SAFETY ASSESSMENT OF GENETICALLY MODIFIED POTATO SPUNTA: DEGRADATION OF DNA IN GASTROINTESTINAL TRACT AND CARRYOVER TO RAT ORGANS

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Received for Publication November 7, 2005
Accepted for Publication December 12, 2005

ABSTRACT

Diet groups for albino rats were (group 1) control basal, (group 2) control plus 30% freeze-dried nongenetically modified potatoes Spunta, (group 3) control plus 30% freeze-dried Spunta genetically modified potato (GMP) G2 and (group 4) control plus 30% freeze-dried GMP G3. After feeding ad libitum for 30 days, no statistical differences were found in food intake, daily body weight gain and feed efficiency. However, there was a small but significant difference in final body weight between the control (group 1) and experimental treatments (groups 2–4). DNA ingested in feed was partially resistant to the mechanical, chemical and enzymatic activities of the rat gastrointestinal (GI) tract (GIT) and was not completely degraded. Modified and nonmodified potato Spunta DNA survival was comparable during feed passage in the rat GIT. Modified constructs from GMP lines G2 and G3 fragment DNA were not detectable in tissue samples.

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INTRODUCTION

Genetic engineering is being used in medicine, fuel production, agriculture and food production. In the field of agricultural biotechnology, genetic engineering has opened new ways in the development of plants (e.g., food, feed, fiber and forest production) with novel traits and also in animal engineering for bioactive compounds (e.g., production of pharmaceuticals). By applying gene modification, potential gains for the world’s agriculture are the production of higher yields with minimal use of herbicides and pesticides (Kohnomurase et al. 1995; Anklam et al. 2002). Furthermore, it offers a promising new tool to develop agronomic traits such as tolerance to harsh environmental conditions as well as improving the nutritional value and quality of crops (Herbers et al. 1996; Nordelee et al. 1996; Spencer et al. 2000; Wyatt et al. 2002; Yang et al. 2002). Genetic engineering provides a precise method to insert a desired trait into an organism by selecting single genes and their products. In contrast, traditional plant breeding results in the exchange of numerous genes (Taylor 2002), and hence, less predictable effects. Apart from the economic advantages for the farmer, “green” engineering provides a means for enhancing the world’s crop production and thus might contribute to avoiding or resolving conflicts derived from an increasing world population, stagnant food production in certain areas and dependency on food imports.

Three targets of gene modification in plants can be distinguished: (1) resistance to insects, virus or fungi; (2) herbicide tolerance; and (3) altered “functional” properties to improve nutritional or processing-related properties (e.g., altered carbohydrate or fatty acid pattern). All types may occur in combination. The functional basis for a genetic modification is frequently the introduction of key enzymes of specific metabolic pathways (Preache et al. 2002). In this manner, e.g., herbicide tolerance is achieved by the expression of specific enzymes degrading glufosinate (Basta) or compensating for the blocking effect of glyphosate (Roundup) on aromatic amino acid metabolism (CP4-EPSPS). Alternatively, modified genes from Bacillus thuringiensis strains encode a toxic, crystalline protein to provide an endogenous resistance to specific insects of the Lepidoptera or Coleoptera.

Starting from 1994, since the FlavrSavr tomato entered the market in the U.S.A., more than 100 transgenic plant lines have been approved for food and/or feed use by authorities worldwide. The global planting of transgenic crops has grown 35-fold from 1.7 million hectares in 1996 to 58.7 million hectares in 2002. It is estimated that in 2002, about 6.0 million farmers in 16 countries are cultivating genetically modified plants (James 2005). Oilseeds like cotton, maize, oilseed rape (canola) and soybean, and also perishable vegetables like chicory, cucumber, papaya, tomato and potato with resistance to viruses and other diseases are of increasing interest especially for develop-
ing countries. Meals, fat/oil, protein and starch produced from genetically modified (GM) maize or soybean can be found in thousands of products like baked goods, snacks and sweets, dietary and supplementary foods, infant formula, sauces, soups and livestock feed (Elsanhoty 2004).

Feeding studies that compare modified soybeans and modified maize with conventional products have not shown the effects on the nutritional assessment of different animals such as rats, chickens, broiler chickens, catfish, dairy cattle, bulls and sheep (Hammond et al. 1996; Aulrich et al. 1998; Brake and Vlachos 1998; Flachowsky and Aulrich 2001). Most of the ingested plant DNA seemed to be inactivated and degraded by the low pH in the stomach or nucleases produced in the saliva and the small intestine (Duggan et al. 2000; Phillips and Beever 2000). Nevertheless, in some cases, small DNA fragments may pass through the gastrointestinal (GI) tract (GIT) because of binding onto soil minerals or proteins (Gallorim et al. 1994), which could act as a protection against degradation. On the other hand, the incorporation of foreign DNA fragments into the body was described for rodents, presumably through Peyer’s patches in the GIT containing membranous cells (M cells) (Doerfler et al. 1997; Schubbert et al. 1997, 1998; Doerfler and Schubbert 1998; Klotz and Einspanier 1998; Einspanier et al. 2001). In this regard for chickens, the appearance of M cells in the gut-associated lymph tissue has been recorded. However, long-term feeding of mice for eight generations did not indicate a germ line transfer of orally ingested foreign DNA (Hohlweg and Derfler 2001).

The objectives of the present study were to investigate the fate of modified (foreign) and nonmodified DNA to monitor the time-dependent degradation of feed-derived DNA in the GIT of rats, and to detect the minimum potential carryover of DNA from feed into rat organs using DNA extraction and polymerase chain reaction (PCR) methods.

MATERIALS AND METHODS

Potato Tuber Samples

Genetically modified potato (GMP) Spunta lines G2 and G3 and their parental nongenetically modified potatoes (NGMP) control line used in this study were field grown, cultivated and harvested under the same conditions in 2001 at the Department of Crop and Soil Science, Michigan State University, East Lansing, MI. Modified lines, designated G2 and G3, have been transformed (Mohammed et al. 2000) with the same vector (pSPUD5), including a gene cassette consisting of CaMV35S promoter–Cry–NOS terminator.

The authenticity of transgenic and nontransgenic potato was checked by DNA analysis as described by Elsanhoty (2004). The potato tubers were
freeze-dried (Christ BETA 2-16, Christ Company, Osterode/Harz, Germany) prior to the preparation of the experimental diets, according to the following conditions: freezing temperature: –10°C; main drying (20 h at 20°C and vacuum of 0.110 mbar); and final drying (4–6 h at 25°C and vacuum of 0.0010 mbar). The final freeze-dried products were analyzed to determine the approximate chemical composition.

Animals, Diet and Feeding Experiment

Forty-eight weanling male albino rats (50 ± 5 g) were obtained from the Faculty of Veterinary Medicine, Moshtoher University, Zagazig, Egypt and were fed in the animal house of the Faculty of Agriculture. All animals were kept under normal healthy conditions on a basal diet for 1 week. The basal control diet and the experimental diets are shown in Table 1. In the treatment diets, 30% freeze-dried potato replaced an equivalent amount of cornstarch. Water and diet were provided ad libitum. After feeding on a basal diet for 1 week, the rats were divided randomly into four groups (n = 12) according to the following scheme: group 1 (the control group, which was fed on basal diet only); group 2 (fed on a basal diet with replacement of 30% cornstarch in control diet by freeze-dried NGMP); group 3 (fed on a basal diet with GMP G2); and group 4 (fed on a basal diet with GMP G3).

During the experimental period (30 days), the rats were kept separately in well-aerated cages. The diet consumed and body weight were recorded daily

<table>
<thead>
<tr>
<th>Ingredient (%)</th>
<th>Control diet*</th>
<th>Experimental diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1</td>
<td>Group 2</td>
</tr>
<tr>
<td>Casein (≥80%) protein</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>55</td>
<td>25</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Spunta control NGMP</td>
<td>–</td>
<td>30</td>
</tr>
<tr>
<td>Spunta GMP G2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Spunta GMP G3</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Reeves et al. (1993).

NGMP, nongenetically modified potatoes; GMP, genetically modified potato.
throughout the experimental period and were used to compute and compare growth performance parameters (body gain, food intake, final body weight, daily body weight gain and feed efficiency). After the end of the experimental period (30 days), all feeds were removed. The animals were slaughtered at 0, 6, 12 and 24 h following feed removal and the internal organs (liver, kidney, spleen, heart and testes) of each rat were removed and weighed. At the end of the experimental period, the excrement was collected from all groups, freeze-dried and ground in an electric mill. After blood collection, the rats were slaughtered and sacrificed. The carcasses were opened under sterile condition to avoid any contamination. The gut was tied off with sterile surgical silk at the distal part of the esophagus and at the end of the rectum. Then, the gut was dissected carefully with further tying off to prevent any exposure of digesta. The gut was removed intact, laid on a flat surface and the digesta from each section (esophagus and stomach, duodenum, jejunum, ileum, cecum and rectum) were carefully squeezed to obtain the digest from different parts of the gut separately. Small parts of liver, kidney, spleen, heart, testes, lung and skin, as well as small parts of the muscles, were collected. All collected samples from the gut contents and organs were stored at −70°C until DNA investigation.

Because the amounts of digesta in different segments of the gut varied appreciably and some sections were completely empty of digesta, pooling of the samples collected at the same time of slaughter within the same group was carried out prior to DNA analyses. Pooling of the organ samples was also applied prior to PCR to minimize the errors associated with individual variation.

DNA Extraction and Purification from Organs, Digesta of Different Parts of the GIT and Feces of Rats

One hundred milligrams wet weight of digesta of different parts of GIT was subjected to total DNA extraction using the hexadecyltrimethylammonium bromide method (Anon 2002) followed by purification of the isolated DNA using a commercial kit (no. 02103-892-230, QIAquick DNA Clean-up, QIAGEN GmbH, Hilden, Germany). The concentration of the isolated DNA was measured fluorometrically using Dynaquant 200 system fluorimeter (Hoefer, San Francisco, CA) according to the manufacturer’s instructions. The quality of extracted nucleic acid was controlled by agarose gel electrophoresis. The DNA concentration was adjusted between 25 and 30 ng/μL prior to PCR.

DNA Extraction and Purification from Feces

DNA from feces was extracted using the QIAamp DNA stool mini kit (cat. no. 02103-892-230, QIAGEN GmbH). All samples were lysed in specific buffer, proteins were digested using proteinase K and DNA was purified on QIAamp spin columns.
Determination of the Concentration and Quality of DNA Extracted from Organs, Excreta and GIT Contents

The concentration of the isolated DNA was measured fluorometrically using a Dynaquant 200 system fluorimeter (Hoefer) according to the manufacturer’s instructions. The quality of nucleic acid extracted was controlled by agarose gel electrophoresis as well. The extracted DNA was diluted prior to PCR to obtain 30 ng/μL, using bidistilled, deionized, sterile water (Fluka, Buchs, Switzerland).

DNA Analysis of Diets, Digesta, Tissue and Feces by PCR

Primer and probes were designed using the software primer designer ABI PRISM, Primer Express version 1.0. using a special software (Mac Vector version 6.0). All primer pairs and probes used with their specific target are listed in Table 2. All primers and probes were synthesized by TIB MOLBIOL (Berlin, Germany) and obtained in a lyophilized state. The primers and probes were dissolved in sterile distilled water (Fluka) to obtain a final concentration of 20 pmol/μL.

Oligonucleotides for the Investigation of Diets

Diets were analyzed for the presence of potato and GMP using primer and PCR systems as described in Table 2.

Oligonucleotides for the Investigation of Digesta and Tissue Samples

Primer and Probes for the Detection of Transgenic Potato Spunta DNA. The presence of GMP G2 and G3 in the GIT and tissue samples was analyzed by using the primer pair 35S1-f/Spu-cryVm1-r with a detection probe which was designed to be suited for analysis on a real-time PCR machine. Two additional primer pairs (RR02/CryVm-Pr; Spu-35S-f/CRYVM-P-r3) (Table 2) have been designed. The position of these primer pairs within the Cry V gene is indicated in Fig. 1. The conditions for the amplification of DNA specific for GMP Spunta lines G2 and G3 by real-time PCR were studied, optimized and applied for further investigation.

Primer and Probes for the Investigation of Plant- and Potato-specific Chloroplast DNA from Tissue. To design a chloroplast-specific primer pair for the detection of potato, DNA from potato, soybean, tomato and maize was extracted and PCR was performed by using the primer pair A1/A2 (Fig. 2 and Table 2). This primer pair has the code number (210/211). The PCR with this primer pair was done under conditions as described by Elsanhoty (2004) at a
<table>
<thead>
<tr>
<th>Primer and probe</th>
<th>Sequence</th>
<th>Fragment length</th>
<th>Target element</th>
</tr>
</thead>
<tbody>
<tr>
<td>MY-f</td>
<td>5′-TTg TgC AAA TCC TgA gAC TCA T-3′</td>
<td>100 bp</td>
<td>Mammals, avian and reptile myostatin gene (aminotes)*</td>
</tr>
<tr>
<td>MY-r</td>
<td>5′-ATA CCA gTg CCT ggg TTC AT-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spu-35S1-f/Spu-cryVm1-r</td>
<td>5′-CTT CgC AAg ACC CTT CCT C 3′ 5′-gCT ggA gAA CgA TgA Tg C 3′</td>
<td>122 bp</td>
<td>CaMV promoter and Cry V gene</td>
</tr>
<tr>
<td>Spu-CryVm-Probe RRO2</td>
<td>5′-TCA TTT CAT TTg gAgA ACA Cgg g 3′ 5′-TCC TTC gCA AgA CCC TTT CTC-3′</td>
<td>504 bp</td>
<td>CaMV promoter and Cry V gene</td>
</tr>
<tr>
<td>CryVm-Pr</td>
<td>5′-ggA gTC gTg gTA gTC AgC CAG-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spu-35S1-f</td>
<td>5′CTT CgC AAg ACC CTT CCT C-3′</td>
<td>1000 bp</td>
<td>CaMV promoter and Cry V gene</td>
</tr>
<tr>
<td>CRYVM-M-P-r3</td>
<td>5′-ggg TgT CgT AgC TCg ggA AC-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cp-po2-f</td>
<td>5′CTC TTT ACA TCg AAA CTT CAg AAA g-3′</td>
<td>111 bp</td>
<td>Potato chloroplast DNA</td>
</tr>
<tr>
<td>CP-po2-r</td>
<td>5′AAA CTA Cgg ATT Cgg gTC g-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cp-Po-Probe</td>
<td>5′AAA AgA ATg AAg TgA Agg ATA AAC gTA TAT A-3′</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Laube et al. (2003).
FIG. 1. SEQUENCE OF Cry V GENE IN POTATO
concentration of 5 pmol/μ each. Amplicons were cut from the agarose gel, DNA was extracted by using the QIA quick gel extraction (commercial kit produced by QIAGEN, cat. 28706) and subjected to sequencing-cycling. The reaction mix and the sequencing-cycling program are listed in Table 3 and Table 4. The DNA resulting from sequencing-cycling was precipitated and

FIG. 2. ALIGNMENT OF THE INTRONS OF THE tRNA LEUCINE GENES ENCODED BY THE CHLOROPLAST DNA FROM POTATO, TOMATO, MAIZE AND SOYBEAN AMPLIFIED WITH A UNIVERSAL PRIMER PAIR

The DNA was isolated, amplified and sequenced. The universal primer used for amplification was A1/A2 as described by Taberlet et al. (1991). For alignment, the MacVector software was used.
cleaned from deoxynucleoside triphosphates (dNTPs) and subjected to sequencing as described by Elsanhoty (2004). Chloroplast DNA alignment to identify differences between potato and tomato, maize and soybean sequences was done in silica (Fig. 3). Figure 4 indicates the potato chloroplast DNA sequence obtained with primer pair A1/A2 and the selected primer pair position Cp-Po2-f/Cp-Po2-r for potato chloroplast-specific detection giving rise to a 111-bp fragment. The sequence of the corresponding newly designed probe is included in Table 2.

**Primer for Amplification Control.** Primer pair (MY-F/MY-R) (Table 2) was used as a control for DNA in blood and tissue samples. The DNA fragment amplified using MY-f/MY-r/MY is of 97-bp size as described by Laube et al. (2003).

**PCR Conditions for the Analysis of DNA Extracted from Potatoes and Diets Using Primer Pairs 35S1-f/Spu-cryVm1-r and A1/A2**

PCR was carried out on a GeneAmp PCR system 2400 (Perkin Elmer, Wellesley, MA). For the primer pair 35S1-f/Spu-cryVm1-r, the master mix contained 2.5 μL PCR buffer (10× concentration, Perkin Elmer), 2 μL MgCl₂ solution (25 mM), 2 μL dNTPs solution (0.2 mM each of 2’-deoxyadenosine 5’-triphosphate [dATP], deoxycytidine triphosphate [dCTP], deoxyguanosine

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount (μL)</th>
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<tbody>
<tr>
<td>API premix</td>
<td>6</td>
</tr>
<tr>
<td>Primer (5 pmol/μ)</td>
<td>1</td>
</tr>
<tr>
<td>Samples DNA (30–100 ng)</td>
<td>5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>8</td>
</tr>
<tr>
<td>Total volume</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table 3.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
<th>Time (min)</th>
<th>Temperatures</th>
<th>Cycles</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Start</td>
<td>2:00</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>0:10</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>0.05</td>
<td>54</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>4</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Cooling</td>
<td>∞</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.**

SAFETY ASSESSMENT OF GMP SPUNTA
FIG. 3. SEQUENCE OF THE INTRON OF THE tRNA LEUCINE GENE FROM POTATO
The primers specific for the intron of the tRNA leucine gene of the potato chloroplast chromosome are indicated by arrows.

FIG. 4. DETECTION OF PLANT DNA USING THE UNIVERSAL PRIMER PAIR A1/A2 IN EXPERIMENTAL DIETS AND POTATO SAMPLES
Lanes 1 + 12: 50 bp DNA ladder; lane 2: DNA from genetically modified potato (GMP) Spunta G2; lane 3: DNA from nongenetically modified potatoes (NGMP) Spunta; lane 4: DNA from GMP Spunta line G2; lane 5: DNA from GMP Spunta line G3; lane 6: DNA from NGMP Spunta; lane 7: DNA from control diet with NGMP Spunta; lane 8: DNA from diet with GMP Spunta G2; lane 9: DNA from diet with GMP Spunta G3; lane 10: polymerase chain reaction control without DNA; lane 11: extraction control.
triphosphate [dGTP] and thymidine 5’-triphosphate [dTTP]), 0.5 μM of each primer, 0.4 μL AmpliTaq Gold polymerase (Perkin Elmer) and 2 μL of extracted template DNA (25–30 ng/mL) completed to 25 μL with water (Fluka). For the primer pair A1/A2, the master mix contained 2.5 μL of 10× PCR buffer (Perkin Elmer), 2 μL MgCl₂ (25 mM MgCl₂), 2 μL dNTP solution (0.2 mM of each dATP, dCTP, dGTP and dTTP), 0.5 μM of each primer, 1 Unit AmpliTaq Gold polymerase (Perkin Elmer) and 2 μL of template DNA (25 ng/μL) or water for control PCR. The PCR was done, and the PCR products were separated on 2% agarose gels.

**Digesta and Tissue Samples**

**PCR Conditions for the Detection of GMP Spunta DNA in Digesta and Tissue Samples by Using Primer Pairs RRO2/CryVm-Pr and Spu-35S1-f/CRYVM-P-r3.** PCR was carried out on a GeneAmp PCR system 2400 (Perkin Elmer). For each series, a master mix was prepared. Each PCR reaction mix of 25 μL total volume contained 2.5 μL PCR buffer (10× concentration, Perkin Elmer), 2 μL MgCl₂ solution (25 mM), 2 μL dNTPs solution (0.2 mM each of dATP, dCTP, dGTP and dTTP), 0.5 μM of each primer, 0.4 μL AmpliTaq Gold polymerase (Perkin Elmer) and 2 μL of extracted template DNA (25–30 ng/mL), and was completed to 25 μL with water. For amplification of both RRO2/CryVm-Pr and Spu-35S1-f/CRYVM-P-r3 systems, the following conditions were chosen: initial DNA denaturation and enzyme activation at 96°C for 10 min, 40 cycles at 96°C for 30 s, 65°C for 30 s, 72°C for 30 s and a final extension step at 72°C for 7 min.

**Real-time PCR Conditions for the Detection of GMP Spunta DNA in Digesta and Tissue Samples Using Primer System 35S1-f/Spu-cryVM1/r/ Spu-CryVm-Probe.** According to Elsanhoty (2004), real-time PCR with the primer pair 35S1-f/Spu-cryVm1-r with Spu-CryVm-Probe was performed on an ABI PRISM 7700 Sequence Detector (real-time TaqMan PCR-Technology) in a final volume of 25 μL per reaction mix, including 5 μL of template DNA, 12.5 μL TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA), 0.25 μL of each primer, 0.125 μL of the corresponding probe and completed to the total volume with sterile bidistilled water (Fluka).

**Real-time PCR Conditions for the Detection of Chloroplast DNA in Digesta and Tissue Samples with Primer System Cp-Po2-f/Cp-Po2-r/Cp-po-probe.** The newly designed primer system Cp-Po2-f/Cp-Po2-r with the probe Cp-po-probe was used on an ABI PRISM 7700 Sequence Detector (real-time TaqMan PCR-Technology) in a final volume per reaction mix of 25 μL, including 5 μL of template DNA, 12.5 μL
TaqMan Universal PCR master mix (Applied Biosystems), 0.25 μL of each primer, 0.125 μL of the corresponding probe and completed to the total volume with sterile bidistilled water (Fluka). The PCR conditions consisted of 50°C for 2-min holding temperature (to prevent contamination, using uracil–DNA glycosylase), then denaturation of DNA at 95°C for 10 min and 45 cycles of 95°C for 30 s followed by annealing temperature at 60°C for 1 min.

PCR products (amplicons) were stained by 2 μL xylene-cyanol dye solution (1-mg xylene-cyanol, 400-mg sucrose and completed to 1 mL with water) and separated on 2% agarose gels containing 0.01% ethidium bromide together with 50-bp DNA marker.

RESULTS

Potato Tubers and Diets

The authenticity of GMP and NGMP was verified by PCR analysis of the DNA from both potato tubers and diets. The primer pair A1/A2 was used with freeze-dried potato ground for feed mixtures. As expected, a 550-bp PCR product was detected in both transgenic and conventional potato, which indicates that the DNA was successfully extracted and amplified (Fig. 4). For the specific identification of GMP Spunta, the primer pair Spu-35S1-f/Spu-cryVm1-r was used resulting in an amplicon of 122 bp. This fragment appeared only in GMP Spunta G2 and G3. Comparable results were observed in the investigation on diets used for feeding in this study (Fig. 5). No cross contamination of nongenetically modified diets with GM material was observed, although the material yielded positive results with the amplification control primer pair A1/A2. The obtained results confirm the observation of Forbes et al. (2000) who mentioned that grinding a plant does not cause significant disruption of DNA.

DNA Extraction

The amount of DNA from different samples was sufficient to give satisfying results after PCR. The results are shown in Fig. 5. DNA from GIT and digesta samples as well as from excreta was extracted as described in the experimental section. Subsequently, the quality of DNA extracted from the GIT samples was tested by using the newly designed potato chloroplast-specific primer pair Cp-Po2-f/Cp-Po2-r. The results are shown in Fig. 6. The quality of extracted DNA from rat organs was tested by PCR using the primer pair My-f/My-r. This primer pair amplifies part of the mammalian and avian chromosomally encoded myostatin gene (Laube et al. 2003). The results are shown in Figs. 6 and 7. These results indicate that the DNA which was
FIG. 5. DETECTION OF GENETICALLY MODIFIED POTATO (GMP) SPUNTA DNA IN DIETS

Analysis was performed by using the primer pair Spu-35S1-f/Spu-cryVm1-r. Lanes 1 + 12: 50 bp DNA ladder; lane 2: DNA from GMP Spunta G2; lane 3: DNA from nongenetically modified potatoes (NGMP) Spunta; lane 4: DNA from GMP Spunta line G2; lane 5: DNA from GMP Spunta line G3; lane 6: DNA from diet with GMP Spunta line G2; lane 7: DNA from diet with GMP Spunta G3; lane 8: DNA from diet with NGMP Spunta; lane 9: DNA from standard diet without potato; lane 10: polymerase chain reaction control without DNA; lane 11: extraction control.

FIG. 6. DETECTION OF POTATO DNA IN SAMPLES FROM THE CONTENTS OF THE GASTRO INTESTINAL TRACT AND EXCRETA

Analysis was performed by using the primer pair Cp-Po2-f/Cp-Po-r. Lane 1: 50 bp DNA ladder; lane 2: polymerase chain reaction control; lane 3: extraction control lane; lane 4: DNA from the content of the stomach; lane 5: DNA from the content of the duodenum; lane 6: DNA from the content of the jejunum; lane 7: DNA from the content of the cecum; lane 8: DNA from the content of the ileum; lane 9: DNA from the content of the rectum; lane 10: DNA from the content of the excreta; lane 11: DNA from genetically modified potato Spunta.
extracted from diets, samples collected from different sections of the GIT and organs of rats can be amplified by using PCR technique.

**Fate of Potato DNA in Rat Organs**

All investigated rat tissue samples revealed positive results in PCR with primer pair MY-F/MY-R (Fig. 7). The obtained results confirmed that the DNA was successfully isolated from different tissues and is amplifiable during PCR. The same samples were examined for the presence of potato chloroplast gene by using the CP-po2-f/CP-po2-r/CP-po-probe real-time primer/probe system, and for GMP Spunta with the Spu-35S1-f/Spu-CryVm-r/Sp-Cryvm real-time primer/probe system. In contrast to the results obtained using the tissue-specific primer pair MY-F/MY-R, all investigated tissue samples gave negative results in GMP-specific real-time PCR. However, the muscles, liver, spleen, kidney, testes and skin samples from groups 2–4, the plant potato DNA (chloroplast DNA) was successfully amplified in the samples collected at 0, 6, 12 and 24 h after feed withdrawal (Fig. 8 and Table 5).

**Fate of Potato DNA in Digesta**

The fate of potato DNA in digesta samples was investigated using potato chloroplast-specific primer system CP-po2-f/CP-po2-r. An additional probe allows a very sensitive online detection of the amplified target DNA, and hence, is best suited for the analysis of DNA in possibly higher degraded state.
The plots generated by real-time PCR represent the standardized \( \Delta R_n \) value (normalized reporter dye fluorescence) as a function of the number of cycles. Cycle threshold (\( C_T \)) is inversely proportional to the number of template copies present in the reaction sample; therefore, the higher the initial amount of genomic DNA tested, the sooner the accumulated product is detected in the PCR process and the lower the value of the \( C_T \).

**TABLE 5.**
DETECTION OF DNA FROM tRNA LEUCINE GENE IN TISSUE SAMPLES (\( n = 3 \)) BY REAL-TIME POLYMERASE CHAIN REACTION (PCR)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Group 2*</th>
<th>Group 3*</th>
<th>Group 4*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slaughtering time (h)</td>
<td>Slaughtering time (h)</td>
<td>Slaughtering time (h)</td>
</tr>
<tr>
<td>0</td>
<td>6</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>Liver</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Kidney</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Testes</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Spleen</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Heart</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Lung</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Skin</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Muscle</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

* The hours indicate the time difference between the last feeding and slaughtering. DNA was extracted and analyzed by real-time PCR as described in the Materials and Methods section.
To investigate the passage and degradation of potato DNA in the different sections of the rat GIT in all groups fed on diet containing potato (2–4), the rats were slaughtered at different times after feed withdrawal. The primer probe system CP-po2-f/CP-po2-r/CP-po-probe gave a positive signal in all collected digesta samples from all groups described previously (data not shown). The relative concentration of potato-specific DNA in stomach was high when the feed was offered continuously until slaughter. In the duodenum, the concentration slightly decreases because the duodenum is the shortest and narrowest part of the intestinal tract and the time of passage in the duodenum is only about 10 min. In addition, digesting enzymes, including DNAse and RNAse, attack the digesta at the duodenal level. At the jejunal and ileal levels, which are the sites of absorption, the relative concentration of the short potato DNA fragments (111 bp) is relatively increased, which indicates that DNA is on the way to degradation. The content in the caeca and rectum might have been affected by the excretion activity of the rats, which frequently occurred and might have influenced the results obtained for this section of the GIT. Even samples collected from the GIT contents of rats slaughtered at 12 h after last feeding (groups 2–4) revealed positive results in potato-specific real-time PCR. However, the results obtained appear at high $C_T$ which may be caused by excretion or partial degradation of the target DNA during this period. From the obtained results, it is clear that potato DNA resists significant degradation or absorption in the intestine and is excreted via the fecal matter. The results are in line with a recent study by Reuter and Aulrich (2003) who concluded that feed-ingested DNA is partially resistant to the mechanical and enzymatic activities of the GIT and is not completely degraded.

In a second step, the digesta samples collected from both groups 3 and 4 fed on GMP Spunta G2 and G3 DNA were also amplified by using the Spu-35S1-f/Spu-CryVm-r/Spu-CryVm-probe system to detect whether GM-specific DNA is present. The obtained results were similar to those using the potato-specific primer CP-po2-f/CP-po2-r/CP-po-probe system. In addition, the primer pairs RR02/CryVm-Pr and Spu-35S1-f/CRYVM-P-R3 were used (Table 2) to detect GMP Spunta DNA in samples collected from different sections of the GIT. These primer pairs are construct specific, binding to the 35S promoter and the Cry V gene, but in contrast to the primer pair spu-35S1-f/SpuCryVm-r they are giving rise to much larger PCR products of 504- versus 1000-bp size. The aim of this additional investigation was to observe if relatively large gene fragments are passing the different parts of the GIT. Fragments up to 1000-bp size of the GMP Spunta DNA were detected in the samples collected at 24 h after feeding withdrawal (Fig. 9), thus resisting digestion and subsequent absorption in rat gut. These results confirm that the absorption of functional large size DNA was low and these findings are in agreement with previous results published by Doerfler et al. (1997) and Beever
and Phipps (2001). A Bt gene fragment was observed in pig GIT at various time up to 48 h after the last feeding of a diet containing Bt maize (Reuter and Aulrich 2003).

**DISCUSSION**

DNA is an essential component of all living organisms and as such, is present in nearly all foods and feedstuffs. In biotech crops, the introduced transgenic DNA molecules possess the same basic chemical components as the endogenous DNA (adenine, guanine, thymine and cytosine). Therefore, the introduction of transgenic DNA into a plant does not introduce any new chemical entities to foods wherein the total DNA in food contributes less than 0.02% to the total dry matter of the food (Watson and Thompson 1988). Similarly, the amount of transgenic DNA in plants manipulated through biotechnology represents a small proportion of the total amount of DNA in a biotech plant (<0.0004% of the total plant DNA) (Beever and Kemp 2000). Furthermore, the protein-coding genetic sequence introduced in a plant by biotechnology is only functional when the complete DNA sequence (gene) is activated in the plant as a complete gene without any degradation. A recent
publication describes experiments that directly tested whether extensive feeding of DNA to mice results in detectable expression of mRNA and protein in organs of the animals (Hohlweg and Derfler 2001). Approximately, 50 mg of DNA, which encoded the green fluorescent protein (GFP), was fed to 21 mice for 3 weeks and in a separate experiment 50 mg of the pEGFP-C1 DNA per day was fed to mice over eight generations. There was no GFP protein or mRNA expression detectable in liver, spleen, blood or intestinal epithelia of animals. Also, fragments of the GFP gene were not detectable by PCR analysis of DNA isolated from the spleen, liver or tail tip samples from either the 3-week feeding study or that extended to eight generations. Another experiment used a gene therapy approach with intramuscular injection into mice of the GFP gene. These gene therapy studies showed clearly that detectable expression of the GFP protein and mRNA occurred only at the site of injection (Hohlweg and Derfler 2001).

It can be concluded from these studies that gene constructs capable of functioning in vivo when administered via a gene therapy procedure (e.g., intramuscularly injection) do not lead to gene expression in somatic cells or detectable integration into the germ line of animals when provided orally. Feed processing procedures and food preparation methods result in significantly degraded DNA, especially techniques that involve heating at high temperatures or acid treatment (Ebbehøj and Thomsen 1991; Meyer et al. 1994; Forbes et al. 2000) or extraction processes (Berger et al. 2003). For example, the stability of transgenic DNA in maize preserved as silage has been studied (Hupfer et al. 1999). The intact transgenic was only detectable during the first 5 days of ensiling, with small fragments (200 bp). The rapid breakdown of DNA during ensiling was expected as concluded by Hupfer et al. (1999). Thus, feed generated by treatments reduces dietary exposure to intact DNA, even before ingestion and further degradation by the digestive system.

CONCLUSION

In this investigation, an endogenous chloroplastic gene was used as an internal control to determine whether uptake into rat organs is possible. The chloroplastic genome is present in very high copy number relative to the transgene being investigated (probably well over 100 copies per cell versus about one transgene copy per cell). This is presumably why the endogenous gene was detected and the transgene was not detected. The control was chosen to ensure that if any uptake into rat organs is possible, then it would be detected by the analytical approaches used in the study. From the results obtained in the present study together with other findings, it can be con-
cluded that feed-ingested DNA is partially resistant to the mechanical, chemical and enzymatic activities of the rat GIT and is not completely degraded. Survival of modified and nonmodified potato Spunta DNA was comparable during feed passage in rat GIT. Small DNA fragments derived from plant feeds (chloroplast DNA) can pass the gut epithelium and enter some organs of rats. Modified constructs from GMP Spunta lines G2 and G3 fragments DNA could not be detected in any investigated tissue samples despite the fact that the diets were offered in the form of mash, without degradation of GMP and chloroplast plant DNA. PCR and primer-amplifying plant-specific (chloroplast DNA) or amplifying modified construct in GMP Spunta fragments could be a practical tool to determine the rate of feed passage and degradation.

REFERENCES


