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Full Length Research Paper

Tending to the issue of level quality exchange from an eating regimen containing hereditarily altered parts into rodent tissues

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Geneticall y 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.

INT RODUCTION

Ag icultural 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 7183-7206	5'-TCCGGAAACCTCCTCGGATTCCAT-3' 5'-GGCATTTGTAGGAGCCACCTTCCT-3'	88	54
GF70	7222 – 7249 7273 - 7293	5'-GCCATCGTTGAAGATGCCTCTGCCG-3' 5'-CACGATGCTCCTCGTGGGGTGG-3'	70	57.5
P-35S	7190-7209 7364-7384	5´- GCTCCTACAAATGCCATCA -3´ 5´-GATAGTGGGATTGTGCGTCA-3´	195	57

‡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 $MgCl_2$, 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 $(NH_4)_2SO_4$, 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:

$$\text{Transfer frequency \%} = \frac{\quad}{\quad} \times 100$$

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

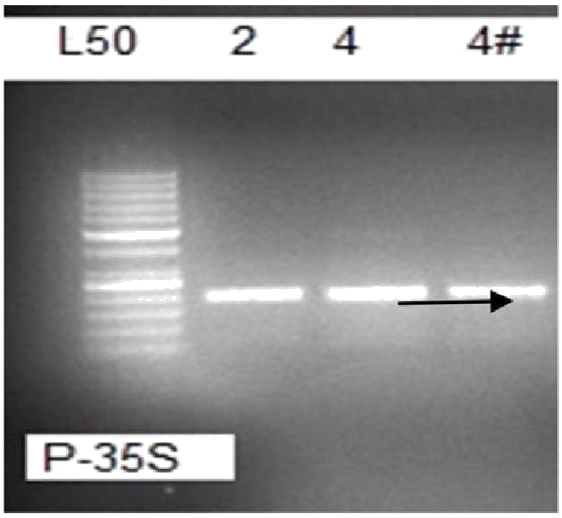

Figure 1. C nfirmation of amplification of CaMV-P35S fragment (195 bp) in the diet used for this experiment. (2, 4 and 4 #) are samples from the lab ratory chow diets used in this study.

(Fi gure 1) in all the sam ples of the used diet. No amplification o curred in the control diet. There are several plant seeds and potato tubers that are genetic ally modified sold for cultivation in Egypt (Oraby et al., 2005). Th ese products are squash, omato, canola seeds, yellow maize and potato tubers w hich are used as diet for hu man and animals. GM varieties of yellow maize and soybeans were reported to be produce d worldwide for animal feed (Nowicki et al., 2010).

Sequencing re sults analysis

As mentioned before and for further confirmation of the presence of Cauliflower mosaic virus-35 (CaMV-P3 S) promoter in th diets used in this stu dy, the internal seq uencing of the PCR a mplicons obtained from he amplification o P-35S promoter primers has been performed. Sequence alignment analysis was carried out using NCBI-BLA STN Program version 2.2.28+. BLASTN seq uence alignment showe d a 100% homology of he amplified segment of the expected size (195 bp) w ith CaMV-P35S pro moter (emb|V00141.1|) at nucleotide (nt) coo rdinate 7190 to 7384 (Fi ure 2). It also shared 100% seq uence homology with a number of binary vectors (for exa mple gb|JX992842.1|, gb|JQ974028.1|, gb|JQ436739.1|, dbj|AB684 33.1|, gb|JQ 305140.1| and dbj|AB752377.1|). Binary ve tors are usually used in he process of ge e transfer de Framon et al., 1983; Hoekema et al., 1983).

PCR amplification of differ nt tissues

PCR amplification using the three primers (GF70, GT88 and P-35S) reve aled amplicons of the expected sizes; 70, 8 8 and 195 bp, respectively in some of the DN A samp les 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 prim ers were amplified in DN A samp les 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 th internal sequencing of t he large amplicon (195 bp) obtained from the amplification of P-35S promoter prime rs in some DNA samples of liver an brain in rats fed o n GM diet was performed against G enBank data base. Results rev eal that these sequences showed 100 simil rity with Cauliflower mosaic virus whole genome (emb|V00141.1|) at the sam e nucleotid coordinates 7190 to 7384 whic h represents 195 nt of th e CaMV-P35 S promoter that gave 100% similarity with the PC R amplicon obtained by the amplification of the same set of prime rs (P-35S) rom the GM -diet. It also aligned with numb ers of binary vectors including those that were aligned with the sequence of the segment obtained fro the diet used in th is study. Th e binary vectors are widely known to be use for gene transfer (de Framond et al , 1983; Hoekema et al., 1983).

Th e total frequ encies of transfer of the GM-target sequences (GF70 , GT88 and P-35S) from the GM-diet into the examined DNA sam ples from bl od, liver and brain tissues in r ats fed on the GM-diet increased fro 33.3 to 37% after feeding rats with GM-diet for 30 or 60 days, respectively, and reache d the highes t level 52.8 after 90 days of the GM -diet regime. ANO VA statistic l 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 se quences significantly (8.0 ± 0.000 0, 12.3±1.2 018 and 16. 7±1.4529, res pectively) by increasing the feeding duratio ns (Figure 6). Mazza et al. (200) reported a progressive decrease in DNA detected in th e target tissues. Therefore, they exclude the possibility that or gans like liver, kidney and spleen elicit an a ccumulation effects; whereas, results of the present work indicate an accumulation effects in blood, liver and brain tissues.

Many research rs reported the presence and the fate of G M-DNA in ga strointestinal tract (GIT) o experiment l animals and how GM-DNA survive digestion in the oral cavit y (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-G diets to different tissues of he experime ntal 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 fragm nts of the *c p4epsps* was detected at low frequencies among GIT tissues, except the oesophagus (Sharma et al., 2006). Our findings

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CaMV-P35S  7190   G TCCTACAAATGCCATCAT GCGATAAAGGAAAGGCCA TCGTTGAAGA TGCCTCTGC CG  7249
                  | |||||||||||||||||| |||||||||||||||||| |||||||||||||||||| ||
GM diet    1      G TCCTACAAATGCCATCAT GCGATAAAGGAAAGGCCA TCGTTGAAGA TGCCTCTGC CG  60
CaMV-P35S  7250   A AGTGGTCCCAAAGATGGA CCCCACCCACGAGGAGCA TCGTGGAAAA AGAAGACGT TC  7309
                  | |||||||||||||||||| |||||||||||||||||| |||||||||||||||||| ||
GM diet    61     A AGTGGTCCCAAAGATGGA CCCCACCCACGAGGAGCA TCGTGGAAAA AGAAGACGT TC  120
CaMV-P35S  7310   C ACCACGTCTTCAAAGCAA TGGATTGATGTGATATCT CCACTGACGT AAGGGATGA CG  7369
                  | |||||||||||||||||| |||||||||||||||||| |||||||||||||||||| ||
GM diet    121    C ACCACGTCTTCAAAGCAA TGGATTGATGTGATATCT CCACTGACGT AAGGGATGA CG  180
CaMV-P35S  7370   C CAATCCCACTATC   738
                  | ||||||||||||
GM diet    181    C CAATCCCACTATC   195
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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|).

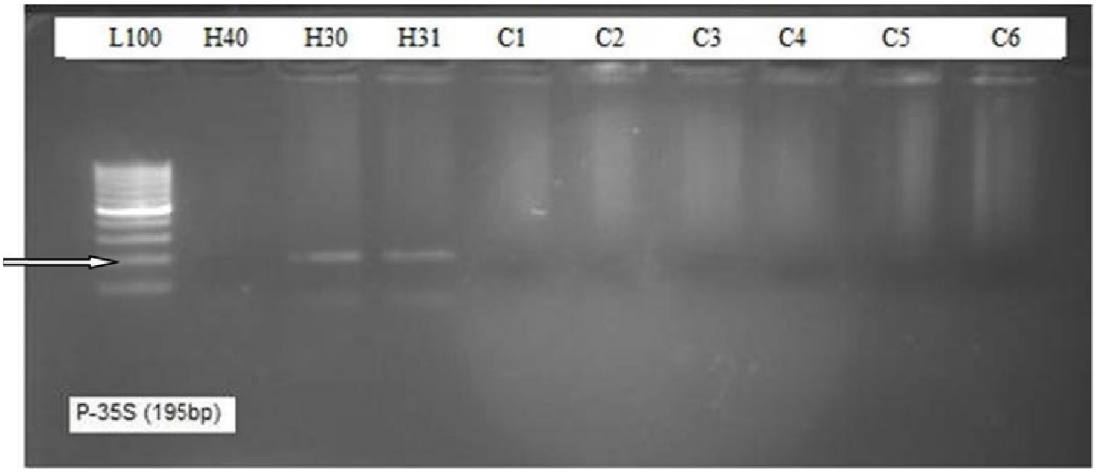


Figure 3. Repre sentative of PCR products of P-35S (195 bp) amplification in blood (H40) and two brain samples (H30, H31) in rats fed n GM diet for 60 days. C1 to C6 are samples from rats fed on the non-GM diet.

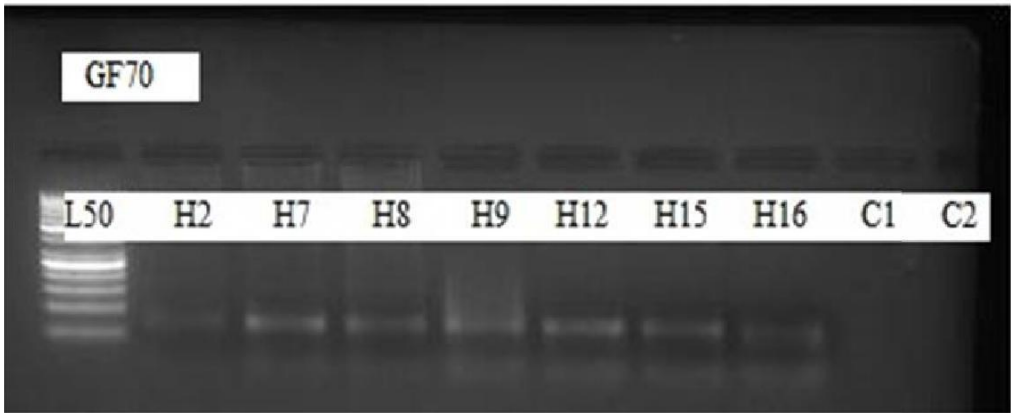
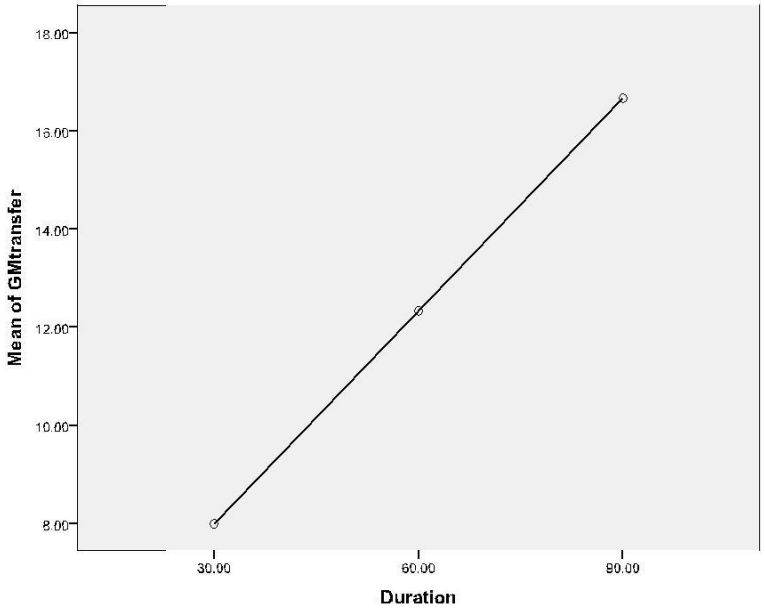


Figure 4. Rep resentative of PCR products of GF70 (70 bp) amplification i blood samples (H2 to H9) and liver samples (H12 to H16) in rats fed on GM diet for 30 days. C1and C2 are blood s amples from control rats.

Figu re 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.



igure 6. Mean transfer of the three GM target sequences (GF70, GT88 nd 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 viru s P-35S promoter do incorporate into blood, liver, and brain tissues of experimental rat s.

The fate of DN A from GM corn and soyb ean has been extensively mo itored in cattle, sheep, pigs and poultry (Aeschbacher et al., 2005; Beagle et al., 2006; Chowdhury et al., 2004; Deaville and M addison, 2005; Duggan et al., 2003; Einspan ier et al., 2001; Mazza et al., 20 5; Sharma t al., 2006). Most of these studies were investigating the transfer of GM-DNA fragments from tra nsgenic gen s (for example *Cry1Ab* and *C4epsps*). Th e authors of hese studies suggested that it is unlikely that small seg ents from a gene will transmit genetic info rmation (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 DN A 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., 20 05; Sharma et al., 2006) and mice (Schubbert et al., 1998). Many GM crops produce an insecticidal protein (for example Cry1Ab) w hich is under the control of the promoter of the Cauliflower mosaic virus.

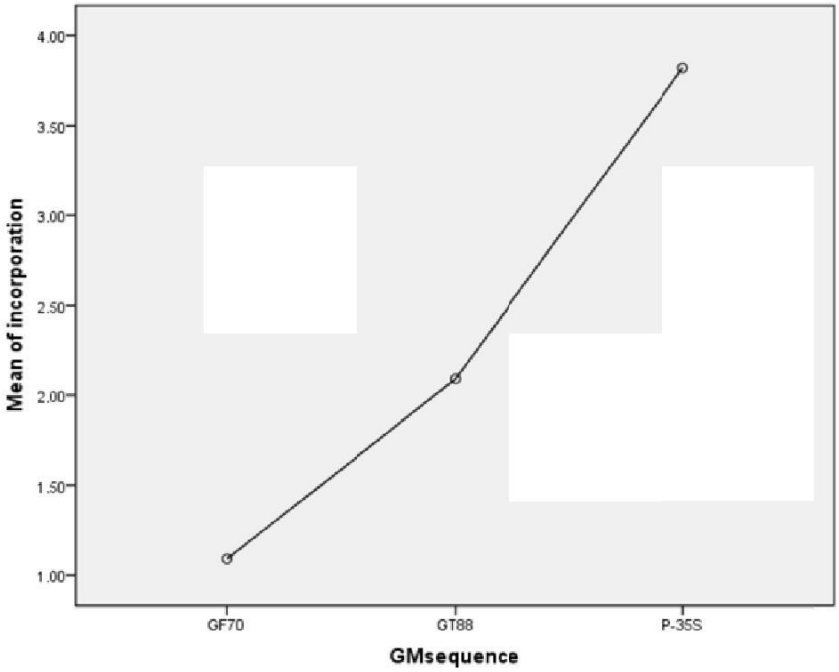


Figure 7. Affinity of incorporation f different GM-sequences (GF70, GT88 and P-35S) into the genome of rats fed on transgenic diet (GM-diet).

I n our investigation, we evaluated he transfer of CaMV-P35S promoter from transgenic d iet into experi-mental animals. For this purpose, we designed th ee pri mers that amplify three segments of CaMV-P35S promoter (acce sion no.emb|V00141.1|) from nucleotide coo rdinate 7117 to 7384 (Table 1) w hich represents ne rly 80% of the whole promoter (34 3 bp). In some cases in the present work, the three seg ments were all amplified in th same anim al. This finding may raise con cern that the whole promoter mig ht have been tra nsferred 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 tra nscription init ation (Odell et al., 1985).

R esults of the present work suggest a health hazards accompanying the inges ion of diets containing Cauliflower mosaic virus 35 promoter since Cauliflo er mosaic virus has been reported to be closely related to hu man hepatitis B virus (Do little et al., 1989, Xiong and Eikbush, 1990). It is also known th at CaMV-P35S promoter can direct the expression of the bacterial ne mycin phosphotransferase II (*NP TII*) gene in *Es cherichia coli* as report d by Assaad and Signer (1990). They al so reported that, it can fu nction in a wide range of organisms (plants and animals). It has also been de monstrated t hat the CaMV-P35S pro oter sequen ce can convert an adjacent tissue- and organ-specific gene promoter into a globally active promoter (Zheng et al., 20 7). Previou ly, Ho and his colleag es (Ho et al., 19 9) deduced that the CaMVP-35S promoter could comb ine to active dormant viruses, create new viruses, and cause cancer by over expression of n ormal 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 bree ing and cultivation of conventional crops.

Comparing tissu e selectivity of the three tissues (blood, liver, and brain) examined thr ugh the who le duration of this work (90 days), no sig nificant diff rences were observed in the r ate of transfer of GM-D N A into these tissu es. GM-DNA transfer to blood tissue was barely highe r (12.66 ± 2.6034) than t he GM-DNA transfer to the other tissues: liver (12.00 ± 3.6055) and b rain (12.00 ± 2.000 0). 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 vess els and involv ed infiltratio n , such as liver and kidney. They concluded that blood is the main tissue involved in the uptake of s hort DNA fragments since it collects macromolecules directly abs orbed by the intestin l epithelium and the cells of the immune system and that DNA molecules m ay be transported in the organism via blood circulation.

Our results have demonstra ted that the frequency of uptake for the larger segment s was greater than that for the smaller segment. This conclusion was based on the fact that the efficiency of tran fer of GM-DNA segments (70 bp) amplified b y GF70 prim ers was the lowest (1.09 ± 0.416 1) compare d 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 GF7

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 Hellingwef (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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