

Full Length Research Paper

Addressing the issue of horizontal gene transfer from a diet containing genetically modified components into rat tissues

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Genetically modified (GM) food crops are considered to have the potential of providing food security especially in developing countries. Scientists have raised concern over the hazards associated with the consumption of genetically modified organisms (GMOs). One of these hazards, which have great controversy reports, is the possible horizontal gene transfer from GM-food or feed to human or animal tissues. Many researches were conducted to investigate the presence of some transgenic sequences in animal tissues fed on GM- crops. Many of the inserted genes in the GM-crops are under the control of the promoter of the Cauliflower mosaic virus (CaMVP35S) and produce insecticidal proteins. Health hazards are suggested to accompany the ingestion of this promoter. CaMVP35S can function in a wide range of organisms (plants and animals). It has also been demonstrated that the CaMV-P35S promoter sequence can convert an adjacent tissue- and organ-specific gene promoter into a globally active promoter. The present work was conducted to evaluate the possibility of horizontal gene transfer from a diet containing DNA segments from Cauliflower mosaic virus -35S promoter (CaMVP-35S) to the cells of different organs of rats fed for three months on diets containing genetically modified components. Analysis of the results revealed that: 1) ingested fragments from the CaMV-35S promoter incorporated into blood, liver, and brain tissues of experimental rats, 2) The total mean of transfer of GM target sequences increased significantly by increasing the feeding durations, and 3) The affinity of different transgenic fragments from the ingested GM-diet, to be incorporated into the different tissues of rats varied from one target sequence to the other.

Key words: Genetic modification, transgenic sequences, GM-crops, gene flow, CaMV P-35S.

INTRODUCTION

Agricultural biotechnology encompasses a variety of technologies used in food and agriculture, for a range of different purposes such as the genetic improvement of

plant varieties and animal populations to increase their yields or efficiency. One of these biotechnologies is genetic modification (FAO/WHO, 2001). Among the many

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Table 1. Primers position on CaMV genome, sequences, amplicon lengths and annealing temperatures

Primer	Position on CaMV genome ‡	Sequences (5'-3')	Amplicon length (bp)	Annealing Temp (°C)
GT88	7117-7138	5'-TCCGGAACCTCCTCGGATTCCAT-3'	88	54
	7183-7206	5'-GGCATTGTAGGAGCCACCTTCCCT-3'		
GF70	7222 – 7249	5'-GCCATCGTTGAAGATGCCTCTGCCG-3'	70	57.5
	7273 - 7293	5'-CACGATGCTCCTCGTGGGGTGG-3'		
P-35S	7190-7209	5'- GCTCCTACAAATGCCATCA -3'	195	57
	7364-7384	5'-GATAGTGGGATTGTGCGTCA-3'		

‡Positions relevant to Ca.

plants that are genetically modified are soybean and maize. Apart from human consumption, soybean and maize are also used in livestock and fish feeds. Genetically modified (GM) varieties of both are extensively traded internationally for this purpose (Nowicki et al., 2010). One of the hazards accompanying consumption of GM- plants is the possible horizontal gene transfer (HGT) which is the transfer of genetic material directly to a living cell or an organism (van den Eede et al., 2004; Keese, 2008). The consequences of horizontal gene transfer may have significant effects on some human-health conditions. It has been shown that ingested DNA from food is not completely degraded by digestion, and that small fragments of DNA from GM foods can be absorbed by either the gut microflora or somatic cells lining the intestinal tract (Netherwood et al., 2004; Nielson and Townsend, 2004; van den Eede et al., 2004). Some of the animal studies support the idea that small fragments of nucleic acids may pass to the blood stream and even get to various tissues (Rizzi et al., 2012).

Recent study (Spisak et al., 2013) found that meal-derived DNA fragments which are large enough to carry complete genes can avoid the total degradation in the gastrointestinal tract, and through an unknown mechanism enter the human circulation system. Foreign DNA fragments were detected by polymerase chain reaction (PCR) based techniques in the digestive tract and leukocytes of rainbow trout fed by genetically modified soybean (Chainark et al., 2008), and other studies reported similar results in goat (Tudisco et al., 2010), pigs (Mazza et al., 2005, Sharma et al., 2006) and mice (Schubbert et al., 1998). Many GM crops produce an insecticidal protein (for example Cry1Ab). The gene coding for this protein is under the control of the 35S promoter of the Cauliflower mosaic virus (CaMV-P35S). This promoter is derived from the common plant virus Cauliflower mosaic virus, which was reported to be closely related to human hepatitis B virus (Doolittle et al., 1989, Xiong and Eikbush, 1990). It can cause cancer through over-expression of oncogenes (Ho et al., 1999).

The CaMVP-35S is a component of transgenic constructs in more than 80% of genetically modified (GM)

plants (Cankar et al., 2008). It can function in a wide range of organisms (plants and animals) and can direct the expression of the bacterial neomycin phosphotransferase II (*nptII*) gene in *Escherichia coli* (Assaad and Signer, 1990). It has also been demonstrated that the CaMV-P35S promoter sequence can convert an adjacent tissue- and organ-specific gene promoter into a globally active promoter (Zheng et al., 2007).

Since most of the studies were investigating the transfer of GM-DNA fragments from transgenic genes for example *Cry1Ab* (Walsh et al., 2012) and *cp4epsps* (Sharma et al., 2006), the aim of the conducted study was to evaluate the transfer of dietary DNA segments from Cauliflower mosaic virus -35S promoter (CaMVP-35S) to the cells of different organs of weanling Wistar Albino rats fed for three months on diets containing genetically modified components. We used three primers to amplify different segments from Cauliflower mosaic virus-P35S (CaMV-P35S) promoter representing nearly 80% of this promoter.

MATERIALS AND METHODS

The experimental material consisted of laboratory chow containing mainly 60% of yellow maize and 34% of soybeans. The presence of genetic modification in this diet was tested in our laboratory using the PCR assay (Rose, 1991). DNA extracted from samples of the laboratory chow diet fed to rats during the three months period of the experiment were screened for the presence of genetic modification; using a pair of primers specific for the Cauliflower mosaic virus (P-35S) promoter that amplify a segment of 195 bp from the CaMV-35S promoter (Hemmer, 1997). DNA was also extracted from another well balanced diet that was fed to the animals of the control group and was tested for the presence of any GM- sequences.

Primer designing

The fact that plant DNA is fragmented to small sizes after the process of digestion lead to the assumption that designing primers to amplify smaller amplicons would increase the chance to detect transgenic fragments, which could transfer to the animal genome. Therefore, two primers GT88 and GF70 (Table 1) were designed using Primer-Blast Program (NCBI). These two primers were

designed to amplify DNA segments of the sizes 88 and 70 bp of the cauliflower mosaic virus promoter, respectively. The Primer 3 software (Rosen et al., 2000) was employed with manual editing as needed, using the Cauliflower mosaic virus (CaMVP-35S) promoter sequence available from GenBank (accession no.emb|V00141.1|). All combinations including forward-reverse primer pair, forward-forward as well as reverse-reverse pairs were avoided by the primer 3 software. Primers were then submitted to BLAST search against bacteria, mice and rat database to confirm the specificity of the designed primers to CaMVP-35S. Along with these two designed sets of primers, a third pair of primers P-35S (Table 1) which amplify a segment of 195 bp from the cauliflower mosaic virus promoter was also used. The P-35S primers were also used for evaluating the possible horizontal gene transfer from the genetically modified diets to the experimental animal's genome applying the polymerase chain reaction technique. All primers were synthesized by Metabion.

Animals, housing and diets

Twenty nine male Wistar Albino rats (*Rattus norvegicus*), obtained from the animal house of the National Research Center immediately after weaning (age of three to four weeks), were divided into two main groups. One group was fed on laboratory chow containing GM ingredient for three months (GM-diet group) and were further divided into three subgroups (6, 6 and 7 animals). Animals from GM-diet groups were euthanized after 30, 60 or 90 days, respectively. The other group (control) was fed on a balanced non-GM diet for the same period (non-GM-diet group). The animals were housed in standard cages and under standard conditions. Temperature was maintained at 22 to 25°C and relative humidity at 55 to 60% during the experiment. Exposure to light was maintained for 12 h. Diet and water were provided *ad libitum*. Animals of the GM-groups were euthanized at three intervals; namely at 30, 60 or 90 days, respectively. Control animals were euthanized at the end of the experiment. The protocol applied throughout this study comply with the NRC Ethical Committee's guidelines (reference: 12142), and all animals received humane care.

Sampling procedures

Three types of tissue samples (blood, liver and brain) were collected from each animal in both groups. Blood samples were collected on EDTA (0.5%) from all animals of both groups at the end of each interval (30, 60 or 90 days) of the experiment. Samples from liver and brain were excised and stored at -20°C.

DNA isolation

DNA extraction and purification from fresh blood samples from GM-diet and non-GM-diet groups were carried out using Wizard Genomic DNA Purification Kit (Promiga) according to the manufacturer's manual. The excised tissue samples of the liver and brain were grinded in liquid nitrogen, and genomic DNA was extracted from tissue samples from animals in both groups using the same extraction kit used above. The quality and concentration of DNA were determined using the Nano Drop 1000/Thermo Scientific spectrophotometer (USA).

PCR conditions and profile

A total of 30 DNA samples extracted from non-GM diet group (three tissue types from 10 animals), and 57 DNA samples extracted from the GM diet group (three tissue types from 19 animals) were

screened for the presence of small segments from the cauliflower mosaic virus 35S promoter using the three sets of primers presented in Table 1. Double-stranded amplifications were carried out in a total volume of 25 µl. Each reaction mixture contained 100 ng template DNA, 2 mM MgCl₂, 50 mM KCl, 200 µM of each dNTP, 2.5 pmol of each primer and 2.5 units of taq DNA polymerase in a reaction buffer containing 75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM (NH₄)₂SO₄, and 0.001% BSA. All polymerase chain reactions were conducted in duplicates and accomplished in TM Thermal cycler (MJ Research PTC-100 thermocycler, USA) programmed to perform a denaturing step at 98°C for 2 min, followed by 40 cycles consisting of 30 s at 95°C for denaturing, 40 s at annealing temperature specific for each primer (Table 1), and 45 s at 72°C for extension. A final extension step of 7 min at 72°C was performed.

Agarose gel electrophoresis

Following completion of the cycling reaction, each PCR product was analyzed by electrophoretic separation of a 10 µl aliquot on a 2% (W/V) agarose gel containing ethidium bromide (1 µg/ml ethidium bromide). PCR products were analyzed, using SYNGENE Bio Imaging Gel Documentation System, for the presence of a fluorescent band of the expected level for each primer (Table 1).

Sequencing analysis

For further confirmation of the presence of CaMV-P35S promoter in the diets used in this study, and to confirm the presence of transgenic DNA in genomic DNA of rats fed on the GM diet, the internal sequencing of the PCR amplicons obtained from the amplification of P-35S promoter primers in the diet DNA and liver and brain tissues of some rats has been performed by MWG-Biotech AG. PCR products of the expected size (195 bp) were purified using total fragment DNA purification kit (MEGA quick-spin, iNtRON Biotechnology, Inc). Sequencing was followed by Blast analysis with the GenBank to test for alignment using NCBI-BLASTN Program version 2.2.28+.

Statistical analysis

Percentages of transfer frequencies of the GM target sequences of the GM-DNA into rats after 30, 60 and 90 days were calculated according to the following equation:

$$\text{Transfer frequency \%} = \frac{\text{No of positives}}{\text{No of samples}} \times 100$$

Multiple analysis of data was conducted applying the analysis of variance (ANOVA) test to evaluate the mean values of the number of GM-DNA segments from the diet that were transferred to different tissues in the experimental GM-diet groups.

RESULTS AND DISCUSSION

PCR amplification of the laboratory chow diet samples

The laboratory chow diet samples used all through this experiment gave positive results when it was screened with primers for the presence of Cauliflower mosaic virus promoter (CaMV-P35S). A band of the size 195 bp was detected when amplifying P-35S promoter primers

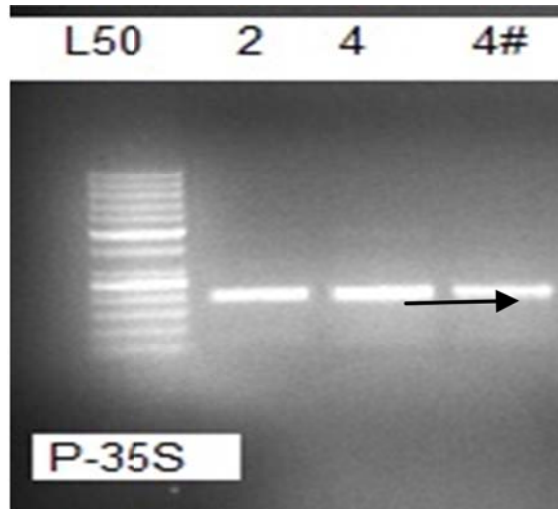


Figure 1. Confirmation of amplification of CaMV-P35S fragment (195 bp) in the diet used for this experiment. (2, 4 and 4#) are samples from the laboratory chow diets used in this study.

(Figure 1) in all the samples of the used diet. No amplification occurred in the control diet. There are several plant seeds and potato tubers that are genetically modified sold for cultivation in Egypt (Oraby et al., 2005). These products are squash, tomato, canola seeds, yellow maize and potato tubers which are used as diet for human and animals. GM varieties of yellow maize and soybeans were reported to be produced worldwide for animal feed (Nowicki et al., 2010).

Sequencing results analysis

As mentioned before and for further confirmation of the presence of Cauliflower mosaic virus-35S (CaMV-P35S) promoter in the diets used in this study, the internal sequencing of the PCR amplicons obtained from the amplification of P-35S promoter primers has been performed. Sequence alignment analysis was carried out using NCBI-BLASTN Program version 2.2.28+. BLASTN sequence alignment showed a 100% homology of the amplified segment of the expected size (195 bp) with CaMV-P35S promoter (emb|V00141.1) at nucleotide (nt) coordinate 7190 to 7384 (Figure 2). It also shared 100% sequence homology with a number of binary vectors (for example gb|JX992842.1|, gb|JQ974028.1|, gb|JQ436739.1|, dbj|AB684633.1|, gb|JQ305140.1| and dbj|AB752377.1|). Binary vectors are usually used in the process of gene transfer (de Framond et al., 1983; Hoekema et al., 1983).

PCR amplification of different tissues

PCR amplification using the three primers (GF70, GT88

and P-35S) revealed amplicons of the expected sizes; 70, 88 and 195 bp, respectively in some of the DNA samples of blood, liver and brain in rats fed on GM diet after 30, 60 or 90 days. Representatives of some of these amplifications are presented in Figures 3, 4 and 5. Whereas, none of these primers were amplified in DNA samples from the second group of rats fed on a non GM-diet. To confirm the presence of transgenic DNA in genomic DNA of rats fed on the GM diet, further analysis of the internal sequencing of the large amplicon (195 bp) obtained from the amplification of P-35S promoter primers in some DNA samples of liver and brain in rats fed on GM diet was performed against GenBank data base. Results reveal that these sequences showed 100% similarity with Cauliflower mosaic virus whole genome (emb|V00141.1) at the same nucleotide coordinates 7190 to 7384 which represents 195 nt of the CaMV-P35S promoter that gave 100% similarity with the PCR amplicon obtained by the amplification of the same set of primers (P-35S) from the GM-diet. It also aligned with numbers of binary vectors including those that were aligned with the sequence of the segment obtained from the diet used in this study. The binary vectors are widely known to be used for gene transfer (de Framond et al., 1983; Hoekema et al., 1983).

The total frequencies of transfer of the GM-target sequences (GF70, GT88 and P-35S) from the GM-diet into the examined DNA samples from blood, liver and brain tissues in rats fed on the GM-diet increased from 33.3 to 37% after feeding rats with GM-diet for 30 or 60 days, respectively, and reached the highest level 52.8% after 90 days of the GM-diet regime. ANOVA statistical analysis of the results of this work revealed that feeding rats with GM-diet for 30, 60 or 90 days increased the mean transfer of GM target sequences significantly (8.0 ± 0.0000 , 12.3 ± 1.2018 and 16.7 ± 1.4529 , respectively) by increasing the feeding durations (Figure 6). Mazza et al. (2005) reported a progressive decrease in DNA detected in the target tissues. Therefore, they exclude the possibility that organs like liver, kidney and spleen elicit an accumulation effects; whereas, results of the present work indicate an accumulation effects in blood, liver and brain tissues.

Many researchers reported the presence and the fate of GM-DNA in gastrointestinal tract (GIT) of experimental animals and how GM-DNA survive digestion in the oral cavity (Duggan et al., 2000), passing the stomach, intestine (Duggan et al., 2003, Sharma et al., 2006) and out with the faeces. Other researchers reported the transfer of DNA fragments from the GM and non-GM diets to different tissues of the experimental animals. Small fragment of transgenic *Cry1Ab* was detected in blood, liver, spleen and kidney of animals raised with transgenic feed (Mazza et al., 2005). Transfer of transgenic fragments of the *cp4epsps* was detected at low frequencies among GIT tissues, except the oesophagus (Sharma et al., 2006). Our findings

CaMV-P35S	7190	GCTCCTACAAATGCCATCATTGCGATAAAAGGAAAAGGCCATCGTTGAAGATGCCTCTGCCG	7249
GM diet	1	GCTCCTACAAATGCCATCATTGCGATAAAAGGAAAAGGCCATCGTTGAAGATGCCTCTGCCG	60
CaMV-P35S	7250	ACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTC	7309
GM diet	61	ACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTC	120
CaMV-P35S	7310	CAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACG	7369
GM diet	121	CAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACG	180
CaMV-P35S	7370	CACAATCCCACTATC	7384
GM diet	181	CACAATCCCACTATC	195

Figure 2. DNA sequence alignment of the PCR products obtained from GM diet DNA together with the sequence of cauliflower mosaic virus (emb|V00141.1|).

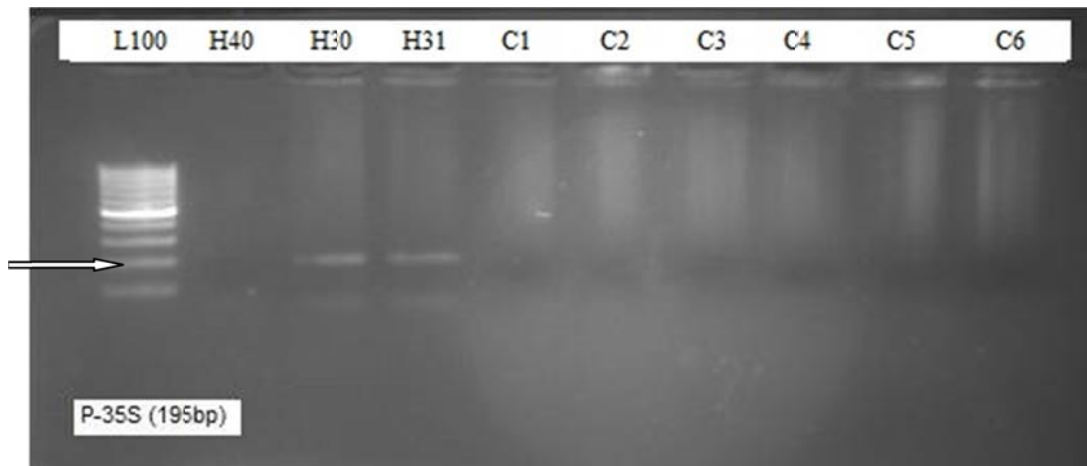


Figure 3. Representative of PCR products of P-35S (195 bp) amplification in blood (H40) and two brain samples (H30, H31) in rats fed on GM diet for 60 days. C1 to C6 are samples from rats fed on the non-GM diet.

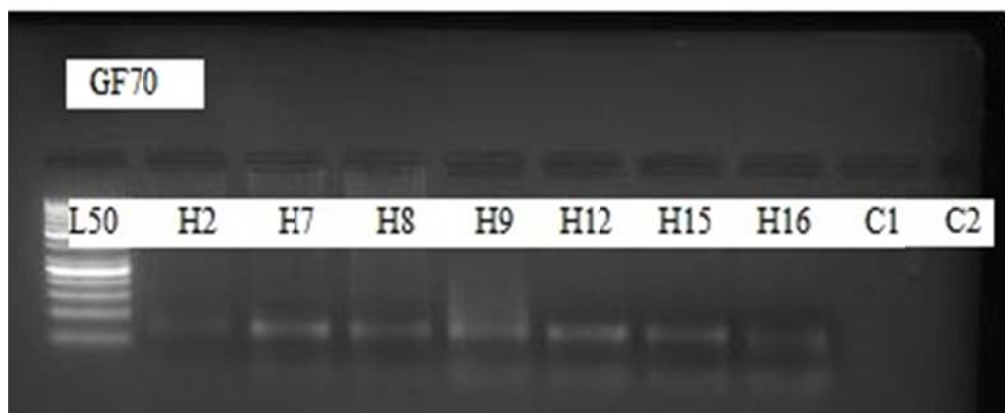


Figure 4. Representative of PCR products of GF70 (70 bp) amplification in blood samples (H2 to H9) and liver samples (H12 to H16) in rats fed on GM diet for 30 days. C1 and C2 are blood samples from control rats.

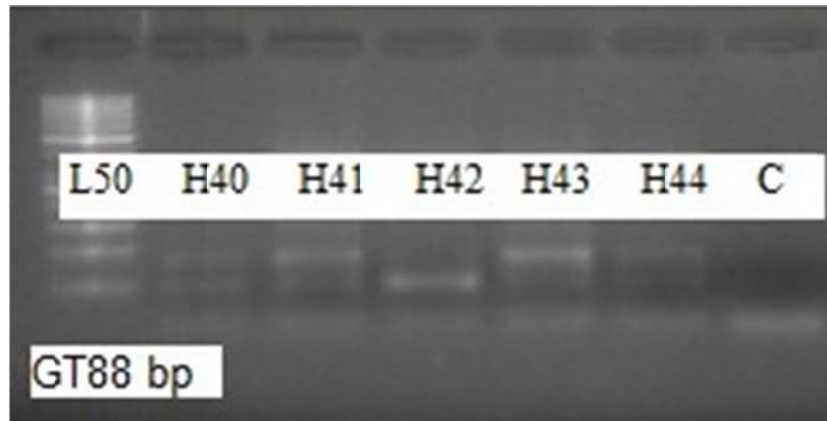


Figure 5. Representative of PCR products of GT88 (88 bp) amplification in blood sample (H40, H41, H43 and H44) of rats fed on GM diet for 90 days, C is a blood sample from a control rat.

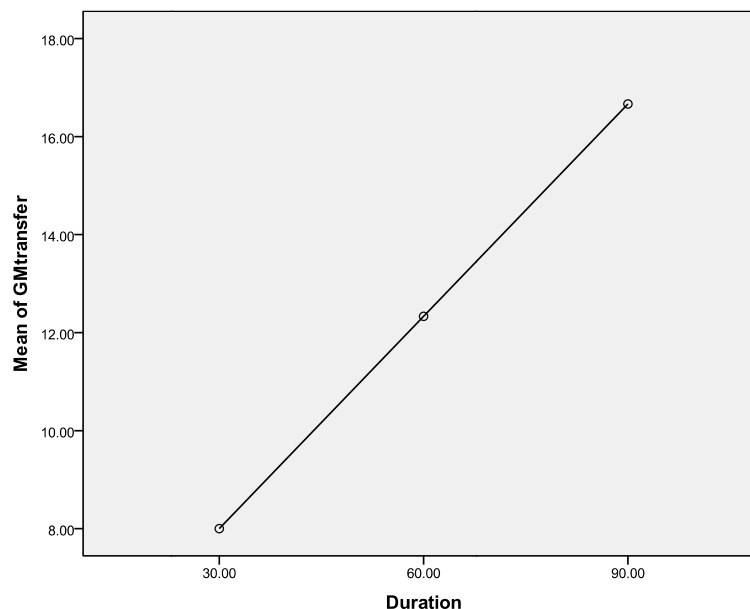


Figure 6. Mean transfer of the three GM target sequences (GF70, GT88 and P-35S) in tissues of rats fed on GM-diet for 30, 60 and 90 days.

moreover, confirm that ingested GM-DNA fragments from the Cauliflower mosaic virus P-35S promoter do incorporate into blood, liver, and brain tissues of experimental rats.

The fate of DNA from GM corn and soybean has been extensively monitored in cattle, sheep, pigs and poultry (Aeschbacher et al., 2005; Beagle et al., 2006; Chowdhury et al., 2004; Deaville and Maddison, 2005; Duggan et al., 2003; Einspanier et al., 2001; Mazza et al., 2005; Sharma et al., 2006). Most of these studies were investigating the transfer of GM-DNA fragments from transgenic genes (for example *Cry1Ab* and *C4epsps*). The authors of these studies suggested that it is unlikely that small segments from a gene will transmit genetic information (Duggan et al., 2003). In a recent study

conducted by Sipsak et al. (2013), it showed that meal-derived DNA fragments are able to avoid the total degradation in the gastrointestinal tract and enter the circulation. They found that DNA fragments large enough to carry complete genes can pass from the digestive tract to the blood. Foreign DNA fragments were detected by PCR based techniques in the digestive tract and leukocytes of rainbow trout fed by genetically modified soybean (Chainark et al., 2008), and other studies reported similar results in goat (Tudisco et al., 2010), pigs (Mazza et al., 2005; Sharma et al., 2006) and mice (Schubbert et al., 1998). Many GM crops produce an insecticidal protein (for example *Cry1Ab*) which is under the control of the promoter of the Cauliflower mosaic virus.

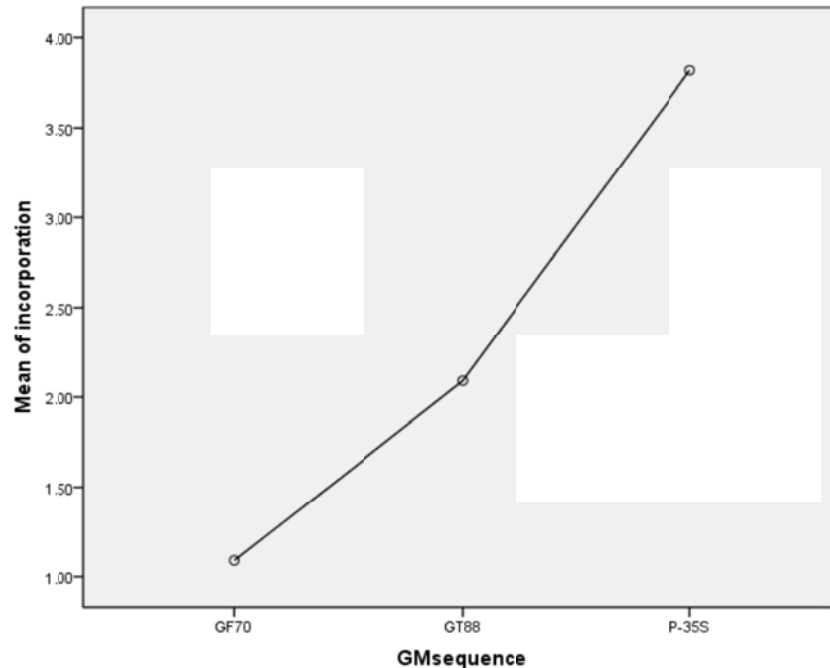


Figure 7. Affinity of incorporation of different GM-sequences (GF70, GT88 and P-35S) into the genome of rats fed on transgenic diet (GM-diet).

In our investigation, we evaluated the transfer of CaMV-P35S promoter from transgenic diet into experimental animals. For this purpose, we designed three primers that amplify three segments of CaMV-P35S promoter (accession no.emb|V00141.1|) from nucleotide coordinate 7117 to 7384 (Table 1) which represents nearly 80% of the whole promoter (343 bp). In some cases in the present work, the three segments were all amplified in the same animal. This finding may raise concern that the whole promoter might have been transferred into the genome of those animals, and this where the risk lies, since, even a promoter containing only 46 bp of 5' sequence from the CaMV-P35S promoter was previously reported to be sufficient for accurate transcription initiation (Odell et al., 1985).

Results of the present work suggest a health hazards accompanying the ingestion of diets containing Cauliflower mosaic virus 35S promoter since Cauliflower mosaic virus has been reported to be closely related to human hepatitis B virus (Doolittle et al., 1989, Xiong and Eikbush, 1990). It is also known that CaMV-P35S promoter can direct the expression of the bacterial neomycin phosphotransferase II (*NPTII*) gene in *Escherichia coli* as reported by Assaad and Signer (1990). They also reported that, it can function in a wide range of organisms (plants and animals). It has also been demonstrated that the CaMV-P35S promoter sequence can convert an adjacent tissue- and organ-specific gene promoter into a globally active promoter (Zheng et al., 2007). Previously, Ho and his colleagues (Ho et al., 1999) deduced that the CaMVP-35S promoter could

combine to active dormant viruses, create new viruses, and cause cancer by over expression of normal genes. Contrary to that, others (Hull et al., 2000) claimed that there is no evidence that the CaMV 35S promoter will increase the risk over those already existing from the breeding and cultivation of conventional crops.

Comparing tissue selectivity of the three tissues (blood, liver, and brain) examined through the whole duration of this work (90 days), no significant differences were observed in the rate of transfer of GM-DNA into these tissues. GM-DNA transfer to blood tissue was barely higher (12.66 ± 2.6034) than the GM-DNA transfer to the other tissues: liver (12.00 ± 3.6055) and brain (12.00 ± 2.0000). In a study conducted by Mazza et al. (2005) blood tissues was found to be the tissue with the highest transfer frequency, followed by organs rich in blood vessels and involved infiltration, such as liver and kidney. They concluded that blood is the main tissue involved in the uptake of short DNA fragments since it collects macromolecules directly absorbed by the intestinal epithelium and the cells of the immune system and that DNA molecules may be transported in the organism via blood circulation.

Our results have demonstrated that the frequency of uptake for the larger segments was greater than that for the smaller segment. This conclusion was based on the fact that the efficiency of transfer of GM-DNA segments (70 bp) amplified by GF70 primers was the lowest (1.09 ± 0.4161) compared to that of P-35S (3.8 ± 0.8069) and GF88 (2.09 ± 0.7318) (Figure 7). The frequency of transfer of GF70 segment was only calculated when GF70

primers were amplified in the animals with no traces of amplification of P-35S primers in these animals. The affinity of incorporation of GF70 segment into the rats' genome was statistically significantly lower compared to that of the P-35S. Palmen and Hellingwerf (1997) and Meier and Wachernagel (2003) postulated that the shorter the fragments are, the lower is the uptake efficiency.

Conclusion

Our results support the suggestion that monitoring for transgenic flow is a way to measure the possible environmental impacts of GMO and to serve as a warning system for deleterious effects (NRC, 2002).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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