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Detection of transgenic DNA in tilapias (*Oreochromis niloticus*, GIFT strain) fed genetically modified soybeans (Roundup Ready)

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Abstract

We used nested-polymerase chain reaction (PCR) to detect Roundup Ready soybean in aquatic feeds and feeding tilapias. A template concentration of 10^{-10} g μL^{-1} DNA solution could be detected with a dilute degree of 0.01%. Most (90.6%) of the aquatic feeds containing soybean byproduct included exogenous DNA segments. We also compared genetically modified (GM) soybean with non-GM soybean diets in feeding tilapias (*Oreochromis niloticus*, GIFT strain) and examined the residual fragments (254 bp) of GM soybeans. Tilapias receiving GM soybean diets had DNA fragments in different tissues and organs, indicating that exogenous GM genes were absorbed systemically and not completely degraded by the tilapia's alimentary canal.

Keywords: aquatic feed, GM soybean, nested-PCR, *Oreochromis niloticus*, tissues, transfer

Introduction

Many genetically modified organisms (GMOs) are approved globally. The main transgenic crops are soybean, maize and cotton, and their principal traits are tolerance to herbicides and insects (Hugo, Martin & Ruben 2002). Considering further growth in genetically modified (GM) constructs, multiplex methods are needed to determine whether feeds and food contain approved or unapproved GM ingredients. Genetically modified constructs are often composed of common elements such as the *35S* promoter, the nos terminator or an antibiotic resistance gene as selection markers (MacCormick, Griffin, Underwood & Gasson 1998). If these elements are detected, GM material may be present. Reliable polymerase chain reaction (PCR) methods with high specificity and sensitivity are needed. Nested-PCR has good detection sensitivity with fewer false negatives. Here, we developed a method for measuring the presence of GMO in aquatic feeds.

The use of GM crops in fish feed has been controversial in Europe (Kok & Kuiper 2003). Some studies have indicated the equivalence of GM soybean to non-GM soybean varieties (Padgette, Taylor, Nida, Bailey, MacDonald, Holden & Fuchs 1996). Here, we measured the residual fragments of GM soybean in tilapias tissues and organs. During the feeding or continued hunger stages, DNA was extracted to detect exogenous DNA and gene transfer to different tissues and organs.

Materials and methods

Soybean samples

Genetically modified soybeans (Roundup ready) were provided by the Qingdao Entry-Exit Inspection and Quarantine Bureau. Non-GM soybeans were obtained from a seed distributor in Qingdao, China. The soybeans were tested to ensure a similar chemical composition, including crude protein, crude fibre, fat, moisture and ash, among others.

Taq DNA polymerase, 10 × Taq buffer with 50 mM $MgCl_2$, dNTPs, DNA marker DL2000 and 10 × loading buffer were purchased from TaKaRa biotechnol-

ogy (19th, Dongbei 2 road, Economic and Technical Development Zone, Dalian, China).

DNA extraction

Soybean samples were homogenized with a warring blender and DNA templates were extracted according to the cetyltrimethylammonium bromide (CTAB) method reported by agricultural trade standard NY/T674-2003.

Preparation of transgenic-DNA templates with different dilute concentrations

DNA concentrations were measured by UV absorption at 260 nm, and DNA purity was assessed by the UV absorption ratio at 260/280 nm. To compare the sensitivity of different amplification methods, DNA samples extracted from transgenic soybean were serially diluted with distilled water (1:10, 1:100, 1:1000, . . ., $1:10^n$) and used as DNA templates with different dilutions (1, 10^{-1}, 10^{-2}, 10^{-3}, . . ., 10^{-n}).

Primer design

The entire exogenous DNA sequence of GM soybean was obtained from the GenBank database (GenBank accession no. AY592954), including an enhanced cauliflower mosaic virus *35S* promoter (derived from the Cauliflower Mosaic Virus), and the 5-enol-pyruvylshikimate-3-phosphate synthase gene (*cp4 epsps*) (derived from Agrobacterium sp. strain CP4, a common soil microorganism). The *35S* promoter sequence was amplified using primers recommended by the European Union (Smith, Deaville, Hawes & Whitelam 2000). Several pairs of primers used for nested PCR were designed according to the exogenous DNA sequence of GM soybean. Primers were designed using DNAstar.

35S promoter primers sequences:

35F: 5′-GCTCCTACAAATGCCATCA-3′,
35R: 5′-GATAGTGGGATTGTGCGTCA-3′
Sequences of primers used for nested PCR:
F1: 5′-GGACCCCCACCCACGAGGAG-3′,
R1: 5′-GAACATGAAGGACCGGTGGGAGAT-3′;
F2: 5′-CATTTGGAGAGGACACGCTGACA-3′,
R2: 5′-CCGGAAAGGCCAGAGGATT-3′;
F3: 5′-TAACAACATGGCACAAGGGATACA-3′,
R3: 5′-CAGAGGATTTGCGGGCGGTTGC-3′.

Figure 1 shows the position of the primers that were used for the nested PCR. The amplified fragments

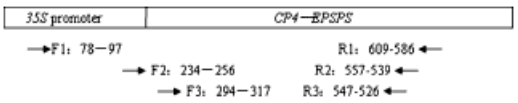

Figure 1 Position of the primers used for nested polymerase chain reaction.

were cloned into the pMD18-T Simple Vector (TaKaRa Biotechnology) and sequenced by Shanghai Sangon Biological Engineering Technology & Services (No. 698, Xiangmin Rd., Chedun Industrial Park, Songjiang, Shanghai, China).

Conditions for amplification

The conditions for the first (conventional PCR) and second amplification step were the same: 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and a final extension at 72 °C for 10 min. The products of the first step were diluted with distilled water (1:100) and used as DNA templates in the second step for nested PCR (van Tuinen, Zhao & Gianinazzi-Pearson 1998).

Polymerase chain reaction amplification of DNA extracted from transgenic soybean

Diluted DNA templates were first amplified with *35S* promoter primer pairs or F1R1 or F2R2 primer pairs. To compare the sensitivity of the different primer pairs, PCR products were analysed using agarose gel electrophoresis. The products of the first PCR amplification using primer pairs F1R1 or F2R2 were diluted 100-fold and used as template DNA for the second PCR reaction using primer pairs F2R2 or F3R3.

Application to aquatic feeds

Aquatic feeds (fish feed, shrimp feed) containing soybean were obtained from various feed and food stores around China. DNA templates were extracted according to the CTAB method and then tested by nested-PCR as described above.

Tilapias rearing

Tilapias with an average weight of 60 g and the same age were used for the feeding studies. They were obtained from a tilapia fry factory in Jiaozhou, Qingdao, China. A total of 160 fish were randomly distributed into eight feeding tanks.

The composition of the experimental diets was 30% soybean, 15% bran, 15% fish meal, 25% flour,

5% shrimp shell powder, 3% yeast powder, 3% aminofusin, 3% medicinal stone and 1% mineral mixture. The non-GM and GM soybeans included in the diets were a full-fat soybean meal and processed through squeezing.

To avoid water pollution, the fish were fed once a day at 8:00 hours, 7 days a week. The feeding trial was conducted in tanks (1 m^3), supplied with fresh water. Water was changed once a day at 16:00 hours. The conditions during the experiment were water temperature 25 ± 2 °C, pH 8.0 ± 0.5, one air stone in each tank, a water oxygen content of 8.34 ± 0.78 mg L^{-1}, ammonia nitrogen content under 0.07 mg L^{-1} and chemical oxygen demand content below 1.34 mg L^{-1}. The feeding was stopped at the end of the 7th week. The remaining fish were fed nothing for 2 weeks.

Before the feeding trial, the fish were fed a non-GM diet for 40 days to allow for adjustment to experimental conditions. Thereafter, they were randomly distributed into two groups of 80 fish with four tanks for each group, the non-GM and the GM dietary groups. Before distribution, the weights of the fish were measured. Their average weight was 77 g.

Application to digestive system contents, faeces, tissues and organs of tilapias

After feeding, the gastric contents, intestinal contents and faeces were sampled from tilapias in the first, second and eighth hour. Tissue samples were dissected carefully from the fish. Special care was taken during the whole procedure to avoid sample contamination and to minimize false-positive results. Most of the equipment used during dissection was only used once. Scissors and micro-homogenizers were washed repeatedly with distilled water and then sterilized by autoclaving to avoid contamination. Gloves were changed after each sampling. Samples were placed in different bags, immediately frozen in liquid nitrogen and stored at -70 °C until DNA analysis. Sampled tilapias were randomly selected from each tank at the 4th and 7th week and after the 2nd week of fasting. DNA was extracted using the CTAB method and analysed as above.

Results

Soybean extracts' purity and concentrations

High-quality DNA could be extracted from transgenic soybean using the CTAB method. All the samples showed a 260/280 nm ratio ranging from 1.6 to 1.9, with DNA concentrations of 3.15×10^{-6} g μL^{-1}.

Conventional PCR amplification of transgenic soybean

All the primer pairs could successfully amplify the DNA sequence of interest, but showed differences in the amplification specificity and sensitivity. The *35S* promoter primers amplified non-diluted DNA to produce a 195 bp fragment. The F1R1 primer pairs amplified 1 and 10^{-1} dilutions to produce a 532 bp fragment, as well as other fragments, indicating low specificity. The highest specificity pair was F2R2, which could amplify 324 bp fragments from 1, 10^{-1} and 10^{-2} dilutions. Template concentrations of 3.15×10^{-8} g μL^{-1} DNA solution could be detected, a dilution ratio of 1% (Fig. 2). Fragments of 532 and 324 bp were sequence confirmed as *35S epsps*.

Nested-PCR amplification of transgenic soybean

Products amplified by F1R1 or F2R2 were diluted 100-fold and used as template DNA for the second PCR reaction using F2R2 and F3R3, respectively, which showed improved specificity and sensitivity. Fragments of 324 bp were amplified by nested-PCR using F1R1–F2R2, with a detection limit of 3.15×10^{-9} g μL^{-1}, a 10-fold lower level than conventional PCR. Fragments of 254 bp were detected with F2R2–F3R3 at 3.15×10^{-10} g μL^{-1}, a 100-fold dilution ratio compared with conventional PCR (Fig. 3). Thus, nested-PCR was more sensitive and specific than conventional PCR, with the F2R2–F3R3 pair being the most sensitive. The fragments amplified were sequence confirmed to be the exogenous gene.

Polymerase chain reaction amplification of DNA extracted from aquatic feeds

We then tested aquatic feeds using conventional and nested-PCR (Fig. 4). Transgenic sequences could not be amplified with the *35S* promoter primer pairs and were rarely detected by conventional PCR with F1R1 or F2R2 pairs, but could be detected with nested-PCR. From 32 different feed brands analysed with

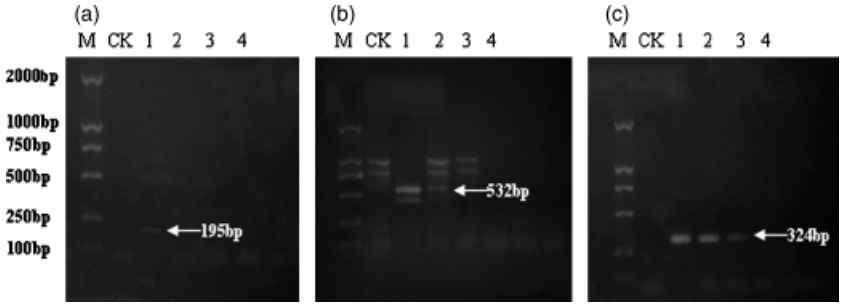

Figure 2 Conventional polymerase chain reaction (PCR) amplification results of transgenic soybean: (a) PCR amplification results of transgenic soybean with *35S* promoter primers; (b) PCR amplification results of transgenic soybean with F1R1 primers; (c) PCR amplification results of transgenic soybean with F2R2 primers. M, DL2000 marker; CK, negative control (soybean without transgenic gene); and 1–4, DNA solution of transgenic soybean with different dilute concentration (1, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}).

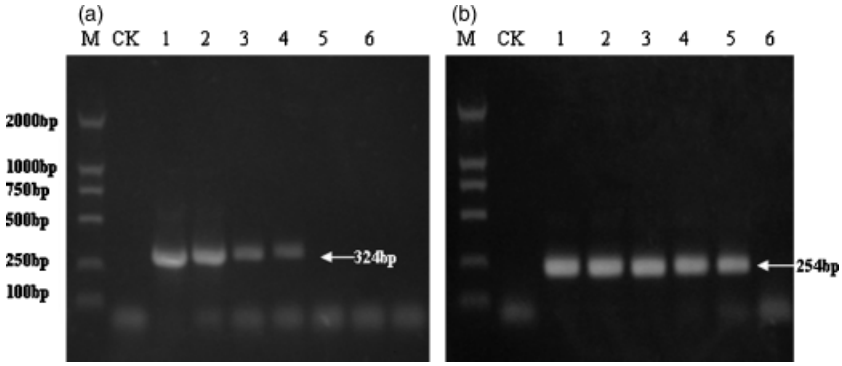

Figure 3 Nested-polymerase chain reaction (PCR) amplification results of transgenic soybean: (a) nested-PCR amplification results of transgenic soybean with F1R1–F2R2 primers; (b) nested-PCR amplification results of transgenic soybean with F2R2–F3R3 primers. M, DL2000 marker; CK, negative control (soybean without a transgenic gene); 1–6, DNA solution of transgenic soybean with different dilute concentrations (1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}).

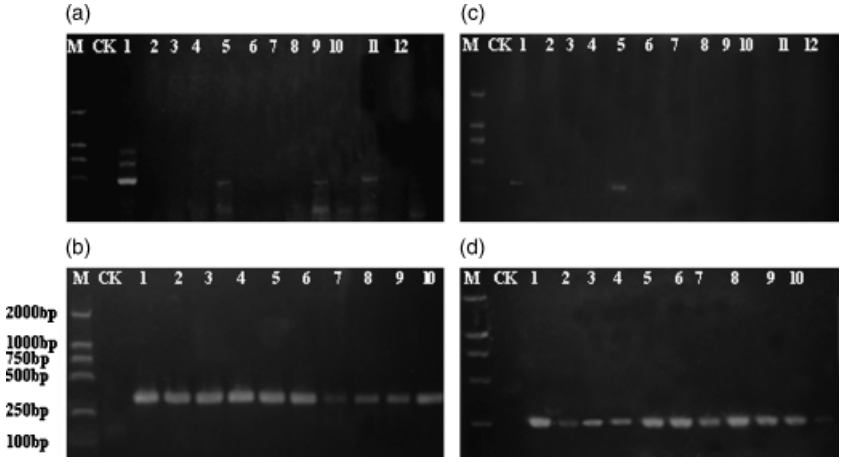

Figure 4 Nested-polymerase chain reaction (PCR) amplification of some aquatic feeds: (a) The first PCR amplification results of aquatic feeds with F1R1 primers; (b) nested-PCR amplification results of aquatic feeds with F1R1–F2R2 primers; (c) the first PCR amplification results of aquatic feeds with F2R2 primers; (d) nested-PCR amplification results of aquatic feeds with F2R2–F3R3 primers. M, DL2000 marker; CK, negative control (soybean without transgenic gene); 1, transgenic soybean; 2–12, different brands of aquatic feeds.

F1R1–F2R2 or F2R2–F3R3, transgenic soybean was detected in 29 samples, or 90.6%.

Degradation of GM soybean by tilapia alimentary canal

Genetically modified DNA fragments (254 bp) could be detected in samples from gastric contents, intestinal contents and faeces (Fig. 5), indicating that exogenous DNA from the GM soybean diet was not degraded by the tilapia alimentary canal.

Transgene DNA in different tilapia tissues and organs

Transgene DNA was measured in tilapia tissues and organs with nested PCR at 4, 7 and 2 weeks after subsequent fasting. Transgenic exogenous DNA fragments were not detected in the heart, liver, stomach, intestines, germen, brain, branchia, spleen, cholecyst or muscle of tilapias fed a non-GM diet. Transgenic DNA fragments were detected in all organs except the cholecyst after 4 weeks of a GM diet. After 7 weeks, transgenic DNA fragments were also detected in the cholecyst. After a 2-week fast, lower levels of transgenic DNA fragments were still detected in all organs, except the spleen and cholecyst (Table 1) (Figs 6–8). Fragments were sequence verified.

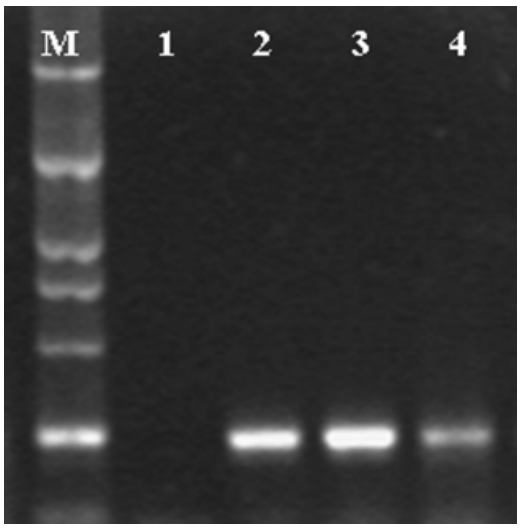

Figure 5 Detection of transgenic DNA fragments by nested PCR in tilapias' gastric contents, intestinal contents and faeces: M, DL2000 marker (2000, 1000, 750, 500, 250 and 100 bp from the top down); 1, negative control (non-GM diet); 2, gastric contents; 3, intestinal contents; 4, faeces.

Table 1 Transgenic detection results of different tissues and organs at 4, 7 and 2 weeks after subsequent fasting of tilapias fed non-genetically modified (GM) and GM soybean diet respectively

	4 weeks			7 weeks			Continued hunger 2 –weeks		
Item	SN	TPSN	TPR (%)	SN	TPSN	TPR (%)	SN	TPSN	TPR (%)
Heart	9	4	44.44	11	8	72.72	11	2	18.18
Liver	15	9	60.00	41	27	65.85	30	8	26.67
Intestines	15	14	93.33	16	15	93.75	21	7	33.33
Stomach	15	10	66.67	28	19	67.86	15	1	6.67
Muscle	14	10	71.42	11	8	72.73	25	7	28.00
Ovary	15	8	53.33	14	6	42.86	17	5	29.41
Spermary	14	7	50.00	14	6	42.86	12	1	8.33
Brain	16	5	31.25	12	5	41.67	12	2	16.67
Branchia	14	8	57.14	14	7	50.00	18	6	33.33
Spleen	15	5	33.33	13	5	38.46	15	0	0
Cholecyst	13	0	0	16	3	18.75	13	0	0

SN, sample number; TPSN, transgenic positive sample number; TPR, transgenic positive ratio.

Discussion

Testing methods for transgenic soybean and aquatic feeds

Conventional PCR has been used to detect transgenic soybeans including amplification of fragments of the *35S* promoter, the *nos* ending and the junction of *35S* promoter and *epsps* coding regions. Other methods such as restricted digestion of the amplified target, Southern hybridization, DNA sequencing, real-time quantitative PCR (Terry, Shanahan, Ballam, Harris, McDowell & Parkes 2002), biosensor technology (Feriotto, Borgatti, Mischiati, Bianchi & Gambari 2002) and microarray technology (De Bellis, Castiglioni, Bordoni, Mezzelani, Rizzi, Frosoni, Busti, Consolandi, Rossi & Battaglia 2002) can increase the specificity of GMO analysis by PCR (Marmiroli, Peano & Maestri 2003). Although these methods are reliable, they are time consuming, may require hazardous reagents such as radioactive probes are expensive as in the case of sequencing.

The nested PCR proposed in this paper produces more accurate results because two primer pairs sequentially amplify the same target. The advantages of this method include high specificity, sensitivity and good repetition. Our primers amplified fragments of 532, 324 and 254 bp, which are small enough to amplify from DNA that has been significantly degraded, and short sequences generally

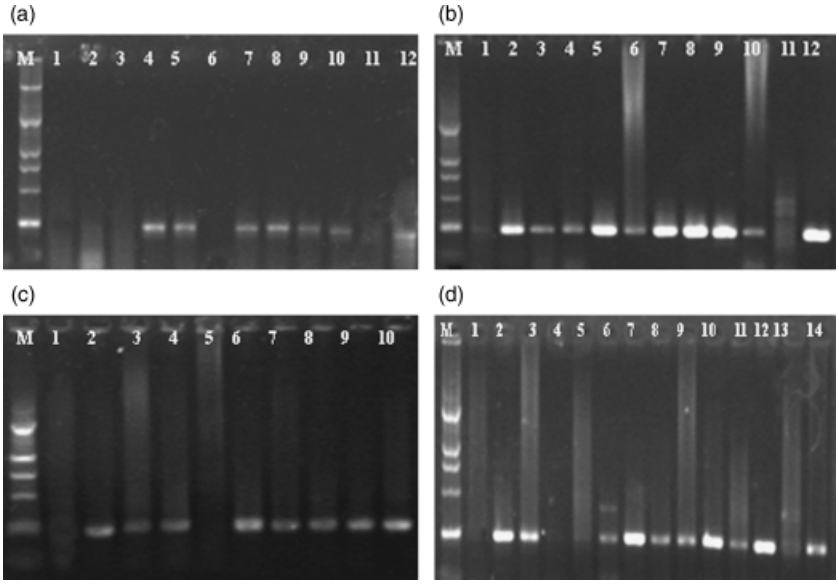

Figure 6 Some transgenic detection results of tilapias at 4 weeks fed with the genetically modified (GM) soybean diet: (a) ovary; (b) intestinal tract; (c) liver; and (d) heart. M, DL2000 marker (2000, 1000, 750, 500, 250 and 100 bp from the top down); 1, tilapias fed with the non-GM diet;and 2–14, tilapias fed with the GM diet.

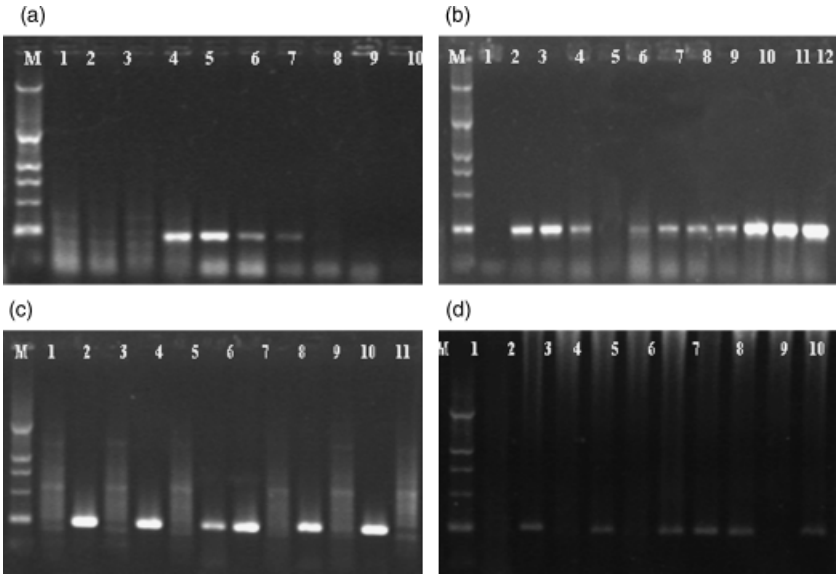

Figure 7 Some transgenic detection results of tilapias at 7 weeks fed with the genetically modified (GM) soybean diet: (a) ovary; (b) intestinal tract; (c) liver; and (d) heart. M, DL2000 marker (2000, 1000, 750, 500, 250 and 100 bp from the top down); 1, tilapias fed with non-GM diet; 2–12, tilapias fed with the GM diet.

produce better PCR results. These relatively small sizes were chosen because they increase the sensitivity of the method as PCR is more efficient at amplifying short sequences of DNA. Nested PCR with F2R2–F3R3 could detect higher dilution ratios than F1R1–F2R2, 0.01% and 0.1% respectively. The sensitivity of nested-PCR for detecting transgenic soybean was 5- to 50-fold higher than the conventional PCR recommended by the European Union. This work demonstrates that nested-PCR can be a useful tool in GMO screening.

In aquatic feeds, transgenic-specific sequences could not be amplified with *35S* promoter primer pairs, and conventional PCR seldom detected transgenic ingredients. In contrast, most aquatic feeds (90.6%) contained transgenic ingredients as detected by nested-PCR, indicating that transgenic soybean is widely used in Chinese aquatic feeds even with our limited sample.

The residual fragments of GM soybean in tilapia alimentary canal

Human intestinal simulations of degradation of transgenic DNA from GM soya and maize showed

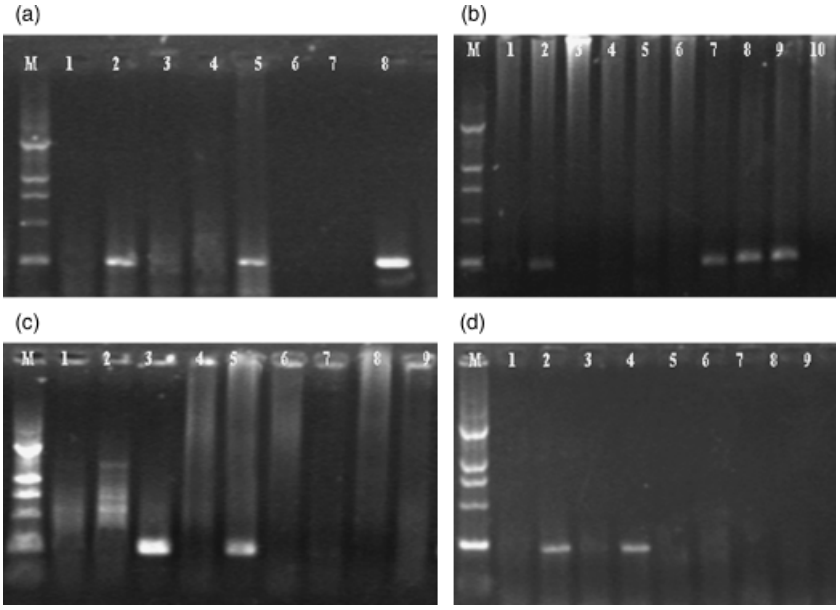

Figure 8 Some transgenic detection results of tilapias at continued hunger for 2-weeks fed with the GM soybean diet: (a) Ovary; (b) Intestinal tract; (c) Liver; and (d) Heart. M, DL2000 marker (2000, 1000, 750, 500, 250 and 100 bp from the top down); 1, tilapias fed with the non-GM diet; 2–10, Tilapias fed with the GM diet.

that some transgenes in GM foods may survive passage through the small intestine (Martín-Orúel, O'Donnell, Ariño, Netherwood, Gilbert & John 2002). In fact, GM studies indicated that foreign DNA ingested by animals is not completely degraded in their gastrointestinal tracts (Chainark, Satoh, Hirono, Aoki & Endo 2008). For example, foreign DNA fragments from defatted GM soybean meal in rainbow trout were not completely degraded. Here, we could detect 254 bp GM fragments from gastric content, intestinal content and faeces at 1, 2 or 8 h after feeding, indicating that exogenous GM DNA was not degraded by the alimentary canal and may be taken up into organs.

Residual fragments of GM soybean in tilapia tissues and organs

DNA from feed is detectable in chickens (Aeschbacher, Messikommer, Meile & Wenk 2005), pigs (Reuter & Aulrich 2003), mice (Schubbert, Hohlweg, Renz & Doerfler 1998), Atlantic salmon (Nielsen, Berdal, Bakke-McKellep & Holst-Jensen 2005; Nielsen, Holst-Jensen, Løvseth & Berdal 2006) and rainbow trout (Chainark *et al.* 2008). DNA can be taken up from the feed and transferred to the blood, liver and kidneys of Atlantic salmon (Nielsen*et al.* 2005, 2006). Intravenous injection instead of feeding caused DNA taken up in the blood to be transported to the liver and kidney, as well as muscle and gonads. Thus, fragments of dietary DNA may be absorbed into the blood stream from the gastrointestinal tract.

We detected DNA fragments of 254 bp in all tissues and organs except the cholecyst after 4 weeks of a GM diet. DNA fragments were detected in all organs after 7 weeks of feeding. After a subsequent 2-week fast, lower levels of transgenic DNA fragments could still be detected in all samples, except the spleen and cholecyst.

Our study supports the hypothesis that genomic DNA, including exogenous GM genes in soybean, can be transferred from the gastrointestinal tract to different tissues through the circulation. Fasting for 2 weeks may have induced gene degradation but GM fragments were still detected. Whether these fragments insert in cellular DNA is unknown. This is the first report of such extensive DNA uptake in cultured animals, for a number of reasons. First, nested-PCR of short fragments is more sensitive than common conventional PCR methods. Second, animals and birds are usually used in feeding studies, and have a different digestive system than aquatic animals. Here, larger DNA fragments could be absorbed and reach the tissues and organs. A 519 bp fragment of the *Cry1A(b)* transgene could be detected by Mazza, Soave, Morlacchini, Piva and Marocco (2005) in the blood, liver, spleen and kidney, which are blood-rich organs, of piglets raised with transgenic feed. Do the exogenous genes exist merely in blood or do they penetrate tissues and organs? Our finding that transgenic *35S epsps* DNA could be found after a 2-week fast suggests

that transgenic fragments exist both in blood and in tissues and organs. However, we are unable to confirm whether these fragments integrated into cellular DNA. In summary, our study will help inform safety assessments of genetic transfer following food consumption. Likewise, it can aid in evaluation of the risks of GM plant use in livestock feed.

Acknowledgments

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