blood, leukocytes and organs of pigs and poultry (Klotz et al., 2002; Phipps et al., 2003; Reuter and Aulrich, 2003; Einspanier et al., 2004), which makes it impossible to exclude the eventuality of transfer or recombination of genes. Experimentally, the presence of transgenic DNA was confirmed in the gastrointestinal tract of pigs (Chowdhury et al., 2003). Agodi et al. (2006) found the presence of modified DNA of soya and maize in milk samples collected from animals fed GMO in diets; the authors indicated, however, the possibility of committing a methodical error and contamination of the research material with bacteria in which the modified genes occur (*Agrobacterium* sp. and *Bacillus thuringiensis).*

The probability of horizontal gene transfer from a GMO constituting an ingredient of the diet to the microflora living in the gastrointestinal tract is considered small although not impossible to exclude. Transfer of the recombined DNA of plants to a bacterial organism would require the presence of intact DNA molecules of the appropriate size. The results of recent studies, (Kharazmi et al., 2003; Weiss et al., 2007) and of our own experiments indicate considerable degradation of endogenous genomic DNA. It is considered as being highly improbable to induce gastrointestinal tract resistance to antibiotics due to expression of the transferred genes. In *in vitro* experiments using cultures of tissue Caco-2 and jejunum bacteria of mammals, *Lactobacillus plantarum* and *Salmonella typhimurium*, subjected to transformation by a gene expressing a protein that determines resistance to neomycin, the transfer of resistance to analogues of the mentioned antibiotic from the cells of prokaryote to eukaryote (Netherwood et al., 2004) was detected. In spite of the small risk of gene transfer, contemporary technologies of obtaining transgenic plants are focused on the possibility of eliminating the use of marker genes of resistance to antibiotics, which is consistent with the requirements of the procedure for GMO approval for commercial application in the European Union.

CONCLUSIONS

The presence of a transgenic protein, thaumatin, in the content of intestines and faeces of the rats fed a diet with 10% of lyophilizate of GM cucumbers was not detected. Transgenic DNA was also not detected in the liver and kidney tissues of rats receiving GMO in their diet. An increase of resistance of gastrointestinal tract microflora to antibiotics, which might be the effect of marker gene transfer from the GMO-containing diet, was not observed.

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