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Detection of transgenic and endogenous plant DNA in blood and organs of Nile tilapia, *Oreochromis niloticus* fed a diet formulated with genetically modified soybean meal

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Abstract. Anxiety regarding the fate of ingested transgenic DNA in farmed fish fed genetically modified (GM) soybean meal (SBM) has been raised with regard to human consumption. The objective of this study was to detect possibility of gene transfer of transgenic and endogenous DNA fragments in Nile tilapia (*Oreochromis niloticus*) blood and organs after consumption of a GM SBM diet. Nile tilapias with an average weight of 75.0 g were fed diets containing 48% GM or non-GM SBM for 21 days. During this period, a GM SBM diet was fed to fish for 12 days, and then switched to feed with non-GM SBM for 9 days for determining the residual span of the transferred *cauliflower mosaic virus* (CaMV) 35S promoter fragment. Blood, spleen, liver, intestine, kidney, and muscle tissues were taken (n = 10) every three days during the feeding period. Total DNA was extracted from the samples and analyzed by polymerase chain reaction (PCR) for determining the presence of a 108-bp fragment of the CaMV 35S promoter and a 144-bp fragment of the soybean chloroplast-specific DNA. Low-copy chloroplast-specific DNA fragment was detected in all organ and tissue samples and the majority of intestinal samples of fish fed GM SBM diet. Similarly, a low number and faint signals of the CaMV 35S promoter fragments were detected in all organ samples except muscle of fish fed the GM SBM diet, while none were detected 3 days after changing to a non-GM SBM diet. A very low frequency of transmittance to muscle and organs of fish was confirmed. It is recognized that the low copy number of transgenic DNA in the GM SBM diet is a challenge to their detection in tissues. These results suggested that transgenic DNA would be processed in the gastrointestinal tract in a similar manner with conventional plant DNA.
Key Words: diet, DNA transfer, genetically modified soybean, Nile tilapia, transgenic DNA.

Introduction. The increasing use of genetically modified (GM) plants has been the topic of considerable scientific debate and public concern. Issues addressed include the feeding of GM plants to animals in relation to effects on animal performance and health, the possible transfer and accumulation of foreign DNA in animal products, and the safety aspects associated with humans consuming foods derived from animals receiving GM diets (Beever & Phipps 2001). The studies that examine the fate of recombinant feed DNA in the gastrointestinal (GI) tract of animals and its potential absorption and transfer to other tissues are essential to fully address these safety concerns. A number of studies have been conducted in which transgenic DNA has not been detected in these food products derived from animal receiving GM feed ingredient (Phipps et al 2002; Yonemochi et al 2002; Ash et al 2003; Jennings et al 2003a, 2003b; Sanden et al 2004). In contrast, only few studies have detected recombinant DNA in blood, liver (Mazza et al 2005) or muscle (Chainark et al 2008; Suharman et al 2009).

However, small fragments of plant chloroplast DNA have been detected in some animal tissues like muscle, liver (Einspanier et al 2001; Tony et al 2003; Nemeth et al 2004; Aeschbacher et al 2005; Guertler et al 2008) or blood (Tony et al 2003; Tudisco et al 2006a, 2006b). Since a low number of fish muscles of Nile tilapia (*Oreochromis niloticus*) fed GM soybean meal (SBM) diet were positive for the CaMV 35S promoter (Suharman et al 2009), thus, this study was conducted to evaluate the possible transfer of plant DNA into Nile tilapia tissues (blood, spleen, liver, intestine, kidney and muscle) with a special emphasis on detecting transgenic DNA from GM soybean meal diet.

Material and Method

Experimental diets. The experiment was conducted on September to December 2012. Two experimental diets containing 48% SBM were formulated by replacing fish meal with either GM or non-GM SBM to get approximately 38% of crude protein (Table 1). The diets were freeze-dried until more than 90% dry matter (DM) and stored as carefully as possible to exclude cross-contamination of them at 4^{o}C until use.

Experimental fish and rearing conditions. Nile tilapia with initial weight of approximately 74.6 ± 2.9 g (mean ± SE) were obtained from Laboratory of Fish Culture, Tokyo University of Marine Science and Technology, Japan, and were allowed to adapt for a week, in four 60-L glass tanks, with 20 fish in each tank at the Laboratory of Fish Nutrition, Tokyo University of Marine Science and Technology, Japan. The study comprised three feeding periods (Figure 1). In period I, fish were adapted to the diets containing non-GM SBM over 7 day. For next 12 day (period II), the diets containing GM SBM were fed. From completion of period II onward (period III) for 9 days, feeding of the non-GM SBM diets was resumed. Fish were fed twice daily at approximately 09:00 and 16:00 h to apparent satiation.

Table 1

Formulation and proximate composition of the experimental diets (%)

Ingredients (%)	*Non GM SBM*	*GM SBM*
Jack mackerel meal	20	20
Non GM SBM	48	-
GM SBM		48
Wheat flour	7.9	7.9
α-Starch	10	10
Palm oil	5	5
Soybean oil	2	2
Vitamin premix[a]	3	3
Mineral premix[b]	1	1
$Ca(H_2PO_4)_2$	1	1
Cellulose	2	2
Vitamin E (50%)	0.1	0.1
Proximate composition		
Moisture	3.59	6.44
Crude protein	39.7	39.3
Crude lipid	8.89	9.78
Crude ash	8.68	8.31

[a] The vitamin mix had the following components (mg 100 g^{-1}): thiamin hydrochloride 6, riboflavin 10, pyridoxine hydrochloride 4, cyanocobalamin 0.01, ascorbic acid 500, niacin 40, Ca-pantothenate, 10, inositol 200, biotin 0.6, folic acid 1.5, *p*-aminobenzoic acid 5, vitamin K_3 5, vitamin A acetate 4000 IU, vitamin D_3 4000 IU. [b] P-free mineral mixture (g 100 g^{-1}): NaCl 5.0, $MgSO_4 \cdot 7H_2O$ 74.5, $FeC_6H_5O_7 \cdot nH_2O$ 12.5, trace element mix 5.0, cellulose 3.0. Trace element mix compositions (mg g^{-1}) -$ZnSO_4 \cdot 7H_2O$ 353, $MnSO_4 \cdot 5H_2O$ 162, $CuSO_4 \cdot 5H_2O$ 31, $AlCl_3 \cdot 6H_2O$ 10, $CoCl \cdot 6H_2O$ 1, KIO_3 3, cellulose 440.

Figure 1. Feeding scheme in which fish were assigned to non-GM SBM or GM SBM diets. The type of soybean meal included in the non-GM and GM SBM diets in each period is indicated.

Sample procedures. During the sampling procedure special care was taken to avoid accidental contamination from environment or from the surgical tools. The working place and the tools were accurately cleaned with 70% alcohol. Disposable gloves were changed between each organ sampling. On the last day of period I (i.e. before any GM SBM diet was fed), approximately 1 mL blood samples were collected from the caudal veins of each fish by a medical syringe (2.5 mL, TERUMO®; Tokyo, Japan), transferred to a sterile Eppendorf tubes containing EDTA. The blood was frozen on dry ice and stored at -80°C until use. The other organs (spleen, liver, intestine, kidney and muscle) were removed or dissected, transferred in Eppendorf tubes, immediately frozen on dry ice and stored at -80°C until DNA analysis. On days 3, 6, 9 and 12 of period II (after feeding on GM SBM diet), blood samples and other organs (spleen, liver, intestine, kidney and muscle) were collected as described before. Afterward, blood samples and other organs (spleen, liver, intestine, kidney and muscle) were sampled on the days 15, 18 and 21 of period III (when feeding of the non-GM SBM diet had resumed).

DNA extraction and quantification. DNA extraction from the experimental diets and fish organs (spleen, liver, intestine, kidney and muscle) were done according to Murray & Thompson (1980) with minor modifications: 1 mL CTAB (hexadecyltrimethyl-ammonium bromide) buffer (20 g L^{-1} CTAB, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, and 20 mM EDTA) was added to 0.2 g sample, mixed by vortex and incubated in a water bath at 60°C for 60 min. Then, the solution was centrifuged at 21,840 x *g* for 3 min at 4°C. The supernatant was transferred to a new tube, filled with 200 µL phenol:chloroform:isoamyl alcohol (PCI, 25:24:1), mixed gently by inverting for 5 min, and centrifuged at 21,840 x *g* for 3 min at 4°C. The upper layer was transferred to a 1.5-mL tube and 200 µL PCI was added, mixed gently by inverting for 20 min, and centrifuged at 21,840 x *g* for 10 min at 4°C. The upper layer was gently mixed with 300 µL 2-propanol for 30 s, and centrifuged at 21,840 x *g* for 5 min at 4°C. The DNA pellet was washed with 800 µL 75% ethanol, centrifuged at 21,840 x *g* for 5 min at 4°C, and dried at room temperature. The DNA pellet was dissolved in 50 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) as a stock DNA solution, and stored at -20°C.

A commercial DNA extraction kit (Dr. GenTLE™; Takara Bio. Inc. Japan) was used for extraction of DNA from whole blood containing EDTA samples according to the manufacturer's protocol. The concentrations of the extracted DNA were measured fluorometrically using the GeneQuant™ 100 (Biochrom® England).

Oligonucleotide primers. The primer pairs (Table 2) were synthesized by Invitrogen (Carlsbad, CA, USA) and diluted with an appropriate volume of distilled water to a final concentration of 30 pmol μL^{-1} and stored at -20°C until use. Three primer pairs framing specific target sequences were used. The primer pair CaMV1/CaMV2 (GenBank accession no. V00141) was used to amplify the CaMV 35S promoter fragment. The primer pair Chlo1/Chlo2 (GenBank accession no. Z95552) was used to amplify the soybean chloroplast gene fragment. The primer pair ß-actin1/ß-actin2 (GenBank accession no. AY116536) used to confirm the feasibility of PCR amplification of the extracted DNA from blood and other organ samples.

Table 2

Primers used in this study

Name	Sequence (5' – 3')	Target sequence	Amplicon size (bp)
1. CaMV promoter			
CaMV1	GATTGATGTGATATCTCCACTGACG	CaMV 35S promoter[1]	108
CaMV2	GTGTCCTCTCCAAATGAAATGAACT		
2. Chloroplast			
Chlo1	CACGGAATCCAAGTTGAAAGAG	Glycine max chloroplast gene[2]	144
Chlo2	GGTAAAATCAAGTCCACCACGA		
3. ß-actin			
ß-actin1	TGTCTGGCTTACTCGGAATGT	Nile tilapia promoter gene[3]	194
ß-actin2	CATACCGGATCCGTTGTCAAC		

[1] Wolf et al (2000) Eur Food Res Technol 210, 367-372; [2] GenBank accession no. Z95552;
[3] GenBank accession no. AY116536.

Polymerase chain reaction (PCR). PCR reaction was carried out in a reaction mixture (20 µL) containing 2 µL 10x *Taq* buffer with 20 mM $MgCl_2$, 1.5 µL dNTP mixture, 1 µL primers, 0.05 µL Taq polymerase (Takara, Japan), 14.45 µL distilled water and 1 µL DNA template. The PCR conditions were: 94°C for 10 min (initial denaturation), 35 cycles of 95°C for 25 s (denaturation), 60°C for 30 s (annealing) and 72°C for 45 s (extension), followed by 72°C for 7 min (final extension). The PCR products were separated on a 2% (w/v) agarose gel containing 1% ethidium bromide. Electrophoresis was carried out at approximately 100 V for 35 to 45 min, visualized under ultraviolet (UV) light at 254 nm and photographed using a charge-coupled device (CCD) camera (SFC Instruments, Japan).

DNA sequencing. The PCR product was labeled with Thermo Sequenase Cycle Sequencing Kit (Amersham Biosciences) for sequencing using the CaMV 35S primers. This PCR product was sequenced by DNA Sequencer (Applied Biosystems 3130*xl* Genetic Analyzer, Hitachi, USA). Sequence data was then pasted into the BLAST test similarity of sequence within the database.

Results and Discussion. Amplication of soybean specific DNA fragments from both soybean lines and diets was detectable using the primer pair Chlo1/Chlo2 (144 bp). The PCR products were detected in both non-GM SBM and GM SBM. This indicates that the DNA was successfully extracted and it was amplified during PCR (Figure 2A). For the specific identification of transgenic soybean, the primer pair CaMV1/CaMV2 (108 bp) was used. The CaMV 35S promoter fragments appear only in transgenic soybean (Figure 2B).

To exclude the possibility of cross-contamination between non-GM and GM SBM diets during preparation, samples from both diets were also subjected to DNA extraction and PCR techniques using the same primer pairs as mentioned above. All samples from non-GM and GM SBM diets have shown positive results with primer pair Chlo1/Chlo2 (Figure 3A). While the primer pair CaMV1/CaMV2 only revealed positive results with samples from the diet containing the CaMV 35S promoter (Figure 3B), which confirm that there was no cross-contamination between non-GM and GM SBM diets.

The transfer of transgenic and endogenous plant DNA fragments was examined in blood and organs of fish fed GM or non-GM SBM diets. Initially, the primer pair *ß* actin1/*ß* actin2, designed to detect the gene encoding Nile tilapia promoter (194 bp), were used to confirm the feasibility of PCR amplification of the extracted DNA from the blood, spleen, liver, intestine, kidney and muscle samples. From samples examined, expected 194 bp *ß* actin gene fragment was obtained (data not shown). Chloroplast plant DNA fragments were detected in all organs and tissue samples and the majority of intestinal samples of

fish fed the GM SBM diet. The results of detectable chloroplast DNA fragments are summarized in Table 3.

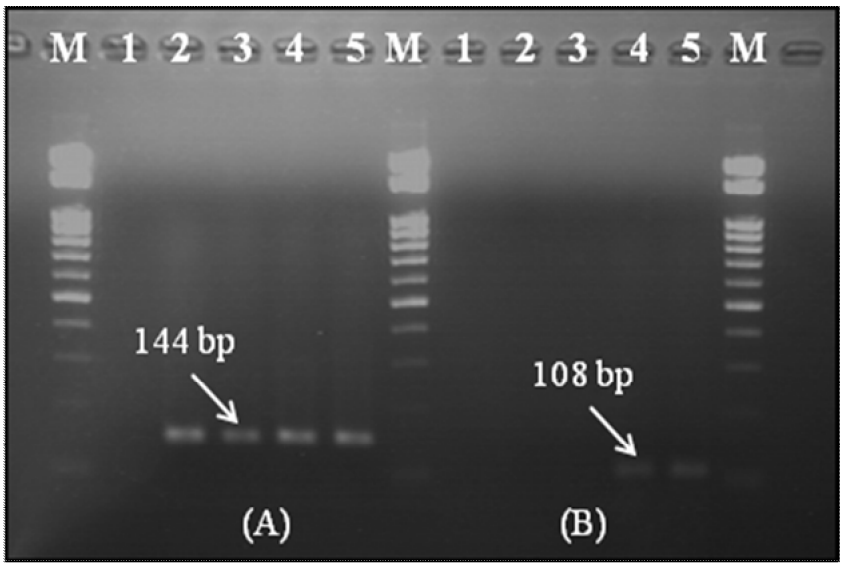

Figure 2. DNA investigation in soybeans. M: 100 bp DNA Marker, 1: negative control (without DNA), 2-3: non-GM SBM samples, 4-5: GM SBM samples; (A): primer pair Chlo1/Chlo2 (144 bp), (B): primer pair CaMV1/CaMV2 (108 bp).

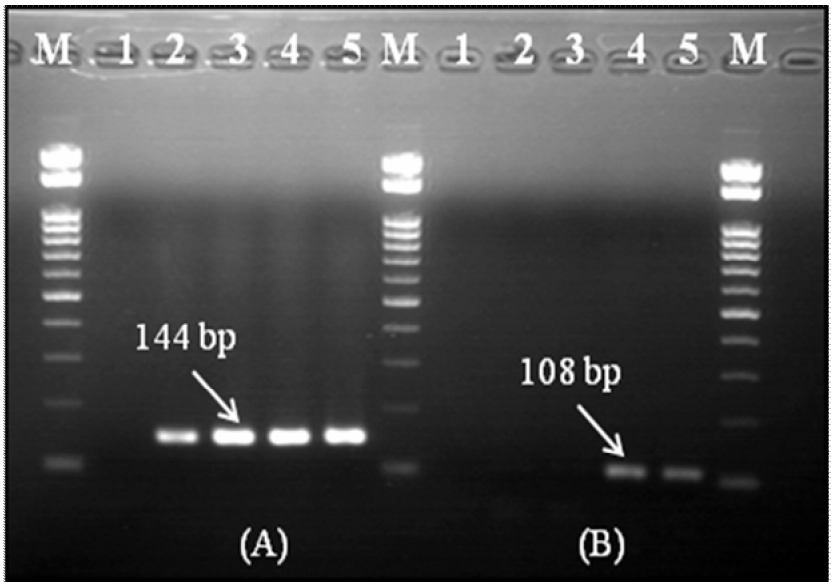

Figure 3. DNA investigation in diets. M: 100 bp DNA Marker, 1: negative control (without DNA), 2-3: non-GM SBM diets, 4-5: GM SBM diets; (A): primer pair Chlo1/Chlo2 (144 bp), (B): primer pair CaMV1/CaMV2 (108 bp).

A low number and faint signals of 108-bp CaMV 35S promoter fragments were detectable in all organ samples except muscle of fish fed the GM SBM diet. Additionally, the promoter fragment was not detected in all organs by third day after changing to non-GM SBM diet. The result of detection of the CaMV 35S promoter fragments are summarised in Table 4 and selected result is shown in Figures 4 and 5. The identity of the CaMV 35S PCR product of fish blood sample was verified by sequence analysis, showing it to be identical to the CaMV 35S promoter reference sequence (GenBank Accession no. V00141) (data not shown). The sequenced amplicons from intestinal sample of fish fed GM or non-GM SBM diet showed 100% homology when compared with the targeted DNA sequence of the chloroplast gene (GenBank Accession no. Z95552).

Table 3

Number of detectable fragment of chloroplast DNA in different tissues of fish (n = 10) fed experimental diets at different sampling day

Diets	*Sampling day*	*Sampled tissues*					
		Blood	*Spleen*	*Liver*	*Intestine*	*Kidney*	*Muscle*
Non-GM SBM diet	0	1	2	2	5	2	4
GM SBM diet	3	0	1	1	0	0	1
	6	0	0	0	2	1	1
	9	0	1	1	4	1	0
	12	0	0	0	2	0	1
Non-GM SBM diet	15	1	2	0	3	0	0
	18	0	2	0	0	0	0
	21	0	0	0	0	0	0

Table 4

Number of detectable fragment of CaMV 35S promoter in different tissues of fish (n = 10) fed experimental diets at different sampling day

Diets	*Sampling day*	*Sampled tissues*					
		Blood	*Spleen*	*Liver*	*Intestine*	*Kidney*	*Muscle*
Non-GM SBM diet	0	0	0	0	0	0	0
GM SBM diet	3	1	1	0	3	1	0
	6	0	1	0	1	0	0
	9	1	1	0	1	1	0
	12	1	0	1	0	0	0
Non-GM SBM diet	15	1	0	0	3	0	0
	18	0	0	0	0	0	0
	21	0	0	0	0	0	0

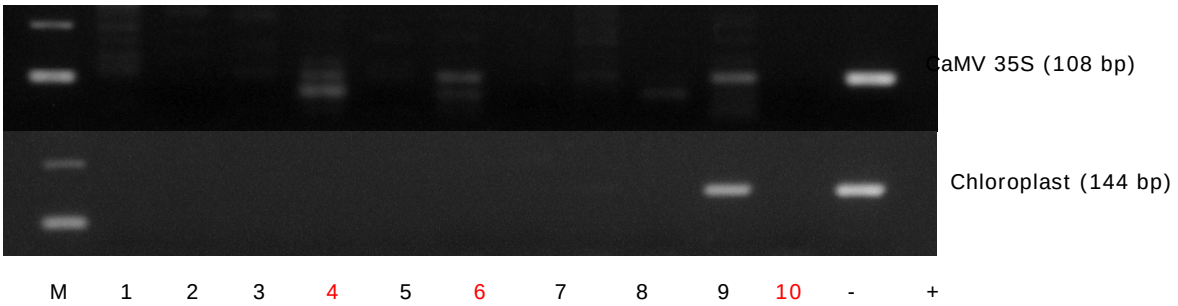

Figure 4. Detection of CaMV 35S promoter and chloroplast DNA fragments in blood of fish fed experimental diets on day 9.

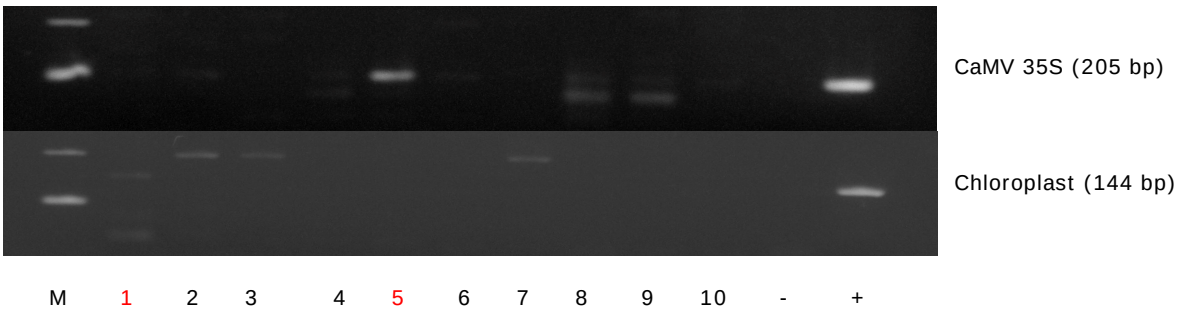

Figure 5. Detection of CaMV 35S promoter and chloroplast DNA fragments in blood of fish fed experimental diets on day 15.

The present study was accomplished to investigate distribution and fate of plant-chloroplast DNA and recombinant DNA from GM soybean in the blood, spleen, liver, intestine, kidney and muscle of fish fed GM or non-GM SBM diet. Regarding distribution of DNA, endogenous plant DNA fragments were found in blood, spleen, liver, intestine and kidney samples of fish fed GM SBM diet. Moreover, a chloroplast gene fragment was detected in muscle samples of fish fed GM SBM diet. One explanation for these results is that the chloroplast gene fragment was detected because of its greater abundance compared to the single copy gene of CP4 EPSPS in Roundup Ready soybean (Nielsen et al 2005). The possibility that DNA detected in muscle could be due to the presence of blood could not be ruled out.

In the present study, a low frequency of fish blood samples receiving GM SBM diet was positive for the CaMV 35S promoter fragment. These results indicate that ingested DNA is not completely degraded by the enzymatic activity in the gastrointestinal (GI) tract of Nile tilapia. Our results are similar to findings in feeding studies with Atlantic salmon *Salmo salar* (Nielsen et al 2006) and rainbow trout *Oncorhynchus mykiss* (Chainark et al 2008) where small chloroplast DNA fragments were found in blood. These experiments are some examples supporting the hypothesis that GI tract does not completely block the uptake of fragments of ingested DNA into the bloodstream (Nielsen et al 2006). Detection of foreign DNA in blood suggests that the remaining DNA in the digestive tract might be absorbed by the organism and detected in the blood.

Investigation of the CaMV 35S promoter residue in muscle of fish fed GM SBM diet; all of the muscle samples were negative for the CaMV 35S promoter fragments. These results are similar to those published by Sanden et al (2004), who reported that GM soy fragment (195 bp) was not detected in the muscle of Atlantic salmon fed a GM SBM based-diet. It has been also reported that transgenic DNA was not detectable in a number of sample tissues from laying hens fed GM SBM (Ash et al 2003). Similarly, fragments of transgenic corn was not detectable in the muscle of broiler and pig fed GM corn (Yonemochi et al 2002; Jennings et al 2003a; Chowdhurry et al 2003). We assume that most of foreign DNA was rapidly degraded to fragments smaller than 180 bp in the digestive tract, being below the level of detection. These results are also in agreement with studies on rainbow trout (Kitagima et al 2013). In addition, Nile tilapia has high capacity to digest dietary plant protein (Watanabe et al 1996). This ability is attributed to the very low stomach pH in this species, which allows them to digest and extract the cellular content even without breaking the cellulose in cell wall (Bowen 1981). In terms of a small number of fish muscle samples were positive for the CaMV 35S promoter, one possible explanation would be attributed to intra-individual difference. However, the foreign DNA is thought to be rather slowly degraded in the GI tract in some fish. Mazza et al (2005) suggested that the DNA fragments are rapidly degraded before reaching peripheral organs. Moreover, feedstuffs with relatively greater digestibility, such as soybean meal, are likely to have their DNA degraded more rapidly, reducing possibility of absorption (Alexander et al 2007).

The present results suggest that blood is the main tissue responsible for uptake of short DNA fragments. DNA molecules may be transported in the organism via blood circulation. The lower detection frequency found in organs may account for the transitory nature of DNA fragments that are progressively degraded before reaching peripheral organs. Therefore, it is thought that foreign DNA transfer to fish muscle is rarely occurred.

Conclusions. Based on this study, feed-derived foreign DNA fragments are not completely broken down and might be taken up into organs through the gastro-intestinal tract. However, the foreign DNA fragments were not detected after the withdrawal period. Therefore, the results presented here support and strengthen the conclusion made in earlier studies that GM soybeans can be used as an equivalent and safe substitute for conventional soybeans in feeds for Nile tilapia.

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