Detection of genetically modified food in digesta and organs of rats fed transgenic potato

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ABSTRACT. Transgenic potato plants harbouring *cry1Ab* gene, resistant to potato tuber moth, were used to examine the persistence of recombinant DNA and proteins in the digesta, gastrointestinal tract (GIT) tissues and visceral organs of rats in a 90-day feeding trial. Native plant DNA (chloroplastic gene, *cp*) and fragments of *cry1Ab*, *nptII* and *nos* promoter were tracked in rat organs and tissues by polymerase chain reaction (PCR). Although complete sequences (1200 bp) of *cry1Ab* gene and its decreased size (400 bp) were detected in the rat diets, the presence of these sequences was not confirmed by PCR analysis in the GIT contents of rats fed diets containing transgenic potato. Moreover, the 400 bp sequence of the *nptII* gene and 300 bp of the *nos* promoter were detected in the caecum, stomach and rectum contents of some rats fed transgenic potato; however, these sequences were not detected in all rats. Immunoassay showed that Cry1Ab protein is detectable in the GIT contents of rats fed transgenic potato, but quantitative assay by ELISA confirmed that the Cry1Ab protein was partially degraded (80.3–83.7% digestibility) after passing through the GIT. Similarly, ELISA assay of NPTII showed that the protein can be digested (76.8–84.36% digestibility) in the GIT. So, although ingested native and recombinant DNA and protein did not totally degrade in the GIT, it can be indicated that there is no need for concern about the effects of recombinant *cry1Ab* and *nptII* genes and their products on animals and humans.

Introduction

Consumers have many difficulties in avoiding genetically modified (GM) products due to the ubiquitous presence of GM crops produced worldwide (Buzoianu et al., 2012). Although regulatory agencies issued precise frameworks for adoption of transgenic crops, concerns about the safety of transgenic products still remain (Walsh et al., 2011). Among the most discussable aspects of transgenic crops are toxicity and allergencity (Dona and Arvanitoyannis, 2009). However, the risk of horizontal transfer of transgens from such products is also raised (Świątkiewicz et al., 2011). The probability of horizontal transfer of antibiotic resistance genes used in the process of producing transgenic crops can induce antibiotic resistance in the human/animal gastrointestinal tract (GIT) microbiota and soil microflora (Nielsen et al., 1998; Kosieradzka et al., 2010). Therefore, the evaluation of potential risk related to consumption of recombinant protein and DNA is a substantial part of studies on the

safety of GM crops used in human and animal diets (Kosieradzka et al., 2010; Swiatkiewicz et al., 2014).

The analysis of adverse effects of recombinant DNA absorption from transgenic foods requires intact and stable transgenic DNA sequences passing through human/animals GIT. It appears that the low pH in the stomach, and nucleases produced in the saliva and small intestine can inactivate and degrade most ingested plant DNA and proteins (Duggan et al., 2000). Sometimes, however, small DNA fragments when bound to soil minerals or proteins can be absorbed in the GIT (Gallori et al., 1994). This process could protect them from degradation (El-Sanhoty et al., 2006).

The passage of food and feed DNA through the GIT barrier is natural; endogenous plant genes were detected in animal tissues and products (Reuter and Aulrich, 2003; Tudisco et al., 2006). The fate of recombinant feed and food DNA in the GIT of animals and its potential absorption and transfer to other tissues was reported by many researchers. The digestive fate of recombinant DNA and protein from transgenic plants has been studied in rats, fish, cattle, sheep, poultry and pigs (El-Sanhoty et al., 2006; Sharma et al., 2006; Walsh et al., 2011; Swiatkiewicz et al., 2014). In the studies using transgenic crops (maize, rice, potato, soyabean) no adverse effects on the nutrition of rats, chickens, broiler chickens, catfish, dairy cattle, bulls and sheep have been shown (Duggan et al., 2003; He et al., 2009; Snell et al., 2012). Nevertheless, the possible survival of recombinant plant DNA fragments in the GIT has been reported, possible hazardous effects and the biological relevance of this data has not yet been substantiated (Mazza et al., 2005; Sharma et al., 2006).

Transgenic potato plants harbouring *cry1Ab* gene for potato tuber moth (*Phthorimaea operculella* (Zeller)) resistance have been developed in the Agricultural Biotechnology Research Institute of Iran (Ghasimi Hagh et al., 2009). After field trials, the possible use of this transgenic potato as food and feed will be reported. Although targeted expression of *cry1Ab* gene, using a light-inducible phosphoenolpyruvate carboxylase (PEPC) promoter, may alleviate biosafety concerns (e.g., Cry1Ab expression in non-edible parts), fears about the possible adverse effects of the genes and recombinant protein still remain (Świątkiewicz et al., 2011; Swiatkiewicz et al., 2014; Koch et al., 2015).

So, the objective of the present study was to determine the fate of recombinant DNA and proteins in the GIT and verify its possible transfer to tissues and organs of rats fed transgenic potatoes in a 90-day feeding study.

Material and methods

Potato tuber samples

The transgenic *Bacillus thuringiensis* (Bt) potato (Marfona line B8) and its near isogenic non-transgenic parental line (as the control) were grown under greenhouse conditions at the Agricultural Biotechnology Research Institute of Central Iran in Isfahan in 2010. The plants were grown and harvested under the same conditions. The transgenic line was generated using *Agrobacteium tumefaciens* AGL01 harbouring the binary vector pBPEPCry1Ab (Ghasimi Hagh et al., 2010). The binary vector includes a gene cassette consisting of PEPC promoter, *cry1Ab* gene and *nos* terminator (Figure 1). A kanamycin resistance gene (*nptII*) was used in the cassette as a selectable marker gene. The authenticity of transgenic and non-transgenic potato lines was confirmed by polymerase chain reaction (PCR) analysis.
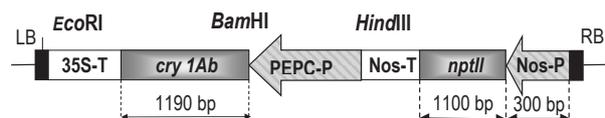

Figure 1. pBPEP-Cry1Ab vector used to generate transgenic potato plants

RB – right border; LB – left border; *nptII* – neomycin phosphotransferase II gene; Nos-P – neopalin synthase gene promoter; Nos-T – neopalin synthase gene terminator; PEPC-P – phosphoenolpyruvate carboxylase promoter; *cry1Ab* – structural gene; *Eco*RI, *Bam*HI and *Hind*III – restriction enzymes

After harvesting, the transgenic and non-transgenic potato tubers were divided in two groups. One group was kept under light (60 μmol · m^{-2} · s^{-1}) for 7 days. The other was kept in the dark at 25 °C for 7 days. The light was used in order to allow the expression of Cry1Ab protein in the transgenic potato tubers and therefore study the fate of Cry1Ab protein in the GIT of the examined rats. The potato tubers were sliced and dried by incubation at 45 °C. The dried potato chips were ground in a grinder and the powder was used in experimental diets.

Animals, diet and feeding trials

The experimental protocols were approved by Biosafety and Bioethics Committee in Agricultural Biotechnology Research Institute of Iran (ABRII). In total, 16 adult male and 16 adult female Wistar rats (130–180 g) were used in the study. Animals purchased from Razi Institute (Karaj, Iran) were grouped randomly into 16 standard cages with two males or two females in each cage. All animals were

Table 1. Composition of experimental diets: potato powder (%) added to the basic diet (NRC, 1995) in four groups

Compound	Groups			
	1	2	3	4
Dark-treated non-transgenic potato (DN) flour	-	-	-	20
Light-treated non-transgenic potato (LN) flour	-	-	20	
Dark-treated transgenic potato (DT) flour	-	20	-	-
Light-treated transgenic potato (LT) flour	20	-	-	-

kept under normal healthy conditions (23 ± 2 °C, 12 h light/dark cycle, 50–60% humidity) and fed a basal diet for 1 week. The basal and experimental diets were formulated according to National Research Council (NRC) guidelines (NRC, 1995). The basic diet contained, %: wheat flour 15, barley flour 8, maize flour 16.5, wheat bran 2, soyabean meal 13, alfalfa meal 2, NaCl 0.8, multivitamin 0.1, vitamin E 0.7, vitamin D_3 0.2, vitamin C 0.2, milk powder 2.5, meat meal 17 and soya oil 2. In the experimental diets, 20% potato flour replaced an equivalent amount of maize-starch (Table 1). Each diet was assessed by PCR analysis to confirm the absence or presence of the *cry1Ab*, *nptII* and *nos* transgenes.

During feeding treatments, the cages were divided into four groups, each containing two cages with male (M) and two cages with female (F) rats. Animals were fed diet containing light-treated transgenic potato (LT; group 1), dark-treated transgenic potato (DT; group 2), light-treated non-transgenic potato (LN; group 3), and dark-treated non-transgenic potato (DN; group 4). All rats had *ad libitum* access to feed and water for 90 days. The animals were observed and monitored daily for signs of possible toxicity resulting from feed and water consumption.

Tissue collection

At the end of 90-day feeding trial, the rats were euthanized and blood samples were collected into polypropylene tubes containing ethylenediaminetetraacetic acid (EDTA) and then stored at −70 °C for further analysis. The rat carcasses were opened under sterile conditions. The gut was carefully dissected and laid out on a flat surface. The digesta from each section (stomach, caecum and rectum) were carefully removed, collected in 1.5 ml tubes and stored at −70 °C to await DNA and protein analysis. Small samples of the brain, liver, kidney, spleen, heart, testes, stomach, duodenum, jejunum, ileum, caecum, rectum and blood were collected and stored like digesta samples. The digesta samples from each GIT segment and organ from all animals in each group were pooled prior to DNA analysis.

DNA extraction and PCR analysis

DNA extraction. A Magnan Blood DNA Extraction Kit (Gil NanoGene Biotech, Tehran, Iran) for DNA extraction from blood was used according to manufacturer's instructions. A Magnan Plant DNA Extraction Kit (Gil NanoGene Biotech, Tehran, Iran) was used to extract the DNA from the potato tubers and diets given to rats. A QIAamp DNA Stool Mini-Kit (#51504, Qiagen, Hilden, Germany) and a DNeasy Blood and Tissue Kit (#69506, Qiagen, Hilden, Germany) were used for DNA extraction from digesta and all other tissue samples, respectively.

The concentration of isolated DNA was measured using a Nanodrop (Hoefer, San Francisco, CA, USA) according to manufacturer's instructions. The quality of the extracted nucleic acid was controlled using agarose gel electrophoresis. The DNA concentration was adjusted to 25–30 ng · μl⁻¹ for PCR analysis.

Oligonucleotide primers. Two pairs of oligonucleotide primers were used to detect intrinsic control gene, one for potato (*cp,* gene encoding chloroplastic gene; primers Pot-cp, product length 111 bp) and the second one for rat (*Mstn,* gene encoding myostatin; primers Mstn, product length 97 bp) (El-Sanhoty et al., 2006). The next four pairs of primers were used to detect the recombinant genes: *cry1Ab, nos* and *nptII* (Table 2). The NptII and Nos primers were designed to amplify recombinant *nptII* and *nos* promoter sequences, respectively. Cry400 and Cry1200 primers, respectively, were designed to amplify a short (400 bp) and a long (1200 bp) sequence of synthetic *cry1Ab* gene *B. thuringiensis* subsp. Kurstaki used in production of transgenic potato plants. All primers were synthesized by Metabion International AG (Martinsried, Germany)

Table 2. Primer pairs used for polymerase chain reaction (PCR)

Primer	Sequence of primer	Length of PCR product, bp
Pot-cp-F	5'-ctctttacatcgaaacttcagaaag-3'	111
Pot-cp-R	5'-aaactacggattcgggtcg-3'	
NptII-F	5'-gctattcggctatgactgggcac-3'	400
NptII-R	5'-tcatcctgatcgacaagaccggc-3'	
Mstn-F	5'-ttgtgcaaatcctgagactcat-3'	97
Mstn-R	5'-ataccagtgcctgggttcat-3'	
NosP-F	5'-tcatgagcggagaattaagggag-3'	300
NosP-R	5'-aggtgcagattatttggattgagagtg-3'	
Cry400-F	5'-ggacatcctgaacagcatcacc-3'	400
Cry400-R	5'-ggcctgcacgtacacgctcag-3'	
Cry1200-F	5'-atcgagaccggctacaccc-3'	1200
Cry1200-R	5'-gaggtggcacgttgttgttc-3'	

and obtained in a lyophilized state. The primers were dissolved in sterile distilled water to obtain a final concentration of 20 pmol · ml^{-1}.

PCR conditions. PCR reactions were performed using 25 ng of template DNA in 25 µl of 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl$_2$, 200 µM dNTPs, 50 pM of each primer and 1 U Taq DNA polymerase (Roche, Mannheim, Germany). Amplification was carried out in a thermocycler (Techne, Staffordshire, UK) under thermal and cyclic conditions (Table 3). The PCR products were separated by electrophoresis onto 1% (w/v) agarose gel followed by a post staining step with Gelred where visualized under UV light. Samples with positive results at least in two replicates were considered positive (Chowdhury et al., 2003).

Table 3. Cycling conditions for amplifying polymerase chain reaction (PCR) products

Primer	Initial activation	Denaturation	Annealing	Extension	Cycles	Final elongation
Pot-cp	4 min at 94 °C	30 s at 94 °C	30 s at 60 °C	30 s at 72 °C	36	5 min at 72 °C
NptII	5 min at 94 °C	30 s at 94 °C	30 s at 60 °C	30 s at 72 °C	36	5 min at 72 °C
Mstn	5 min at 94 °C	30 s at 94 °C	30 s at 60 °C	30 s at 72 °C	36	5 min at 72 °C
NosP	4 min at 94 °C	30 s at 94 °C	30 s at 60 °C	30 s at 72 °C	36	5 min at 72 °C
Cry1200	5 min at 94 °C	60 s at 94 °C	60 s at 58 °C	90 s at 72 °C	36	5 min at 72 °C
Cry400	4 min at 94 °C	30 s at 94 °C	30 s at 60 °C	30 s at 72 °C	36	5 min at 72 °C

Cry1Ab and NptII protein detection

Lateral flow strip method. A lateral flow test strips (Bt-Cry1Ab/1Ac Immuno-Strip; Agdia, Elkhart, IN, USA) were used according to manufacturer's instructions to assay the presence of Cry1Ab protein in potato tubers, rat diet, GIT contents and tissues. Protein was extracted from 500 mg samples using sample extraction buffer 4 (SEB4). The solid material was allowed to settle in the tubes for 1–2 min and then the test strips were inserted into the tubes and left until the control line developed.

Enzyme-linked immunosorbent assay. Determination of the Cry1Ab and NptII proteins was carried out using a commercially available enzyme-linked immunosorbentassay (ELISA) kits according to manufacturer's instructions (Bt-Cry1Ab/1Ac ELISA; complete kit PSP 06200/0288 and *nptII* ELISA: complete kit PSP 73000/0288; Agdia; Elkhart, IN, USA). The samples were ground under liquid nitrogen using a mortar and a pestle. Total protein was extracted from the ground samples using phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, 0.08% Tween 20, pH 7.4). The control Cry1Ab and NPTII proteins were diluted and used in the following concentrations to create a standard curve: 0, 0.5, 1.0, 1.5 and 2 ng · ml^{-1}.

The ELISA plate was loaded with 100 µl of extracted protein, standard protein or control samples and incubated for 2 h at room temperature. After washing with PBS buffer, 100 µl of antibody enzyme conjugate was added to each well and then incubated for 2 h. The plates were washed using PBS buffer and then 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB; provided by kit supplier) was added and incubated for 20 min at room temperature. Plates were read at 650 nm in an ELISA reader (Thermo-Max; Molecular Devices, Sunnyvale, CA, USA). The amount of Cry1Ab and NPTII proteins was expressed as ng of Cry1Ab or NPTII protein per g of wet sample. Each sample was assayed in triplicate.

Statistical analysis

All statistical analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). The significance of differences has been tested using the parametric analysis of variance (ANOVA). The differences were considered significant at $P \leq 0.05$.

Results

Rat performance

The animals from all groups were in good health throughout the study. There were no significant differences in general health and growth rate of the rats between groups (data not shown).

Potato tubers and diets

PCR analysis using specific primer pairs for *cry1Ab*, *nptII* and *nos* sequences confirmed the authenticity of transgenic and non-transgenic potato tubers and diets (Figure 2). This analysis also identified *nos*, *nptII* and *cry1Ab* genes in all diets containing transgenic potato tubers.

Detection of recombinant DNA in GIT contents

In all experiments, the quality of each DNA sample extracted from the diet and GIT contents was first verified using *cp* gene. The primer pairs detected potato DNA fragments in the GIT contents of rats fed transgenic and non-transgenic potatoes (Figure 3A). The quality of the DNA
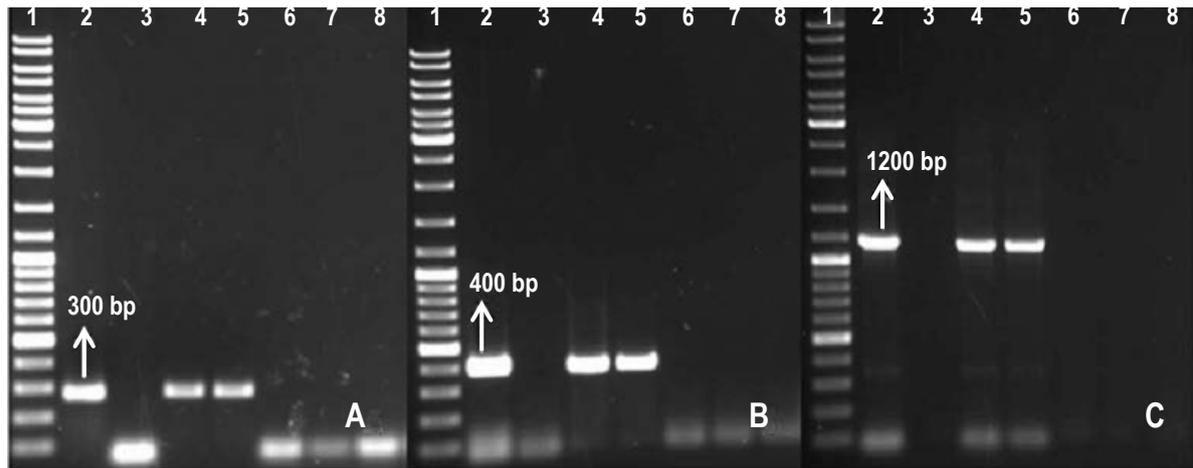
Figure 2. Confirmation of transgenic and non-transgenic potato tubers and diets by PCR analysis using NosP (A), NptII (B) and Cry1200 (C) primers (see Table 2)

Lines: (1) ladder (GeneRuler, #SM0333, Fermentas, Waltham, MA, USA); (2) pPEP-Cry1Ab plasmid (positive control); (3) water (negative control); (4) diet containing LT potato; (5) diet containing DT potato; (6) diets free of potato (control diet); (7) diet containing LN potato; (8) diet containing DN potato; T – transgenic; N – non-transgenic; L – light-treated; D – dark-treated

extracted from rat organs was verified by PCR using the Mstn primer pairs to amplify a segment of the mammalian and avian gene encoding myostatin (El-Sanhoty et al., 2006). The results showed that the DNA extracted from the diets and samples collected from different sections of the GIT and organs of rats had sufficient quality for detection of DNA fragments in PCR analysis (Figure 3B).

The stomach, caecal and rectal contents were positive for the *cp* gene in >95% of rats fed transgenic and non-transgenic potatoes (data not shown).
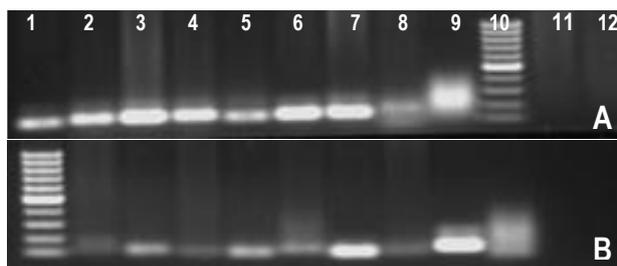

Figure 3. PCR analysis for verification of extracted DNA by (A) Pot-cp primers in diets and digesta from: (1) M-NT stomach; (2) F-LT caecum; (3) diet containing DT potato; (4) M-LT jejunum; (5) M-LT ileum; (6) diet containing DN potato; (7) diet containing LT potato; (8) F-DT rectum; (9) F-LT duodenum; (10) ladder (GeneRuler #SM0333, Fermentas, Waltham, MA, USA); (11) water (negative control); (12) stomach tissue (negative control); and (B) Mstn primers in rat organs: (1) ladder (GeneRuler #SM0333, Fermentas, Waltham, MA, USA); (2) M-LN testes; (3) F-LT liver; (4) M-DN rectum; (5) F-LN caecum; (6) F-LT spleen; (7) M- LN lung; (8) F-DT rectum; (9) F-DN stomach; (10) M-DT kidney; (11) rectum content (negative control); (12) water (negative control); F – female; M – male; T – transgenic; N – non-transgenic; L – light-treated; D – dark-treated

Although, PCR analysis confirmed the presence of *cry1Ab* gene in rat diets, it did not show positive results for Cry1200 or Cry400 primer pairs in any sample of GIT contents. The results indicated that *cry1Ab* gene sequences had degraded into small segments by digestion in the GIT (Figure 4A; Table 3).

PCR analysis of GIT contents using NptII primers confirmed the presence of *nptII* gene segments in the caecal contents of M-DT rats. The *nptII* gene was also detected in the stomach of F-DT and M-LT rats. PCR analysis of rectal contents for the *nptII* gene was positive for F-DT rats (Figure 4B, Table 4). None of the control rats exhibited positive results for the NptII primer pairs.

The *nos* sequence was detected in the cecal contents of F-LT and M-LT rats and in the rectal contents of F-DT and M-LT rats. PCR analysis confirmed the presence of *nos* sequences in the stomach contents of F-DT and F-LT rats. No *nos* promoter sequence was detected in the control rats (Figure 4C; Table 4).

Detection of recombinant DNA in rat organs and tissues

The *Mstn* gene (as internal control) was detected by PCR analysis in all rat tissues (Figure 3B). The results confirmed the successful extraction of DNA from rat tissues and their amplification by PCR. The same tissues were assayed for the presence of *cry1Ab*, *nos* and *nptII* genes using PCR analysis. The results showed no amplification for the tested samples; however, *cp* gene segments were detected in
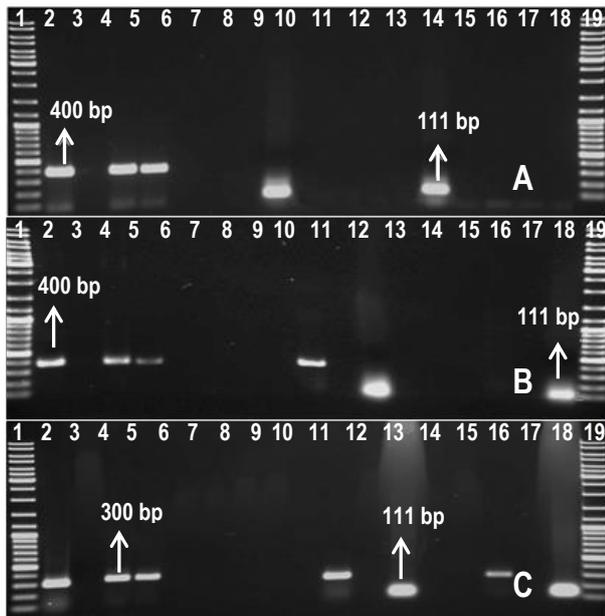
Figure 4. Detection of (A) *cry1Ab*, (B) *nptII* and (C) *nos* genes in rectum contents

Lines: (1), (19) ladder (GeneRuler #SM0333, Fermentas, Waltham, MA, USA) (2) pBPE-Cry1Ab plasmid (positive control); (3) water (negative control); (4) DT diet; (5) LT diet; (6) control diet (no potato); (7) LN diet; (8) DN diet; (9A), (14A), (12B), (18B), (13C), (18C): positive control (*cp* gene expression); (10A), (10B), (11C): F-DT; (11A), (16B), (16C): M-LT; (12A), (9B), (9C),: M-DT; (13A), (11B), (10C): F-LT; (15A), (13B), (12C): F-DN; (16A), (14B), (14C): M-LN; (17A), (15B), (15C): M-DN; (18A), (17B), (17C): F-LN; F – female; M – male; T – transgenic; N – non-transgenic; L – light-treated; D – dark-treated

Table 4. Detection of recombinant DNA fragments (*cry1Ab*, *nptII*, *nos*) in rat gastrointestinal tract (GIT) contents and tissues and other organs using polymerase chain reaction (PCR)

Sample	Animal groups							
	M-DT	F-DT	M-LT	F-LT	M-DN	F-DN	M-LN	F-LN
GIT tissues								
stomach	–	–	–	–	–	–	–	–
duode-num	–	–	–	–	–	–	–	–
jejunum	–	–	–	–	–	–	–	–
ileum	–	–	–	–	–	–	–	–
caecum	–	–	–	–	–	–	–	–
rectum	–	–	–	–	–	–	–	–
liver	–	–	–	–	–	–	–	–
spleen	–	–	–	–	–	–	–	–
GIT contents								
stomach contents	–	+*nptII*, +*nos*	+*nptII*	+*nos*	–	–	–	–
caecum contents	+*nptII*	–	+*nos*	+*nos*	–	–	–	–
rectum contents	–	+*nptII*, +*nos*	+*nos*	–	–	–	–	–
Other tissues								
blood	–	–	–	–	–	–	–	–
brain	–	–	–	–	–	–	–	–
heart	–	–	–	–	–	–	–	–
lung	–	–	–	–	–	–	–	–
kidney	–	–	–	–	–	–	–	–
testes	–	–	–	–	–	–	–	–

DN – dark-treated non-transgenic potato, LN – light-treated non-transgenic potato, DT – dark-treated transgenic potato, LT – light-treated transgenic potato, F – female rats, M – male rats

two out of three replications in the duodenum tissue of rats fed transgenic potato (data not shown). This could be attributed to pollution of the sample with the contents of the duodenum.

Detection of Cry1Ab protein

Immunochromatographic strip assay for detection of Cry1Ab protein was positive in the diets containing light-treated transgenic potato (Figure 5). Immunoassay detected Cry1Ab protein in the stomach, caecum and rectum contents of rats fed transgenic potato (Table 5). Diets containing DT or non-transgenic potato and all GIT tissues and other organs in rats fed non-transgenic or DT potato showed no positive reaction to immune strip tests.

The amounts of Cry1Ab protein detected by ELISA were: in the LT diet – 49.3 ng · g⁻¹ (fresh weight), and in the rectal contents of M and F rats – 8.3 and 9.7 ng · g⁻¹ (fresh weight), respectively. The digestibility of Cry1Ab protein was estimated to be 80.3% for F and 83.7% for M rats. The Cry1Ab protein was detected in the contents of the stomach, caecum and rectum (Table 5). None of the GIT contents of the control rats showed a positive reaction for Cry1Ab. The Cry1Ab protein was not detected neither blood, spleen, brain, duodenum, ileum, jejunum, heart, stomach, liver, testes nor in kidney and lung samples (data not shown).

Detection of NPTII protein

With the use of ELISA kit the NPTII protein was detected in all GIT contents of rats fed LT and DT potatoes (Table 5). There were no significant differences between LT and DT potatoes for NPTII in the three sections of the GIT tested. The amounts of NPTII protein in diets containing LT and DT potato were 55.15 and 53.97 ng · g⁻¹ (fresh weight), respectively, and the rectal contents of M and F rats were between 8.62 and 12.8 ng · g⁻¹ (fresh weight). Based on NPTII content, the digestibility of the protein was from 76.8 to 84.36% in rats fed transgenic potato. The GIT contents of rats fed non-transgenic potato and all other tissues were negative for NPTII protein.
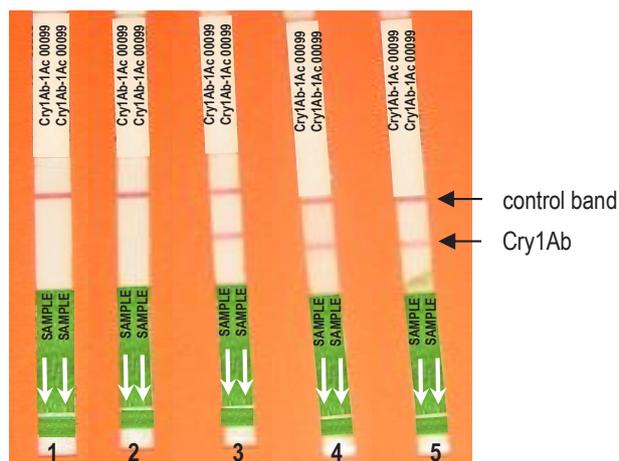
Figure 5. Immunochromatographic strip assay for detection of Cry1Ab protein in the diets containing: (1) non-transgenic potato (N), (2) dark-treated transgenic (DT) potato and (3) light-treated transgenic (LT) potato; and in the gastrointestinal tract contents of rats fed experimental diets: (4) stomach contents of rats fed LT diet and (5) caecum contents of rat fed LT diet

The control band confirms the correct function of the immune-strips; the Cry1Ab band shows the presence of Cry1Ab protein

Table 5. Cry1Ab and NPTII proteins concentration in the diets containing transgenic potatoes and in the gastrointestinal tract (GIT) segments contents of rats fed these diets

Sample	Diet/Animal group	Cry1Ab ng · g⁻¹	NPTII
Diet containing light-treated transgenic potatoes	LT	49.8 ± 3.9[a]	55.15 ± 2.4[a]
Diet containing dark-treated transgenic potatoes	DT	0[d]	53.97 ± 2.1[a]
Stomach contents	F-LT	22.5 ± 2.4[b]	31.1 ± 1.8 [b]
	M-LT	18.6 ± 1.3[b]	22.25 ± 1.45[cd]
	F-DT	0[d]	20.35 ± 1.44[d]
	M-DT	0[d]	25.6 ± 1.46[c]
Caecum contents	F-LT	12.5 ± 1.26[c]	14.4 ± 0.91[ef]
	M-LT	11.5 ± 1.32[c]	15.5 ± 1.1[e]
	F-DT	0[d]	13.8 ± 0.96[ef]
	M-DT	0[d]	12.8 ± 1.03[efg]
Rectum contents	F-LT	9.1 ± 1.83[c]	10.1 ± 0.86[fg]
	M-LT	8.3 ± 0.64[c]	12.8 ± 0.73[efg]
	F-DT	0[d]	8.62 ± 0.38[fg]
	M-DT	0[d]	10.8 ± 1.03[fg]

All samples were determined in duplicate; Data are presented as mean values ± SE; [a–g] – values with different superscripts within each column are significantly different at $P ≤ 0.05$; F – female rats, M – male rats

Discussion

In all raw and unprocessed foods and feeds there are present large numbers of DNA molecules. Food/feed processing often decreases the size of the DNA; therefore, depending on the extent of processing, a defined DNA molecule (e.g., gene) may be of different size in different products (Nielsen and Daffonchio, 2010). Statistically, humans ingest from 0.1 to 1 g of DNA per day (Doerfler, 2000). Moreover, dead microorganisms and shed intestinal cells continually release DNA into the GIT (Nielsen and Daffonchio, 2010).

Although transgenic crops contain novel sequences of DNA, the transgenes are thought to be chemical equivalent to other genes in the food because they have the same basic components as other DNA segments (El-Sanhoty et al., 2006). Therefore, the risk connected with transgene is mainly focused on the novel genetic composition of the recombinant DNA and not on the overall chemical structures (Nielsen and Daffonchio, 2010).

It is assessed that DNA comprises less than 0.02% of the total dry matter in the food (Watson and Thompson, 1988), therefore the quantity of any transgene ingested will be a minor fraction of the total DNA consumed per human per day. Pre-consumption food processing and digestion in the GIT alter the size of a defined gene as less than the length of a single transgene with a complete protein coding sequence. Therefore, introduction of a complete and functional sequence of a transgene to prokaryotic or eukaryotic cell is a rare phenomenon in the GIT. In the present study *nos*, *nptII* and *cry1Ab* genes were identified in all diets containing transgenic potato tubers using PCR analysis. It was concluded that the potato grinding and heating (for diet preparation) did not cause a significant degradation of DNA (El-Sanhoty et al., 2006).

It was also shown that complete sequences (1200 bp) and those decreased in size (400 bp) of the *cry1Ab* gene were not detected in the GIT contents or in other tissues; however, 400 bp sequence of the *nptII* gene and 300 bp of the *nos* promoter and control *cp* gene were detected in the caecum, stomach and rectal contents of some rats fed transgenic potato. They were not detected in all rat tissue.

It was also indicated that feed-ingested DNA is partially resistant to the mechanical, chemical and enzymatic activities of the rat GIT and does not completely degrade. Therefore, recombinant DNA is processed in rat GIT in the same manner as are endogenous feed-ingested genetic materials.

El-Sanhoty et al. (2006) showed that segments from transgenic potato lines could not be found in tissue samples although the diets were offered in the mash form without degradation of the transgenic potato and chloroplast plant DNA. Broll et al. (2005) detected no plant specific DNA or recombinant DNA in the organ of pigs fed transgenic potato.

These authors also found chloroplast-specific DNA in the digesta of the duodenum, jejunum, colon and rectum, and reported no evidence for integration transgenes in the host.

The presence of recombinant DNA from transgenic plants in the organs and tissues of animals fed transgenic feed has been previously reported as rare (Chowdhury et al., 2003; Broll et al., 2005; Mazza et al., 2005; Sharma et al., 2006; Swiatkiewicz et al., 2014). Sharma et al. (2006) reported that native plant DNA and the *cp4-epsps* transgene can be detected in the GIT digesta and in tissues and organs of fish and pigs fed transgenic Roundup-Ready canola meal. It was concluded that there was no evidence to suggest that transgenes would be processed in the gut differently from ingested native feed genetic material (Sharma et al., 2006). Walsh et al. (2011) detected transgenic DNA (*cry1Ab*) and protein only in the GIT digesta and not in the tissues (kidney, liver, muscle, heart or blood) of pigs fed Bt MON810 maize.

Grønsberg et al. (2011) reported that the low level of plasmid DNA introduced in feed was transiently detected in organs of young, growing rats; however, there was no indication of increased DNA uptake levels in the GIT of growing rats (Grønsberg et al., 2011). Chowdhury et al. (2003) detected endogenous gene segments of maize (zein, 242 bp; invertase, 226 bp; rubisco, 1028 bp) in the GIT contents of pigs fed Bt and non-transgenic maize. The presence of *cry1Ab* gene segments (110 bp and 437 bp) was found in the GIT contents of the pigs fed Bt maize, but not in the control pigs. Also, PCR analysis did not reveal intrinisic maize or *cry1Ab* gene fragments in the peripheral blood (Chowdhury et al., 2003).

The results of the present study suggest that *cry1Ab* gene sequences may be degraded into small segments while digested in the GIT; however, the primer pairs for detection of a shorter length (<400 bp) of *cry1Ab* gene increased the chance of detection of the *cry1Ab* gene in the GIT contents.

The potential for horizontal transfer of antibiotic-resistant selectable marker genes used to generate transgenic plants into GIT microflora is of a common concern. If so, the distribution of antibiotic resistance in GIT microflora may be at risk. Approximately 31.7% of all transgenic events approved for commercialization contain *nptII* gene as a selectable marker (Breyer et al., 2014). In the present study the 400 bp sequence of *nptII* gene in the caecum, stomach and rectum contents of rats fed transgenic potato was detected; however, there was no signs of concern about horizontal transfer of *nptII* gene to GIT microflora.

International regulatory agencies have approved the commercial release of transgenic potato, tomato, canola, maize, chicory, flax, and cotton containing the *nptII* gene (Breyer et al., 2014). The GMO panel of the European Food Safety Authority (EFSA) has accepted marketing of GMO potato containing the *nptII* gene (EFSA, 2007). The EFSA believes that the frequency of gene transfer from plants to bacteria is extremely low (Devos et al., 2014). Basing on many reports (Ramessar et al., 2007; Breyer et al., 2014) EFSA concluded that the presence of antibiotic-resistance marker genes, in particular the *nptII* gene, in transgenic plants does not bring any disadvantages to human or animal health or to the environment (EFSA, 2007, 2009).

The results of the present study show that Cry1Ab protein can be detected in the GIT contents of rats fed LT transgenic potato. The Cry1Ab protein in the DT diets and GIT contents of rat fed DT potato was not detected since dark conditions could not activate the light inducible promoter (PEPC) to the expression of Cry1Ab protein in the transgenic potato. Proteomic analysis confirmed that the amount of Cry1Ab protein in the stomach, caecum and rectum contents was lower than in the uningested transgenic diets (Table 4). It was concluded that Cry1Ab protein was partially degraded by passing through the GIT. Positive immunoassay of Cry1Ab in GIT suggested that, although ingested protein had mostly degraded, the antigenicity of the Cry1Ab was retained in the GIT. The amount of Cry1Ab in the rectum contents was lower than in the primary GIT sections. Moreover, there was no significant difference between F and M rats in digestion of Cry1Ab protein. Chowdhury et al. (2003) reported similar results for calves fed Bt11 maize. At least a dozen reports confirmed the safety of Cry1Ab proteins in mammalian gut epithelial cells because they lack Cry protein receptors (Kuiper et al., 2001).

In the present study the NPTII protein was found in all GIT contents of rats fed transgenic potato, but it was observed that NPTII was digested in 84.6% while passing through the GIT. There are no reports indicating such adverse effects of either NPTII or the *nptII* gene on humans, animals or the environment (Breyer et al., 2014; Devos et al., 2014).

In mice, purified NPTII degrades rapidly in simulated gastric and intestinal fluids (Fuchs et al., 1993). Most (if not all) NPTII can be inactivated or degraded by the acidic environment and digestive enzymes in the stomach and small intestine (EFSA, 2004). The EFSA concluded that the therapeutic use of kanamycin would not be compromised and that allergic responses would be unlikely (EFSA, 2004).

Conclusions

Endogenous and recombinant DNA exhibit similar stability and persistence in the gastrointestinal tract (GIT). Although ingested native and recombinant DNA and protein were not totally degraded in the GIT, it is believed that there is no concern for animal and human consumption of recombinant *cry1Ab* and *nptII* genes and their products.

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