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Degradation of transgenic DNA from genetically modified soya and maize in human intestinal simulations

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The inclusion of genetically modified (GM) foods in the human diet has caused considerable debate. There is concern that the transfer of plant-derived transgenes to the resident intestinal microflora could have safety implications. For these gene transfer events to occur, the nucleic acid would need to survive passage through the gastrointestinal tract. The aim of the present study was to evaluate the rate at which transgenes, contained within GM soya and maize, are degraded in gastric and small bowel simulations. The data showed that 80 % of the transgene in naked GM soya DNA was degraded in the gastric simulations, while no degradation of the transgenes contained within GM soya and maize were observed in these acidic conditions. In the small intestinal simulations, transgenes in naked soya DNA were degraded at a similar rate to the material in the soya protein. After incubation for 30 min, the transgenes remaining in soya protein and naked DNA were 52 (SEM 13·1) % and 34 (SEM 17·5) %, respectively, and at the completion of the experiment (3 h) these values were 5 % and 3 %, respectively. In contrast to the soya transgene, the maize nucleic acid was hydrolysed in the small intestinal simulations in a biphasic process in which approximately 85 % was rapidly degraded, while the rest of the DNA was cleaved at a rate similar to that in the soya material. Guar gum and tannic acid, molecules that are known to inhibit digestive enzymes, did not influence the rate of transgene degradation in soya protein. In contrast guar gum reduced the rate of transgene degradation in naked soya DNA in the initial stages, but the polysaccharide did not influence the amount of nucleic acid remaining at the end of the experiment. Tannic acid reduced the rate of DNA degradation throughout the small bowel simulations, with 21 (SEM 5·4) % and 2 (SEM 1·8) % of the naked soya DNA remaining in the presence and absence of the phenolic acid, respectively. These data indicate that some transgenes in GM foods may survive passage through the small intestine.

Genetically modified foods: Intestinal degradation: Quantitative polymerase chain reaction

The recent development of technologies that enable arable crops to be genetically modified (GM) has resulted in the inclusion of GM foods in the human diet. Many of the modifications introduced into plants by this technology are designed to confer resistance to herbicides and pesticides. Typical examples are the glyphosphate-resistant variants of soya (Padgette *et al.* 1995), and pesticide-tolerant maize strains that express *Bacillus thurinogenesis* δ-toxin (Bt maize; Koziel *et al.* 1993). One of the major concerns in respect of GM foods is their possible threat to human

Abbreviations: bp, base pairs; Bt maize, pesticide-tolerant maize expressing *Bacillus thurinogenesis* δ-toxin; GG, guar gum; GM, genetically modified; MM, maximizer maize; PCR, polymerase chain reaction; QC-PCR, quantitative competitive polymerase chain reaction; RRS, Roundup Ready soya; TA, tannic acid.

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health. Some of the current GM foods contain antibiotic resistance genes, which could contribute to the spread of pathogenic micro-organisms that are immune to the antimicrobial agents currently available. In addition, there is a general concern that the transfer of plant-derived transgenes to the resident intestinal microflora could have safety implications.

Intuitively one would predict that the chance of vertical gene transfer from plants to micro-organisms in the gastrointestinal tract is extremely slight, due to the high levels of the pancreas-derived DNAase in the small intestine. This view is supported by Maturin & Curtiss (1976) who showed that bacterial DNA was rapidly degraded in the rat intestinal tract. In contrast, mammalian DNA infused into the duodenum of calves was detected in the distal ileum (Maturin & Curtiss, 1976). Further evidence indicating that a proportion of dietary DNA can survive passage through the gastrointestinal tract is provided by two recent studies (Schubbert *et al.* 1994, 1997), which showed that bacteriophage M13 DNA, fed to mice, was detected in the faeces, indicating that a proportion of the DNA survives in the gastrointestinal tract. In addition, this group detected the phage DNA in the liver demonstrating that the nucleic acid was able to pass through the intestinal epithelium into the systemic circulation. These studies cast doubt on the assumption that plant-derived transgenes will not transfer to the intestinal microflora because the nucleic acid will be rapidly and completely degraded by the digestive enzymes.

In evaluating the safety of GM foods there is need to determine the stability of the transgenes in food within the gastrointestinal tract. The objectives of the present study were to investigate (1) the rate at which two heterologous genes, present in GM soya and maize, were degraded when exposed to *in vitro* gastric and small intestinal simulations, and (2) how components of the diet that are known to influence intestinal function, affected this process.

Material and methods

In vitro digestions

The sources of transgenic DNA were seeds of Bt maize (Maximizer maize; MM) kindly supplied by Monsanto UK Ltd., London, and a commercial texturised soya protein containing 10 % of GM herbicide (glyphosate)-resistant soyabeans (Roundup Ready soya; RRS). These were chosen as examples of GM foods consumed in the USA and in Europe. A 50 g sample of each food source was ground to a fine powder, checking frequently for overheating of the samples.

Guar gum (GG) and tannic acid (TA) were purchased from Sigma-Aldrich Company Ltd. (Fancy Road, Poole, Dorset, UK) and dissolved in 120 mM NaCl to a final concentration of 8 mg/ml and 1 mg/ml, respectively. The proteinase, porcine pepsin (800–2500 units/mg protein), was purchased from Sigma Chemical and used without further preparation. Ileal digesta was obtained from five ileostomised volunteers and the material subsequently lyophilised and pooled. Shortly before use, 100 mg pepsin were dissolved in 2·5 ml of 120 mM NaCl. Peptic and intestinal digestions were carried out in an orbital incubator.

The *in vitro* simulation of human digestion was modified from that used by Glahn *et al.* (1996). A 50 mg sample of ground maize seeds or texturised soya protein, or naked genomic-DNA extracted from an equivalent amount of soya protein (approximately 48 μg genomic DNA) was resuspended in 500 μl of 120 mM-NaCl, NaCl containing GG and NaCl containing TA. To these mixtures, HCl and pepsin were added to final concentrations of 15 mM and 2 mg/ml, respectively. The tubes were then capped, placed horizontally and incubated in an orbital incubator at 37°C and 150 rpm for 60 min. Once the peptic digestion was completed the pH was neutralized by adding $NaHCO_3$ to a final concentration of 50 mM followed by 50 mg of freeze-dried ileal digesta and 200 μl of Milli-Q water to simulate intestinal digestion. Samples were incubated for 5, 10, 15, 30, 60, 120 and 180 min (separate tubes for each time point). At the completion of each digestion time, 800 μl of 6 M guanidine hydrochloride were added to stop enzymic activity. The capacity of this concentration of guanidine hydrochloride to inhibit enzymic degradation of DNA was tested previously (data not shown). To evaluate the degradation of transgenic DNA after digestion with pepsin-HCl, guanidine hydrochloride was added to one reaction after pH neutralisation and ileal digesta was added subsequently. In the time 0 sample, all the components of the reaction, including guanidine hydrochloride, were added to the tube before the addition of the DNA-containing material. As a negative control one tube with all the reactants except the DNA-containing material was treated in the same way as the test samples. Once the reactions had finished and been treated with guanidine hydrochloride, they were placed on ice until DNA was extracted.

Incubations of soya protein and naked DNA with and without GG or TA were always performed in the same batch. Each incubation was repeated at least twice in different experiments.

Sample DNA extraction and purification

Naked DNA from GM soya protein, used in the *in vitro* digestions, was extracted from 100 mg of ground material resuspended in 400 μl of water and 600 μl of 6 M guanidine hydrochloride and incubated at 58°C for 3 h with vortexing every 15 min. The mixture was centrifuged at 13 000 ***g*** for 10 min and DNA in the soluble fraction was purified from 500 μl of the supernatant fraction (i.e. equivalent to half of the starting material) using the Wizard DNA clean-up system Protocol (Promega, Madison, WI, USA). The DNA was eluted in 50 μl of sterile Milli-Q water. The DNA from the *in vitro* pepsin-HCl and ileal incubations was extracted and purified following the same protocol.

Primer oligonucleotides

Primers, which were synthesised by the Central Molecular Biology Facility, University of Newcastle upon Tyne, were obtained in a lyophilized state and stored at −20°C. The sequences of the primers used to construct internal DNA standards and to perform quantitative competitive

polymerase chain reactions (QC-PCR; Raeymaekers, 1993) are listed in Table 1 where italicised bases denote restriction sites.

Quantitative polymerase chain reaction

All polymerase chain reaction (PCR) amplifications were carried out on a PTC-100 Programable Thermocontroller (M.J. Research, Inc., Waltham, MA, USA). For each series of reactions, a master-mix was prepared comprising 5 μl 10 × PCR buffer (Perkin Elmer, Vienna, Austria), 10 μl 25 mM $MgCl_2$, 0·4 μl 100 mM deoxynucleoside triphosphate mix (Gene Kraft, Germany), 0·5 μl 25 mM primer, 0·25 μl Amplitaq Gold Polymerase (5 U/μl, Perkin Elmer, Germany), 1 μl 1 mg/ml bovine serum albumin (Boehringer Mannheim, Germany) and 22·4 μl of water. Each reaction contained 5 μl of a 1:5 dilution of the DNA purified from each sample, 5 μl of DNA competitors and 40 μl of master mix. The competitor DNA, in a volume of 5 μl, was added at amounts ranging from 8×10^1 to 25×10^5 copies. As negative controls for the DNA extractions and the amplification reactions, two PCR were carried out; one contained material extracted from ileal contents that had not been incubated with GM soya or maize, the other reaction contained 10 μl of water instead of DNA. After initial denaturation at 95°C for 7 min, fifty cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 45 s were carried out, with a final extension step of 7 min at 72°C. Following the PCR amplification, 15 μl of each reaction mix were electrophoresed on 2% (w/v) agarose gels, stained with ethidium bromide and visualized using a u.v. transiluminator.

Construction of the internal standards

Strategies for construction of the competitor sequence for the QC-PCR are presented in Fig. 1. The complete synthetic *cryIA (b)* gene was amplified from GM-maize DNA using the primers Cry01 and Cry02 described by Hupfer *et al.* (1998), and cloned into pCR-Blunt (Invitrogen, Leek, The Netherlands) to generate pBNS1. The 509 (bp) product, obtained from GM-soya DNA using the primers RR01 and RR02 described by Studer *et al.* (1998), was cloned into the vector pCR2·1 (Invitrogen) to generate pBNS2. The plasmids containing the competitor DNA

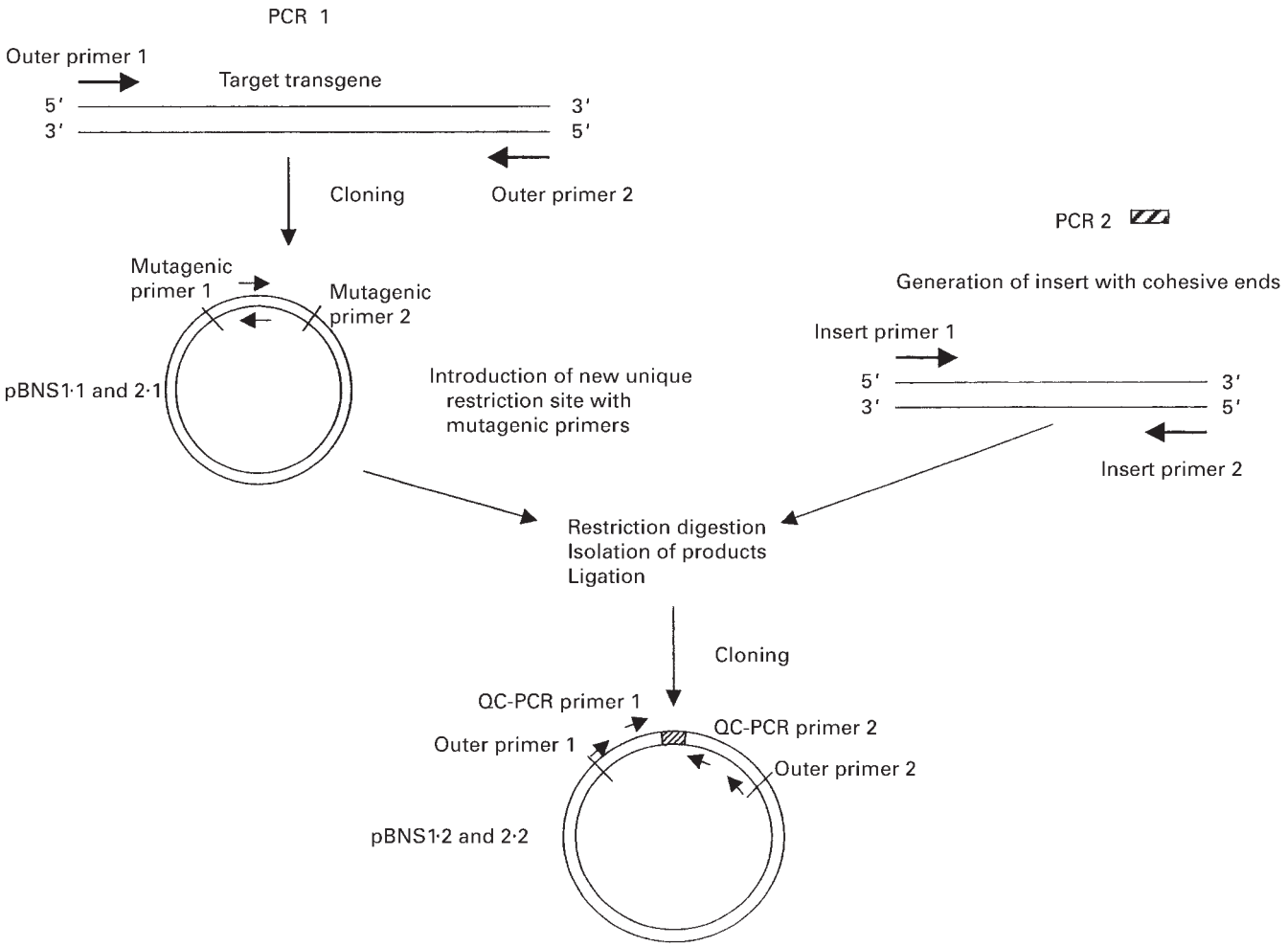

Fig. 1. Scheme for the construction of competitor for use in quantitative competitive polymerase chain reactions (QC-PCR). The primers used to amplify the transgenes and to introduce the restriction site into this sequence are listed in Table 1. The 100 base pairs (bp) sequence inserted into the soya and maize transgene were derived from pET16b and pCR-Blunt, respectively. The sequence denotes the 100 bp insert.

Table 1. Primers for construction of DNA standards and used in quantitative competitive polymerase chain reaction (QC-PCR)

	Name	Sequence (5′-3′)*	Target DNA	Size (bp)	Reference
To construct DNA competitiors					
Maize					
Outer primer	Cry01	ATG GAC AAC AAC CCC AAC ATC	cryIA (b)	1914	Hupfer *et al.* (1998)
	Cry02	GGT CTT CAG GCC GAT CTG GTT			
Mutagenic primer	MTG1	GCC TAG CGG ACA T*CA TAT G*GG GCA TCT TCG GC	pBNS1	5427	Present study
	MTG2	GCC GAA GAT GCC C*CA TAT G*AT GTC CGC TAG GC			
Insert primer	INS1	CAT CAT *CAT ATG* GCC CCA GTG CTG C	pET 16b	112	Present study
	INS2	CAT CAT *CAT ATG* CGC TCG GCC CTT CCG			
Soya					
Outer primer	RR01	TGG CGC CCA TGG CCT GCA TG	CP4 EPSPS, petunia EPSPS CTP	509	Studer *et al.* (1998)
	RR02	CCT TCG CAA GAC CCT TCC TCT ATA	E35S promoter		
Mutagenic primer	MTG3	CGT GAA GCA TGC AGG CT*G TCG AC*A CTG ATG CTG AAA TCC	pBNS2	4416	Present study
	MTG4	GGA TTT CAG CAT CAG T*GT CGA C*AG CCT GCA TGC TTC ACG			
Insert primer	INS3	CAT CAT *GTC GAC* CAG TGC CGT TCC GG	pCR-Blunt	109	Present study
	INS4	CAT CAT *GTC GAC* GAA GTC CCG GGA G			
To perform QC-PCR					
Maize	BT1	TCG ACA TCA GCC TGA GCC TG	cryIA(b)	151	
	BT2	TGG TTG ATC AGC TGC TCG ATC			
Soya	RR04	CCC CAA GTT CCT AAA TCT TCA AGT	E35S promoter petunia EPSPS CTP	180	Studer *et al.* (1998)
	RR05	TGC GGG CCG GCT GCT TGC A			

bp, base pairs; EPSPS, 5-enolpyruvylshikimate-3-phosphatesynthasse.
* Italicised bases denote restriction sites.

were generated by introducing a unique restriction site, *Nde* I for maize (pBNS1·1) and *Sal* I for soya (pBNS2·1), in the middle of each of the cloned amplicons. This was achieved using the Quick Change Site-directed Mutagenesis kit (Stratagene Europe, Amsterdam Zuidoost, The Netherlands) and the mutagenic primers listed in Table 1. Inserts comprising 100 bp flanked by either *Nde* I or *Sal* I restriction sites were generated by PCR using primers listed in Table 1, using pET16b and pCR-blunt vectors, respectively, as DNA templates. Restricted and purified fragments, derived from the PCR, were ligated with previously restricted and dephosphorylated pBNS1·1 and pBNS2·1 (alkaline phosphatase; Boehringer Mannheim-Roche). Plasmids containing the inserted DNA derived from pBNS1·1 and pBNS2·1 were designated pBNS1·2 and pBNS2·2, respectively. The plasmids pBNS1·2 and pBNS2·2 were isolated using the Qiagen Plasmid Midi Kit (Qiagen, Crawley, West Sussex) and sequenced. Plasmid DNA concentrations were determined by measuring A_{260}.

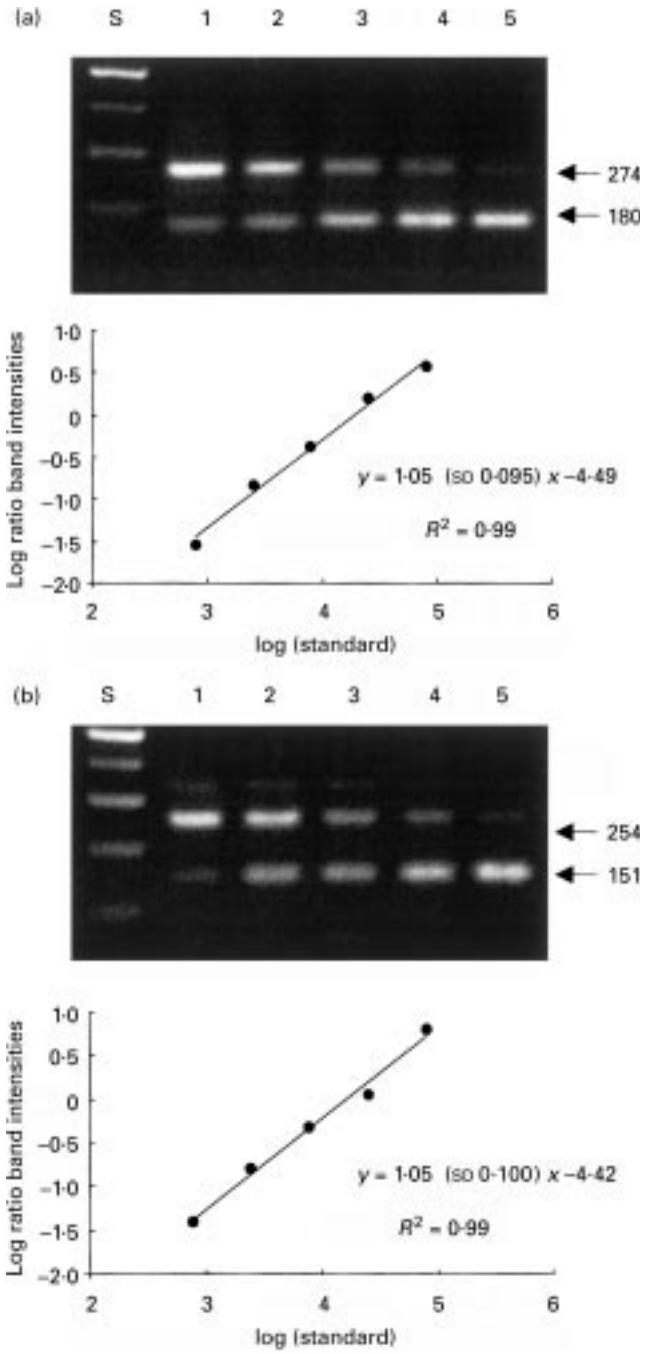

Fig. 2. Determination of transgenic DNA. Content of soya protein at time 0 of incubation (a) and maize meal incubated for 1 h with pepsin-HCl and 2 h with intestinal content (b). The polymerase chain reaction products were subjected to electrophoresis on a 2 % (w/v) agarose gel. Lane S 100-bp ladder, lanes 1–5 competitor added to the sample at 79×10^3, 25×10^3, 79×10^2, 25×10^2 and 790 copies, respectively. For details, see p. 534.

Quantification of polymerase chain reaction products

Gel pictures were digitalized using a charge-coupled device camera. The intensities of the bands were determined by image processing software (Quantity ONE, version 4.0, Bio-Rad, Cambridge, UK) and corrected for their molecular weight. The equivalence point where competitor and sample DNA concentrations were the same was calculated by the linear regression between the logarithm of the ratio of corrected band intensities of the two DNA species, and the logarithm of the initial amount of target DNA. An example calculation is shown in Fig. 2.

Experimental design

Four batches of incubations containing soya protein and four with naked soya genomic DNA were carried out. In two of them, samples were incubated simultaneously with and without TA and in the other two with and without GG. For maize, four duplicates were carried out in four independent batches.

Results of degradation of transgenic soya protein and maize, and naked genomic DNA were statistically analysed using the General Linear Model in SAS (SAS Institute, 1996). Before analysis, data were log-transformed as necessary to achieve equal variance. The model used was:

$$Y_{ijkl} = \mu + P_i + I_j + T_k + (P \times I)_{ij} + (P \times T)_{ik}$$

$$+ (P \times I \times T)_{ijk} + E_{ijkl},$$

in which P, the protective effect of the food (soya protein *v.* naked DNA); I, the inhibitory effect of GG or TA; T, the time of incubation were considered as fixed effect factors (Sokal & Rohlf, 1995). The residual error term derived from variation between replicates was used to test each of the fixed effect factors and their interactions.

Results

Quantitative competitive polymerase chain reaction

To detect and quantify transgenic DNA from RRS and MM in the *in vitro* gastric and intestinal digesta, a QC–PCR method was developed where the two competitor DNA molecules contained an extra 100 bp of sequence. The region of the transgenes amplified from the soya and maize were 151 and 180 bp, respectively. This relatively

small size was chosen because it increases the sensitivity of the method as PCR are more efficient at amplifying short sequences of DNA, particularly where fragmentation of the DNA is expected to be high. The primers BT1 and BT2 amplified a fragment of the synthetic *cryIA(b)* gene introduced into maize, while the RR04 and RR05 primers amplified a fragment of the transgene inserted into the soya genome comprising regions of the CP4 5-enolpyruvylshikimate-3-phosphatesynthase gene from *Agrobacterium* sp. strain CP4 and the 5-enolpyruvylshikimate-3-phosphatesynthase *Petunia* CTP gene. To confirm that the DNA amplified by the QC-PCR comprised the target sequence, the PCR product was sequenced directly using the primers BT1 and BT2, and RR04 and RR05 for the maize- and soya-derived transgenes, respectively. The data (not shown) revealed that the amplified DNA contained the appropriate sequence, confirming that the QC-PCR amplified the target transgenes. To ensure that no false positive PCR products, due to contamination of reagents or extraction buffers with target DNA, were detected, two negative controls were carried out, neither of which generated a PCR product in any of the assays performed.

An example of the quantification method for each of the transgenes is shown in Fig. 2. The validity of the two detection systems is indicated by the high regression coefficient value (r^2), which was $>0{\cdot}99$, and the slope of the regression line that was close to unity. To further validate the QC-PCR method, the linearity of the system was tested by using DNA derived from 50 or 5 mg of soya or maize in the QC-PCR. The data showed that the total copy number for each sample was 21×10^6 and 25×10^5 for soya and 10×10^7 and 9×10^6 for maize, for 50 and 5 mg respectively. The detection limit of the assay was assessed by performing PCR on serial dilutions of the competitors. For both systems approximately eighty copies of the transgene could be detected clearly. Given that $>100\,000$ copies of the transgenes were present at time 0, the QC-PCR could detect down to about 0·08 % of added transgenic DNA.

Incubation of DNA with pepsin-HCl

The GM foods were incubated with the gastrointestinal simulations, without any additional treatments, to investigate protection of the DNA by the food matrices. To study the persistance of naked DNA in gastric and small-intestinal environments, nucleic acid extracted from the RRS was used as the source material. Prior to incubation under the simulated gastrointestinal conditions, the total amount of transgene present in these samples was determined. RRS contained approximately 10-fold less transgene per mg DNA than did MM maize. This is likely to reflect the source of the material. RRS was purchased from supermarkets and is thus a blended product containing only a small proportion of transgenic material. In contrast, maize seeds were obtained directly from Monsanto and were completely transgenic.

The size of DNA extracted from soya and maize were quite different. The maize nucleic acid was of high molecular weight while the soya-derived material had been extensively fragmented, and had a molecular weight that ranged from 100 to 1000 bp. The low molecular weight of the soya DNA is likely to be the result of extensive

Table 2. Degradation of transgenic DNA from soya or naked genomic-DNA incubated *in vitro* with pepsin-HCl with (+GG) or without (−GG) guar gum and with (+TA) or without (−TA) tannic acid as potential DNA protectors*

	Soya		Naked DNA		Statistics	
Time (min)	−GG	+GG	−GG	+GG	Effect	Significance
Initial	5·34	5·26	4·98	5·08	P	†
Digested	5·26	5·34	4·18	4·49	I	NS
					T	NS
RSD = 0·422					I × T	NS
					P × I	NS
					P × T	†
					P × I × T	NS
	Soya		Naked DNA		Statistics	
	−TA	+TA	−TA	+TA	Effect	Significance
Initial	5·26	4·81	4·29	4·12	P	†††
Digested	5·29	4·79	3·67	3·86	I	†
					T	†
					I × T	NS
RSD = 0·193					P × I	†
					P × T	†
					P × I × T	NS

P, protective effect of the food (soya *v.* naked DNA); I, inhibitory effect of GG or TA; T, time of incubation effect.

*Tabulated data are log of copies of the target sequence detected by quantitative competitive polymerase chain reaction. Each polymerase chain reaction included the DNA extracted from 0·3 mg of soya or the equivalent naked genomic-DNA.

†$P<0{\cdot}05$; †††$P<0{\cdot}001$.

processing to generate the soya protein product (KeShun, 1999). The incubation of RRS and MM with pepsin-HCl did not result in a significant decrease in transgene DNA (Table 2). In contrast, when naked DNA was incubated in the stomach simulations there was a large decrease in the amount of transgene DNA with approximately 80 % of the material being degraded. This degradation was not specific to the transgenes as ethidium bromide staining of extracted DNA showed that less total DNA was present after incubation in pepsin-HCl.

Incubation of DNA with ileal digesta

The degradation of GM food-derived DNA in the ileal samples was evaluated by agarose gel electrophoresis of total DNA extracted from the incubations, and by the quantification of transgenic DNA using QC-PCR. The data, presented in Table 3 and Fig. 3, showed that there was progressive degradation of total DNA and the transgenes. Surprisingly, the naked soya DNA was degraded at a rate similar to the transgene in the RRS; after incubation for 30 min the transgenes remaining in RRS and naked DNA were 52 (SEM 13·1) and 34 (SEM 17·5) %, respectively. At the completion of the experiment, after soya protein and naked soya DNA had been incubated with ileal digesta for 3 h, approximately 5 % and 3 %, respectively, of the transgenes (present at the start of the ileal incubations) could still be detected. These data suggest that the food stuff protected DNA against degradation in acidic conditions, but not against digestive enzymes in the small intestine. In contrast to soya DNA, the degradation of the maize nucleic acid in the small-intestinal simulation was a biphasic process in which approximately 85 % was rapidly degraded, while the rest of the DNA was cleaved at a rate similar to that for the transgene in soya (Table 3).

Effects of tannic acid and guar gum on DNA degradation

To investigate the influence of other components of food on the rate of degradation of the transgenes in the intestines, GG and TA were included in the incubations (Table 3). Addition of GG to the incubations did not result in a detectable change in the rate of DNA degradation from soya protein. However, surviving transgenic-DNA was higher in samples of naked-genomic DNA in which GG was added (interaction P × I, $P=0{\cdot}12$; Table 3). This protective effect was apparent only in the initial stages of the incubations (up to 30 min). The amount of DNA remaining at the end of the experiment (180 min) was approximately 3 % and independent of whether GG was added.

The effect of TA on DNA integrity was different between the nucleic acid in soya-protein and the naked genome (interaction P × I, $P<0{\cdot}001$; Table 3). TA promoted a higher apparent rate of degradation of transgenic DNA from RRS which remained constant with time of incubation. In contrast, the rate of naked DNA cleavage was lower when TA was included in the incubations, with differences ranging from $10^{0{\cdot}2}$ to $10^{1{\cdot}6}$ dependent on

Table 3. Degradation of transgenic DNA from soya or naked genomic-DNA incubated *in vitro* with intestinal enzymes for 5–180 min with (+GG) or without (−GG) addition of guar gum and with (+TA) or without (−TA) tannic acid as potential DNA protectors*

	Soya		Naked DNA		Statistics	
Time (min)	−GG	+GG	−GG	+GG	Effect	Significance
0	5·26	5·34	4·18	4·49	P	†††
5	5·27	5·25	4·17	4·57	I	NS
10	5·00	4·97	3·81	4·44	T	†††
15	5·14	5·29	3·89	4·41	I × T	NS
30	4·87	4·94	3·66	4·38	P × I	NS
60	4·73	4·69	3·51	4·14	P × T	NS
120	3·95	4·09	2·79	3·52	P × I × T	NS
180	3·80	3·84	2·35	2·83	RSD = 0·601	
	Soya		Naked DNA		Statistics	
	−TA	+TA	−TA	+TA	Effect	Significance
0	5·29	4·79	3·67	3·86	P	†††
5	5·29	4·76	3·48	4·03	I	††
10	5·21	4·63	3·27	4·03	T	†††
15	5·29	4·78	3·21	3·99	I × T	NS
30	5·06	4·60	2·92	3·76	P × I	†††
60	4·77	4·32	2·80	3·46	P × T	NS
120	4·26	3·91	1·98	3·20	P × I × T	NS
180	3·99	3·52	1·62	3·17	RSD = 0·246	

P, protective effect of the food (soya *v.* naked DNA); I, inhibitory effect of GG or TA; T, time of incubation effect.

*Tabulated data are log of copies of the target sequence detected by quantitative competitive polymerase chain reaction. Each polymerase chain reaction included the DNA extracted from 0·3 mg of soya or the equivalent naked genomic-DNA.

††$P<0{\cdot}01$; †††$P<0{\cdot}001$.

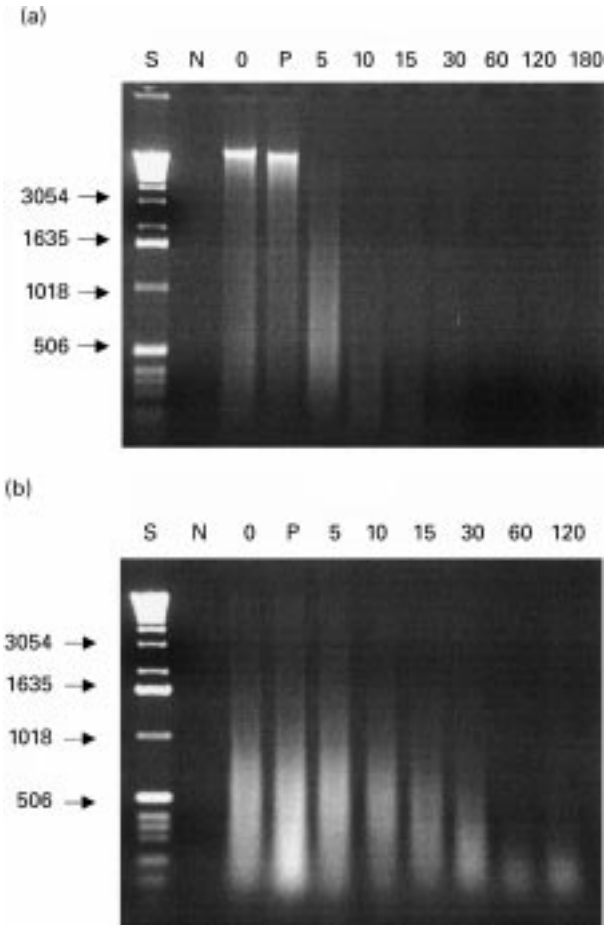

Fig. 3. Degradation of maize and soya DNA. Maize (a) or soya DNA (b) was incubated for 1 h with pepsin-HCl (P) and subsequently for 5, 10, 15, 30, 60, 120 and 180 min with ileal digesta. DNA was separated by electrophoresis on a 1 % (w/v) agarose gel using 10 μl samples. A 1 kb ladder (S) was used as marker and 0 is the DNA at time zero of incubation. For details, see p. 534.

the time of incubation. Naked genomic-DNA nucleic acid remaining after 180 min of incubation was 11·2 (SEM 1·65) % when TA was included compared with 0·4 (SEM 0·23) % in its absence. When values of surviving DNA were compared with the DNA that resisted gastric simulation, percentages of degradation were 20·8 (SEM 3·1) and 2·4 (SEM 1·8) % when genomic-DNA was incubated with ileal digesta in the presence or absence of TA respectively.

Discussion

The objective of the present study was to evaluate the extent to which transgenes in GM foods can survive in the upper gastrointestinal tract. Such information is important when assessing the perceived risk that gene transfer from GM foods to the intestinal microflora could have on human health. In our study we have used two GM foods, MM and RRS, that are freely available to the general public, and QC-PCR to determine the survival of the transgenes. In assessing the validity of the PCR methodology used in the present study, the possible contamination of the samples with minute traces of experimental DNA (especially at the end of the incubations) was investigated by including two negative controls in each batch of incubations, extractions, purifications and PCR. No DNA was detected in the negative controls indicating that the DNA detected was not due to contamination.

The present study shows that naked DNA was extensively degraded in the simulated acid stomach, consistent with the view that acid conditions mediate extensive depurination of nucleic acid (Raw, 1983). In contrast, the nucleic acid in soya protein and maize seeds did not appear to be affected by the acidic conditions, suggesting that the DNA contained within these foods was resistant to depurination. It is possible that the food matrix, in some way, had a buffering effect such that the proton concentration in the micro-environment of the DNA was not as high as in the bulk of the reaction.

Mammalian DNAases from the small bowel have pH optima in the range 4·5–7·7 (Nakayama *et al.* 1981; Nagae *et al.* 1982; Anai *et al.* 1983) and so should have been active at the neutral pH used in the digestion model used in the present study. Degradation of DNA in both maize and soya protein occurred in the small-intestinal simulations, but approximately 4·5 and 0·5 % of soya and maize DNA, respectively, survived after 3 h of these incubations. The kinetics of DNA degradation were different for the two foods; the MM nucleic acid was hydrolysed in a biphasic process, while DNA in the RRS decreased exponentially. These data suggest that the maize DNA existed in two forms; a component that was particularly susceptible to degradation, with the remaining material extensively protected against DNAase action. It is likely that protection of the DNA to enzyme attack is mediated by its encapsulation within the maize cells, with the nucleic acid becoming available for degradation only as the plant cell wall is ruptured. If this hypothesis is tenable, then it is likely that a component of the maize seeds has been disrupted such that the intestinal DNAase can access the DNA contained within these seeds. In contrast, the RRS has already been extensively processed (KeShun, 1999), and the more uniform structure of this food product may explain the uniform sensitivity of the nucleic acid to DNAase attack. Given the extent of processing that led to the production of soya protein, it might be expected that the DNA in this material would be more sensitive to DNAase action than the maize nucleic acid. It would appear, therefore, that the soya processing did not significantly disrupt cell wall structure and thus the plant cell wall could continue to act as an effective barrier to DNA degradation by intestinal enzymes. However, if accessibility was the major factor limiting DNA degradation in the GM foods, it is somewhat surprising that naked soya DNA was hydrolysed, in the small-intestinal simulations, at a rate similar to the nucleic acid in the RRS and the bulk of the MM. A possible explanation for these data is that the depurination of the naked nucleic acid in the pepsin-HCl incubations destroyed the DNA that was particularly susceptible to DNAase attack, i.e. about 85 % of the material. The remaining 15 % of DNA may have been extensively modified within the soya seeds, such that it was resistant to acid and enzyme attack. Modifications

could include very tight association with histones within the highly structured heterochromatin regions of the genome. The heterochromatin structure may not have been disrupted during DNA purification, and would thus be present in the naked DNA used in these experiments. Alternatively, the DNAase-resistant DNA may comprise hypermethylated regions of the nucleic acid, as such regions may be resistant to both depurination and enzymic degradation.

There are very few studies that have investigated the fate of foreign DNA ingested with food in the gastrointestinal tract. Monitoring the fate of naked DNA (thymus) infused into the duodenum of calves showed that only 3 % was detected in the distal ileum (McAllan, 1980). Similarly Maturin & Curtiss (1976) reported that rat small-intestinal contents rapidly degraded bacterial naked DNA with only 0·1 % of DNA remaining after 4 h. These authors also showed that the DNA was degraded much more slowly in the rat large bowel contents, although these assays were not carried out under the anaerobic conditions necessary to ensure viability of anaerobic bacteria which dominate the large bowel. In the Maturin & Curtiss (1976) study, similar results were obtained when the intestinal contents from gnotobiotic and conventional rats were used, suggesting that, under the conditions of their studies, DNA degradation was caused by mammalian enzymes and not microbial DNAases. More recently Schubbert *et al.* (1994) fed M13 bacteriophage DNA to mice and could detect, by dot-blot hybridization, about 2–4 % of the administered DNA in the faecal excretions of mice 1–7 h after feeding. They also detected about 5 % of the phage DNA after incubation with rat intestinal contents *in vitro* for 8 h. A further study by Schubbert *et al.* (1997) reported that 95 % of naked phage DNA was degraded after gastric passage, and 0·7–2·2 % of the nucleic acid remained in the small intestine 1–8 h after feeding. Notwithstanding differences in methodology between these studies, these data suggest that eukaryotic DNA may survive longer in the gastrointestinal tract than prokaryotic-derived nucleic acid. It is possible that the hypermethylation of plant and mammalian DNA, its tight association with histones, and, especially for plant DNA, the protection against ingress of DNAases afforded by the cell wall may provide more protection against DNAase attack than is the case with prokaryotic DNA.

In addition to measuring DNA degradation *per se*, the present study was also designed to evaluate how components in the human diet could influence DNA degradation. The two food sources evaluated were TA and GG. GG is a soluble galactomannan non-starch polysaccharide used widely as a stabiliser and for other technological reasons in many foods. It was chosen as a potential protector of the DNA as it has been reported to retard the digestion and absorption of several nutrients (Cameron-Smith *et al.* 1994; Ehrlein & Stockmann, 1998). TA is a phenolic compound present in numerous foods including green beans, lentils, tea and coffee and is also used as a food additive. It was selected because of its ability to form complexes with proteins including digestive enzymes (Horigome *et al.* 1988), inhibiting their activity (Quesada *et al.* 1996). TA has also been reported to bind strongly to DNA (Khan *et al.* 2000) and this property could, potentially, confer protection to the DNA. The data showed that GG protected naked DNA from degradation during the initial stages of the incubations, but not the nucleic acid in the soya protein. These results are consistent with the capacity of GG to inhibit the action of digestive enzymes. That GG did not protect against nucleic acid degradation in soya protein suggests that the major factor limiting cleavage of this DNA is the integrity of the food matrix, which was not influenced by this polysaccharide.

TA reduced the rate at which naked DNA was degraded, and this protective effect continued throughout the incubation. It is likely that TA mediates its effect by binding to both DNAase directly, thus reducing its catalytic activity, and to DNA making it less available to attack from the intestinal digestive enzymes. In contrast, TA appeared to increase the rate at which DNA in RRS was degraded. The decrease observed in the amount of transgenic DNA after the addition of TA, particularly at time 0, could be due to a reduction in the efficiency of extraction of DNA from these samples. To examine this possibility, the influence of TA on the extraction process was tested by extracting nucleic acid from samples of soya protein and naked genomic-DNA in the presence and absence of the acid. The data (not shown) showed that TA did not affect the extraction process.

Important unresolved questions emanating from the present study include how much of the surviving transgene DNA will be available to intestinal bacteria and whether any of this residual transgenic DNA is taken up by commensal bacteria or by gut epithelial cells. Although it is likely that a high percentage of the DNA will remain intact within unbroken cells in food particles, or will bind strongly to macromolecular complexes, it is probable that much of the nucleic acid will be released during the fermentation of the food residues in the colon. This process takes several hours involving a consortium of prokaryotic enzymes, which degrade plant cell walls (Mathers, 1991) making accessible, at least in part, the DNA, which could then be available for uptake by micro-organisms or colonocytes. While simple juxtaposition of DNA and cells does not lead automatically to incorporation of the DNA into the cellular genome, there is evidence that exogenous DNA can be taken up by bacteria of the intestinal tract (Mercer *et al.* 1999).

Conclusions

The present study showed, as predicted, that naked DNA was rapidly degraded in the *in vitro* gastrointestinal simulations. However, transgenic DNA could be detected readily by QC-PCR at the end of each incubation. Several factors were shown to have an effect on the degradability of the DNA. The food matrix protected the DNA from digestion and GG afforded some protection to naked DNA extracted from soya. TA also increased the survival of naked genomic DNA in the small-intestinal simulations.

The results from these *in vitro* simulations of intestinal digestion suggest that some transgene DNA may survive in the human stomach and small bowel for up to 4 h and provide insights into some dietary factors that affect the

digestion of DNA in the gastrointestinal tract. It should be noted that the QC-PCR assay used in the present study employed relatively short target sequences and leaves open the possibility that, while transgene DNA survived for a considerable period under these simulated gut conditions, the DNA may be in such small fragments as to be of limited biological significance and thus may represent no apparent health risk. However this conclusion should be treated with some caution because, using primers which amplify the complete transgene, we have detected PCR products (albeit at low levels) in digesta from a human feeding study (T Netherwood, S Gockling, J Graham, AG O'Donnell, HJ Gilbert and JC Mathers, unpublished results). Future studies will be needed to determine the stability or otherwise of transgene DNA *in vivo* and also its persistance in the large bowel. In any event, the data from our study support the hypothesis that a proportion of transgene DNA can survive passage through the small intestine, and would therefore be available for uptake by the intestinal microflora or intestinal epithelial cells. In the light of these results the possibility that plant transgenes could be transferred to the human intestinal microflora should be considered.

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