Food and Chemical Toxicology 124 (2019) 423–430

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Review

Addressing concerns over the fate of DNA derived from genetically modified food in the human body: A review

Muhammad Amjad Nawaz[a,b,1], Robin Mesnage[c,1], Aristides M. Tsatsakis[d], Kirill S. Golokhvast[a,e], Seung Hwan Yang[b], Michael N. Antoniou[c,**], Gyuhwa Chung[b,*]

[a] Education Scientific Center of Nanotechnology, Far Eastern Federal University, Vladivostok 690950, Russian Federation
[b] Department of Biotechnology, Chonnam National University, Yeosu, Chonnam, 59626, Republic of Korea
[c] Gene Expression and Therapy Group, King's College London, Department of Medical and Molecular Genetics, School of Basic & Medical Biosciences, 8th Floor, Tower Wing, Guy's Hospital, Great Maze Pond, London SE1 9RT, UK
[d] Department of Toxicology and Forensics, School of Medicine, University of Crete, Heraklion, Crete, Greece
[e] Pacific Institute of Geography, FEB RAS, Vladivostok 690041, Russian Federation

ARTICLE INFO

Keywords:
Genetically modified
GM food and feed
Transgene integration
Gastro intestinal tract
Integratability of DNA
Experessability of DNA

ABSTRACT

Global commercialization of GM food and feed has stimulated much debate over the fate of GM food-derived DNA in the body of the consumer and as to whether it poses any health risks. We reviewed the fate of DNA derived from GM food in the human body. During mechanical/chemical processing, integrity of DNA is compromised. Food-DNA can survive harsh processing and digestive conditions with fragments up to a few hundred bp detectable in the gastrointestinal tract. Compelling evidence supported the presence of food (also GM food) derived DNA in the blood and tissues of human/animal. There is limited evidence of food-born DNA integrating into the genome of the consumer and of horizontal transfer of GM crop DNA into gut-bacteria. We find no evidence that transgenes in GM crop-derived foods have a greater propensity for uptake and integration than the host DNA of the plant-food. We found no evidence of plant-food DNA function/expression following transfer to either the gut-bacteria or somatic cells. Strong evidence suggested that plant-food-miRNAs can survive digestion, enter the body and affect gene expression patterns. We envisage that this multi-dimensional review will address questions regarding the fate of GM food-derived DNA and gene-regulatory-RNA in the human body.

1. Introduction

Global cultivation of genetically modified (GM) crops for food and feed reached 185.1 million hectares in 2016 with total commercial benefits of 150.3 billion US$ (for 1996–2014) (Brookes and Barfoot, 2016). The vast majority of GM plants have been developed to improve field performance (herbicide tolerance, insecticide production). Depending upon the country and its respective legislation, ingredients such as starch, fats/oils, proteins, corn syrup and other products derived from GM crops can now be found in the human diet (from infant formulas to baked snacks) as well as in livestock feed (Tsatsakis et al., 2017a, 2017b).

Humans and animals are constantly exposed to foreign DNA (GM and/or non-GM) from a broad range of food and feed sources. Until a few years ago it was assumed that ingested DNA is completely degraded in the digestive tract of humans and animals (Rizzi et al., 2012). However, with the global commercialization of GM food and feed, there has been a renewed interest in the fate and effects of GM-derived extracellular DNA in the body of the consumer. This has triggered the need to understand precisely the biological processes involved in degradability, stability, mutagenic potential and expressibility of extracellular food-derived DNA.

The objective of this review is to explore recent developments in understanding the possibilities and mechanisms through which foreign DNA can resist degradation in the digestive system and enter the body of the consumer. Although comprehensive reviews on the topic have already been published (Rizzi et al., 2012), a large number of articles have been published since and an update is necessary to address concerns associated with recent changes in the use GM crops. We are particularly interested in assessing the gastrointestinal tract as a

* Corresponding author.
** Corresponding author.
E-mail addresses: michael.antoniou@kcl.ac.uk (M.N. Antoniou), chung@chonnam.ac.kr (G. Chung).
[1] Authors contributed equally.

https://doi.org/10.1016/j.fct.2018.12.030
Received 7 November 2018; Received in revised form 17 December 2018; Accepted 19 December 2018
Available online 21 December 2018
0278-6915/

possible hotspot for horizontal gene transfer of plant food DNA to gut bacteria, which potentially can lead to dysbiosis and ill-health. We discuss the controversies surrounding the presence of dietary DNA in the blood and other organ systems of the consumer. We also discuss the possibility of genome integration and expression of foreign dietary DNA in the consumer's tissues. Finally, we also briefly consider the potential of plant food-derived miRNA uptake and possible effects on gene function of animals and humans.

2. State of DNA in food products

The amount of DNA present in foods varies depending upon the food source; that is, animals or plants, cell density and type of cells in edible parts of food, processed or raw foods, etc. The relative content of DNA in animal (10–20 g/kg) is higher than plant (0.6–3 g/kg) (Herbel and Montag, 1987; Lassek and Montag, 1990) foods. The DNA content of processed and highly processed foods such as vegetable oil, starch, and sugars is quite low and sometimes undetectable depending upon the physical conditions used during food processing and analytical methods employed. It is well established that higher temperature and pressure, lower pH and chemical processing cause denaturation and fragmentation of DNA molecules and significantly reduces the size of detectable and amplifiable DNA in processed foods. However, even after physical processing conditions, up to 300 bp long DNA fragments were detected in plant derived foods (Bergerová et al., 2011). Furthermore, DNA interacts differently with different types of food additives and compounds used during processing and storage (Srinivasan et al., 2002). Depending upon the nature of compounds added for food processing and the nature and level of enzymes present in each food source, the extent of DNA degradation is variable. The presence of endogenous DNases, nuclease-induced DNA degradation, hydrolysis due to high temperatures, addition of tannic acid and amines, lactic acid fermentation, chopping, cooking, release of endonucleases, and cell wall degradation all contribute to the amount, size and state of DNA that may ultimately be found in food and feed (Gryson, 2010).

Commercially cultivated GM plants can harbour < 5 unique protein-encoding transgenes, which can be 'stacked' in the same plant to confer new traits to the host. It has been estimated that normally humans ingest 0.1 g–1.0 g (more than 100 trillion genes) of foreign DNA on a daily basis (Doerfler, 1998; Doerfler et al., 1998). The transgenes ingested by humans are chemically equivalent (structurally) to any other gene present in food. Physical as well as chemical (hydroxylation, oxidation, deamination) damage to DNA is brought about during multiple physicochemical conditions applied during food processing (Kharazmi et al., 2003). Reduction in fragment length of transgenic maize DNA was reported when 65 °C was applied for 90 min at pH 4.0, although 957 bp long fragments remained detectable (Bauer et al., 2003). Under the combined effect of temperature (120 °C), pressure (0.1 MPa) and low pH (2.25), DNA fragments of < 300 bp were amplified from MON 810 GM maize (Bergerová et al., 2011). DNA of high molecular weight has been reported in semi-processed foods such as ground wheat grains (> 21 kbp), biscuits (100–1500 bp), pop corn and wheat bread (< 800 bp), tomato ketchup and soup (< 400 bp) and fermented silage (< 200 bp) (Rizzi et al., 2003, 2012). In one investigation it was found that in the production of maize or ryegrass silage farm animal feed, DNA remained stable and intact suggesting that if horizontal gene transfer to either gut bacteria or the cells of the animal consumer is an issue, then this feed would pose a significant risk (Forbes et al., 1998). Only following heat treatment to 95 °C for at least 5 min was DNA completely fragmented (Forbes et al., 1998). In another study of the amplification of highly processed dietary DNA, amplification was still observed for amplicons of 351 bp (Hird et al., 2006).

The mechanism of DNA degradation at higher temperatures is based on depurination and deamination and strand scission. In addition, irreversible loss of DNA secondary structure occurs at elevated heat during food processing (Gryson, 2010; Lindahl, 1993). Drying at higher temperatures degrades DNA much more rapidly as was observed in heating maize kernels to 94 °C for 5 min (Chiter et al., 2000) and drying wet-milled gluten fractions at 135 °C for 2 h (Gawienowski et al., 1999). However, a mean size of up to 400 bp was still observed even after heating DNA in solution at 99 °C for 7 h. The degradation of DNA during food processing, cooking, mechanical treatment, enzymatic degradation, irradiation, and sonication is presented elsewhere (Gryson, 2010), and will thus not be detailed here. Autoclaving, sterilization, baking, roasting, and frying notably increased DNA degradability (Allmann et al., 1993; Bauer et al., 2003; Debode et al., 2007). Nevertheless, DNA during all these processing, heating and cooking treatments was still detectable and found to have a fragment length that was amplifiable by polymerase chain reaction (PCR). This is important when considering the possibility of DNA fragments being ingested and present in the human or animal digestive system.

The daily intake of DNA differs greatly based on individual dietary variations. The amount of dietary DNA consumed by humans is much lower compared to cattle, mainly due to the difference in the amount of food ingested. Furthermore, dairy animals often consume the same type of dietary DNA; that is, in silage and/or forage. Humans consuming 170 g of GM soybean, potato and maize will ingest approximately 70 μg DNA, only a very small fraction of which (0.00006%) will be from the inserted transgene. Although humans are consuming largely processed products derived from GM crops, farm animals ingest far greater quantities including raw or only partially processed feeds (Van and Young, 2014). For example, in the case of a 600 kg Holstein dairy cow, the consumption of a diet made of 60% GM maize would result in the consumption of 54–57 g of maize DNA per day (Beever and Phipps, 2001). This results in the consumption of 54 mg of GM transgene per day (0.000094% of total dietary DNA), in a maximum possible exposure situation, which assumes no degradation of the DNA during digestion (Fig. 1A) (Jonas et al., 2001; Phipps et al., 2001; Rizzi et al., 2012). Upon digestion, the size of this dietary GM and/or non-GM DNA will be greatly reduced, and thus the final amount of dietary DNA containing intact gene fragments in the gastrointestinal tract that could participate in horizontal gene transfer will be much lower.

3. Fate of dietary DNA in the gastrointestinal tract

Epithelial cells of the gastrointestinal tract (GIT) lumen are constantly exposed to foreign dietary DNA. These cells are the most likely entry route for foreign DNA into the body of the consumer, since one of the primary functions of the GIT is the absorption of nutrients. In humans, the total mucosal surface of the digestive tract averages 32 m^2 (Helander and Fändriks, 2014) and it has been estimated that the average dietary intake of DNA varies from 0.1 to 1 g per day as part of a normal diet (Doerfler, 1998).

Although deoxyribonuclease I (DNase I) is detected in saliva, it is believed that DNA digestion starts in the stomach (Liu et al., 2015) where histones are separated from DNA by the action of pepsin (the primary enzyme in the stomach) and the acidity of the environment. DNA is further broken down by gastric acid and DNA nucleases along the GIT and thus only small fragments are presented to intestinal epithelial cells. However, even if the proportion of dietary DNA that is capable of being absorbed by intestinal cells is extremely low, possible biological consequences cannot be excluded. Whether foreign DNA can be absorbed and can reach the bloodstream was first investigated by the group of Walter Doerfler (University of Cologne). In a series of experiments, mice consumed large amounts of bacteriophage M13 DNA added to their regular laboratory rodent feed (Schubbert et al., 1994, 1997). These experiments first showed that M13 DNA fragments of up to 1692 bp (out of a total length of 7250 bp) survived passage through the GIT. However, 20 years after these pioneering studies, little is still known about the mechanisms and consequences of DNA uptake.

The intestinal epithelium is not a complete barrier against the intrusion of DNA fragments. Even if the intestinal cell membrane prevents

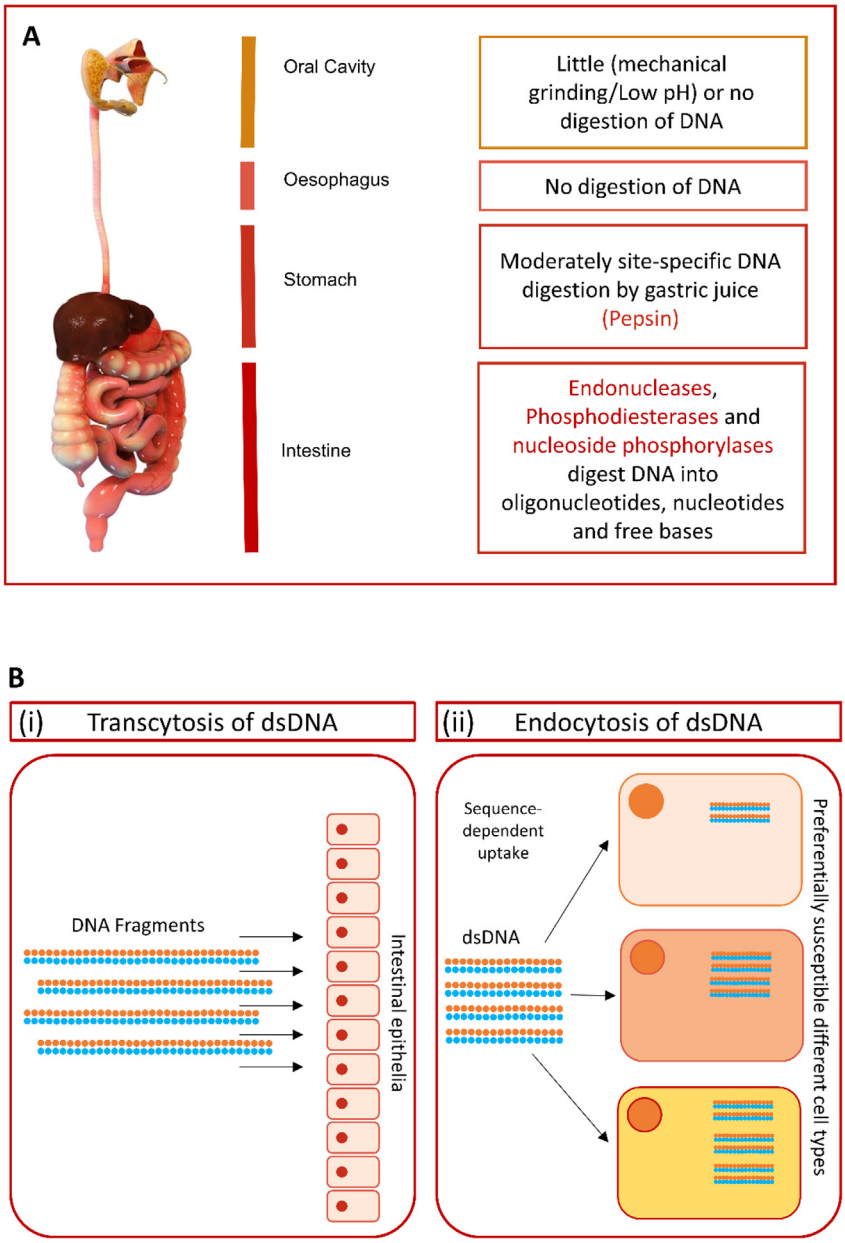

Fig. 1. A. Fate of extracellular DNA in human digestive system. B. Two possible processes involved in extracellular DNA uptake into the cells. (i) Transcytosis of dsDNA: Uptake of DNA fragments across the intestinal epithelia mediated by vesicular transport. (ii) Endocytosis of dsDNA: Naked dsDNA can be spontaneously internalized by sequence-dependent mechanism by which genetic information can enter living cells at significant amounts in a bioactive form. The process is also cell-type dependent.

passive diffusion of DNA fragments into cells, it has more recently been demonstrated that uptake across the intestinal epithelium is mediated by vesicular transport (transcytosis) (Fig. 1B(i)) (Johannessen et al., 2013). Using the human intestinal epithelial cell line CaCo-2 as a model system, it was shown that fragments (278 and 270 bp) of the CP4 variant of 5-enolpyruvyl-shikimate-3-phosphate synthase (CP4-EPSPS) transgene, which is present in glyphosate tolerant GM crops, can be internalized (Sharma et al., 2007). In a follow-up investigation, a total of 0.06% of 633 bp long DNA fragments were found to be transported across polarized CaCo-2 cells (in an apical to basolateral direction) within a 90 min period (Johannessen et al., 2013). Even whole non-degraded DNA fragments of 633 bp were detected in the basolateral compartment. This led the authors to estimate that a standard DNA uptake for an adult human would correspond to approximately 0.5 μg DNA, or 10^{10} DNA fragments of 1000 bp, considering an average daily DNA dietary intake of 1 g (Johannessen et al., 2013). In the case of GM crop-derived food in the diet, only a few thousand copies of transgene fragments would thus likely be absorbed per day (Jonas et al., 2001). It is also important to note that spontaneous uptake of DNA fragments by mammalian cells is a tissue- or cell-type specific process, and is also sequence-dependent (Fig. 1B (ii)) (Lehmann and Sczakiel, 2005). Moreover, interspecies extrapolation should be considered with caution since the longer transit time of digesta through the human intestinal tract (compared with laboratory rodents) probably allows a more complete DNA hydrolysis by intestinal microbial and pancreas-derived DNases. In a study of the survival of transgenic plant DNA from GM soya in the human digestive system, it was found that only a small proportion (a maximum of 3.7%) of the CP4-EPSPS transgene survives passage through the stomach and the upper GIT, but that the transgene was fully degraded by the time it had passed through the large intestine (Netherwood et al., 2004).

It is also interesting to address the question of DNA digestion from

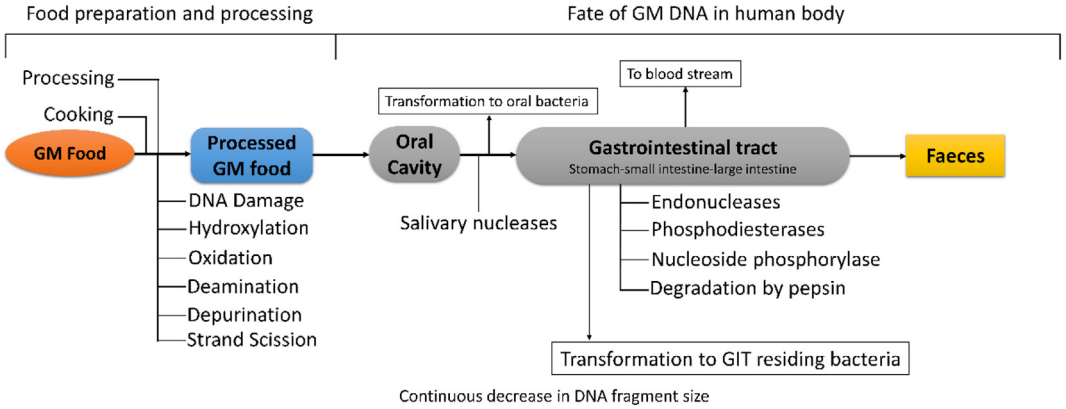

Fig. 2. Summary of effect of food preparation and processing on DNA and the fate of GM-derived dietary DNA in the body of the consumer.

an evolutionary standpoint. Mammals have been exposed to DNA through their diets over millions of years, and it is thus likely that the extent to which the DNA is degraded by the GIT is a specific trait, which could have been evolutionarily selected. The detection of specific DNA fragments could serve as a sensor to detect the presence of pathogens. In fact, the transport of foreign DNA across the intestinal barrier has well known immunostimulatory effects. The GIT hosts several types of immunosensory cells, which are able to detect bacterial unmethylated CpG motifs to trigger innate immunity as a defence mechanism (Krieg, 2002). For instance, dendritic cells strongly express the Toll-like receptor TLR9, which recognizes specific bacterial CpG motifs in order to activate innate immune cells via the Toll/IL-1R signalling pathway (Bauer et al., 2001). As CpG motifs are usually methylated in mammals but remain unmethylated in bacteria, TLR9 can discriminate between self and non-self DNA. Molecular patterns recognized by TLRs include different types of lipids, proteins or nucleic acids derived from bacteria, viruses, parasites and fungi (Ohto and Shimizu, 2016). Fig. 2 summarises the fate of DNA during food processing and in the body of the consumer.

4. The gastrointestinal tract: a hotspot for horizontal gene transfer

The human body is generally studied as a single organism, although it functions more as a complex ecosystem since it hosts trillions of bacteria in different body habitats. The GIT alone is inhabited by 10^{13}-10^{14} bacteria (Sender et al., 2016). There is a gradient in bacterial concentration along the GIT, from low concentrations in the stomach and the duodenum (10^{3}-10^{4} bacteria/g), increasing in the ileum (10^{8} bacteria/g), with the highest bacterial concentrations found in the colon and stools where $\sim 10^{11}$ bacteria/g are present. Dysbiosis of the gut microbiota is implicated in a wide range of diseases such as inflammatory bowel disease, diabetes, cardiovascular disease, or even autism spectrum disorders (Cho and Blaser, 2012). The dynamics of these bacterial communities is complex and will not be fully detailed here. However, one hallmark of these communities, which is relevant to this review, is that bacteria can share different phenotypic traits through a transfer of genetic material. This was first described in 1928 by Fred Griffith, when DNA from a virulent bacterial strain (*Streptococcus pneumonia*) was isolated and mixed with an avirulent form of the bacterium (Griffith, 1928). While neither the isolated DNA from the virulent bacteria, nor the avirulent bacteria, was able to harm mice, the mix of both provoked a chronic infection causing septicemia. This was subsequently found to be caused by a mechanism known as horizontal gene transfer (HGT), by which bacteria can share different traits such as antibiotic resistance (van Schaik, 2015). This phenomenon is very common in bacterial populations and constitutes a strategy for these organisms to adapt to environmental changes.

Concerns have been raised regarding the ability of transgenes from GM crops to be transferred to bacteria inhabiting the GIT (Uzogara, 2000). A particular concern stems from the fact that genes coding for antibiotic resistance have been incorporated into the genome of some GM crops along with the GM trait transgene. These antibiotic resistance genes were used in order to facilitate the selection of transformed plants that were positive for the introduced GM event.

DNA fragments from GM plants can be taken up by single bacterial species. For example, transgene DNA from GM sugar beet (Gebhard and Smalla, 1998) and GM potatoes (de Vries et al., 2001) has been shown to be readily assimilated by *Acinetobacter* sp. However, these experiments were performed using optimised laboratory conditions, which are not likely to be encountered in natural environments. To date, no *in vivo* study has reliably demonstrated integration of DNA fragments from GM crops into the gut microbiota of a mammalian species. For example, an investigation of the incorporation of plasmid DNA encoding for ampicillin resistance by the gut microbiome of mono-associated rats (either with *E. coli* or *B. subtilis*) showed no uptake *in vivo*, even if the plasmid isolated from the intestine was able to transform bacteria *in vitro* (Wilcks et al., 2004). There are however several technical limitations preventing the detection of rare integration events that should be born in mind. It can require years for a bacterial transformant to multiply to numbers that can be detected with currently used analytical methods (Nielsen et al., 2014). For instance, it took several decades for penicillin-resistant *S. pneumoniae* to become 21.5% of the isolates in the US(Doern et al., 2001).

Although the incorporation of DNA fragments from GM plants into bacteria inhabiting the gut microbiome is plausible, only one study of humans has provided evidence of GM soya transgene DNA transfer into the subject's microbiota (Netherwood et al., 2004). In this study 3 out of 7 ileostomists were found to have the CP4-EPSPS transgene within their gut microbiota, and that this did not increase following consumption of a GM soya meal (Netherwood et al., 2004). Generally, HGT into gut bacteria has to date not been completely proven. First of all, complete genes are unlikely to be available given the degradation of DNA fragments during the digestive process. Then, these fragments should preferably include DNA sequences matching those of the bacterium in order to expedite integration by homologous recombination, although some bacteria are able to take up DNA from any source with equal efficiency (de Vries and Wackernagel, 2005). Furthermore, if it happens that a bacterium incorporates a DNA fragment from a GM crop, an intact or functional sub-domain would need to be present. Insertion into the genome of the microorganism would need to be in manner that will allow its expression, and it would need to be selected in order to outgrow wild-type bacteria. As most of the DNA fragments taken up from food will not provide any selective advantage, it is likely that the transformation will impose a physiological price and thus provide a negative fitness. Only genes conferring resistance to antibiotics or herbicides such as glyphosate with antibiotic capability would be

capable of being selected, if the amount of antibiotics or herbicides ingested is sufficient. It is also of note that most studies dedicated to the emergence of antibiotic resistance do not take into account random transformation with linear DNA fragments as a possible contributor to this problem (Gay and Gillespie, 2005). Overall, given the far reaching ramifications to human and animal health that could arise from the generation of antibiotic resistant pathogenic microorganisms through HGT, it is logical that the new generation of GM crops should be created without incorporation antibiotic resistance genes.

5. Controversies on the presence of dietary DNA in the bloodstream

Even if most dietary DNA is degraded during digestion, fragments of a few hundred nucleotides can nonetheless reach the bloodstream. In experiments conducted by Doerfler and colleagues aimed at investigating the fate of M13mp18 plasmid fed to mice, DNA fragments were found in blood (194–976 bp), in about 1 of 1000 peripheral blood leukocytes, as well as in spleen or liver cells up to 24 h after feeding (Schubbert et al., 1997). Moreover, upon extended feeding, M13mp18 DNA was re-cloned from total spleen DNA into a lambda vector, which indicates that the foreign DNA became covalently linked to murine DNA (Schubbert et al., 1997). In a follow-up study, M13 DNA was also found in cells of fetuses and new-born animals when it was administered during pregnancy, demonstrating passage of ingested DNA across the placenta (Doerfler et al., 1998). Further experiments were undertaken by the same group to study the fate of a larger gene, ribulose-1,5-biphosphate (Rubisco), in mice fed soya bean leaves (Hohlweg and Doerfler, 2001). Fragments of the Rubisco gene were recovered in the intestine up to 49 h after feeding, in the cecum up to 121 h after ingestion, as well as in liver and spleen.

Although the persistence of host-derived DNA in blood is well known, the extent of dietary DNA presence in human blood is an area of intense debate. The existence of circulating cell-free DNA was first described in 1948 by Mandel (1948). It is well known that these DNA fragments in the circulation mostly originate from the death of host cells (apoptosis). These circulating DNA fragments surprisingly resist serum nuclease activity because they circulate as nucleoprotein complexes adsorbed to the surface of blood cells (Thierry et al., 2016). In 2013, it was reported that dietary DNA fragments large enough to carry complete genes can enter the bloodstream after the analysis of 1000 human samples from four independent studies (Spisák et al., 2013). However, these findings were challenged in a subsequent study, which suggested that contamination and sensitivity could compromise the integrity of the dataset thus warranting its closer scrutiny (Lusk, 2014). In another investigation the presence of rabbit DNA was assessed in the blood of two human volunteers who had eaten 400–600 g cooked rabbit meat (Forsman et al., 2003). The presence of the high-copy-number rabbit retrotransposon RERV-H in food, and in the blood, was assessed by quantitative PCR. The meal of rabbit meat ingested by these two volunteers contained a total of 10^{14} copies of RERV-H DNA. This study detected a maximum concentration of 200 copies of the transposon per mL peripheral blood one hour after ingestion. This corresponded to an uptake of approximately 10^{6} RERV-H DNA copies.

Overall, sufficient evidence gathered by different studies confirms that the presence of small DNA fragments in the blood originating from the diet is probably a common phenomenon. In relation to foods derived from GM crops, there is no evidence suggesting that the frequency of transgenic DNA is higher than normal plant host dietary DNA in the bloodstream. Thus, the presence in the bloodstream of either whole genes or their intact regulatory elements and their potential to give rise to insertional mutagenesis, remains hypothetical.

6. Biological effects of exogenous dietary RNAs

The controversy surrounding biological effects of dietary nucleic acids was further stimulated in 2012 when Chen-Yu Zhang and colleagues reported that ingested rice miRNAs from rice directly influenced gene expression in mice (Zhang et al., 2012). This has implications for GM foods since crops have started to be approved, which express double-stranded (ds) short-hairpin RNA (shRNA) molecules in order to knock-down expression of specific gene(s) via the RNA interference (RNAi) pathway targeting either plant host genes (e.g., browning-resistant Artic Apples and Innate potatoes (Food and Drug Administration, 2015)) or those of virus or insect pests that may infect or feed on the crop (Zhang et al., 2017). In addition, herbicidal and insecticidal sprays based on shRNAs are being deployed to target weeds and insect pests respectively (Broglie et al., 2017). Thus novel shRNA molecules from these two, relatively new types biotechnology are beginning to enter the food supply raising health concerns from possible off-target effects of these gene regulatory molecules (Heinemann et al., 2013; Lundgren and Duan, 2013; Zhao et al., 2017).

The study by Zhang and colleagues triggered an intense debate, which is still ongoing. A subsequent study conducted by miRagen Therapeutics and Monsanto company failed to replicate the results of Zhang and colleagues (Dickinson et al., 2013). This led miRagen and Monsanto to claim that the results obtained by Zhang were artefactual or spurious. An elevation of plasma LDL-cholesterol level in mice was nonetheless detected as found by Zhang and colleagues, but it was attributed to a nutritional effect rather than to the effects of miRNAs, which were not detected. In response, Zhang and colleagues identified flaws in the RNA-seq procedure used by miRagen Therapeutics and Monsanto (Chen et al., 2013), which could account for their inability to detect rice miRNAs in the tissues of the mice. Further studies attributed the results of Zhang et al. to a cross-contamination issue during sequencing (Tosar et al., 2014).

As plant-derived miRNAs possess a 2′-*O*-methyl modified 3′ end, they can be distinguished from equivalent animal and human sequences leading to them being unequivocally identified in the blood, organs and breast milk of consumers (see Zhao et al., 2017). A recent analysis of human plasma small RNA sequencing datasets has clearly identified numerous plant food miRNA species to be present (Liu et al., 2017). Thus, although the area of cross-kingdom (plant-animal) miRNA communication remains a controversial topic (Witwer and Zhang, 2017), these observations confirm that plant food miRNA species can enter the body of the consumer potentially affecting host gene function (Zhao et al., 2017). In the context of this review, what appears clear from currently available evidence is that shRNAs present in foods (derived from GM crops or not), or from foliar spray application will, in all likelihood, enter the body of the animal and human consumer.

It is not clear if these ingested siRNAs will impact on consumer gene function with negative health consequences. Nevertheless, potential health risks from off-target disruption of gene expression arising from these new sources of miRNA food exposure should be fully taken into account by regulators when considering applications for market approval of these products (Heinemann et al., 2013). This risk assessment could include bioinformatics analysis to identify any targets in humans and other critical organisms, followed by evaluation of the possible biological activity on cell cultures (Heinemann et al., 2013).

7. Integrability and expressability of foreign DNA in host tissues

The cauliflower mosaic virus 35S promoter (CaMV 35S) is the most frequently used transcriptional regulatory element to express transgenes in GM crops (Hull et al., 2000). It has been proposed that transfer and integration of an intact CaMV 35S promoter-enhancer fragment may result in activation insertional mutagenesis; that is, where the CaMV 35S promoter activates host gene function around the site of integration (Ho and Cummins, 2009; Ho et al., 1999; Podevin and Du Jardin, 2012). The CaMV 35S promoter has indeed been shown to be functional in animal and human cells *in vitro*. This was first demonstrated in *Xenopus laevis* oocytes (Ballas et al., 1989) with subsequent

studies employing transient transfection reporter gene assays showing that CaMV 35S is active in both animal and human cells (Kitagima et al., 2013; Myhre et al., 2006; Tepfer et al., 2004; Vlasák et al., 2003; Zanta et al., 1999). Most recently, CaMV 35S has also been shown to be active *in vivo* following intramuscular injection of plasmids harbouring this element in Atlantic salmon (Seternes et al., 2016). However, expression from CaMV 35S was found to be at best 1% of that observed from control constructs using equivalent viral promoter-enhancer elements that were appropriate for the species of cells being used, (for example, CMV or SV40 promoters in mammalian cell lines) (Vlasák et al., 2003). This demonstrates that CaMV 35S is a very weak element in a human cell environment even when it is present on non-integrated, transcriptionally permissive episomal plasmid molecules. In all likelihood CaMV 35S would be even less functional when randomly integrated into the host cell genome where it would come under the sway of epigenetic (e.g., DNA methylation) mediated silencing (Rajeev Kumar et al., 2015).

Although DNA-fragment transport across the intestinal barrier seems to be frequent, integration in the genome of intestinal epithelial or other cell types is likely to be a rare event. Even in the unlikely case of an integration of a DNA fragment such as the CaMV 35S promoter at a location that results in a disturbance of host gene and cellular function, this may be of no consequence if the cell type in question has a high turnover rate as in the case of intestinal epithelial cells. It is not clear if the activation of oncogenes after integration of DNA fragments in intestinal epithelial cells could happen before they undergo turnover.

The fact that dietary DNA can be frequently detected in peripheral white blood cells in mouse feeding experiments (Schubbert et al., 1997), but that this DNA is barely retrievable more than 24 h post-administration, suggests the existence of several mechanisms facilitating the elimination of foreign DNA from the host organism. The major mechanism for protection against the function of foreign DNA element integration in mammalian cell genomes consists of inactivation by methylation. This mechanism has allowed mammalian cells to protect themselves from retroviral infections over evolutionary timespans. Accumulated endogenous retroviruses account for 8% of the human genome (Lander Eric et al., 2001). Animal cells are equipped with a wide range of molecular tools allowing them to silence foreign DNA elements by methylation of cytosine residues at CpG dinucleotides. Silencing of transgene integrations is also a common problem in the development of gene therapy strategies, which led to at least one therapeutic failure in a clinical trial (Neville et al., 2017). Hence there are efforts to develop new tools to confer a stable, site of integration-independent transgene expression (Neville et al., 2017). On the rare occasion when integration of a DNA-fragment takes place, this DNA fragment will probably become methylated and thus transcriptionally silenced. As the CaMV 35S promoter lacks a dominant chromatin remodelling capability, this too would be prone to DNA methylation-mediated silencing (Rajeev Kumar et al., 2015) (see Information Box). In addition, human and animal cells are also equipped with cytoplasmic DNA sensing pathways, such as the STING (for stimulator of interferon genes), which was demonstrated to facilitate IFN production in response to the presence of intracellular foreign DNA (Barber, 2011).

A large number of studies have been conducted to elucidate the mechanisms of foreign DNA integration in order to facilitate clinical or industrial applications using genetic engineering technologies. As previously discussed, DNA does not diffuse passively across cell membranes and its uptake is both sequence and cell-dependent (Lehmann and Sczakiel, 2005). The access to the nucleus is limited by passage through nuclear pore complexes, except when the nuclear envelope disassembles at the end of prophase during mitosis. This was first observed in 1980 by Capecchi who demonstrated that microinjection of the Herpes virus thymidine kinase gene into a murine cell line deficient in this enzyme activity (Capecchi, 1980). Between 50 and 100% of the cells were found to express thymidine kinase activity when the DNA encoding this enzyme was delivered directly to the cell nuclei, but that the gene was not expressed when the DNA was injected into the cytoplasm (Capecchi, 1980). However, further studies have shown that plasmid DNA can translocate into the nuclei of non-dividing cells through the nuclear pores (Dowty et al., 1995). An investigation of SV40 viral genome's capability to enter the nucleus of non-dividing cells, led to the discovery of DNA nuclear targeting sequences, allowing specific import through the nuclear pores (Dean, 1997). The SV40 nuclear localisation signal peptide was subsequently exploited to enhance cell transfection efficiency (Brandén et al., 1999).

If foreign DNA successfully enters the nucleus, its incorporation into the host cell genome is a very rare event with degradation or dilution and loss from cells through successive rounds of mitosis being by far the more usual outcome. However, in some rare instances, the foreign DNA fragment will integrate into the genome with frequencies varying depending on the cell type and sequence. For instance, some fibroblasts (XP2OS-SV40 and HeLa cell lines) have transformation frequencies of 0.1% whereas the simian kidney cell line CV-1 can be stably transformed with a higher frequency of 6% (Gorman et al., 1983). One should notice that these last results came from laboratory transfection studies and may not accurately reflect natural cellular processes, especially *in vivo*.

There are two possible mechanisms by which DNA fragments can become integrated into chromosomes: homology-dependent recombination and illegitimate integration. The outcome from homology-dependent recombination is more predictable but also a much rarer event. Illegitimate integration, which occurs in the vast majority of cases has been estimated to take place between 4 and 1,000,000 times more frequently than homology-directed integration, with these ratios depending on experimental conditions and cell types used (Würtele et al., 2003). Indeed, little is still known about the mechanisms by which foreign DNA integrates in the genome, with insertion sites being generally random and thus unpredictable. Furthermore, DNA fragments can undergo numerous modifications and rearrangements prior to integration. The foreign DNA fragment can be truncated, is frequently concatenated into tandem repeats (direct or inverted) or even interspaced with other DNA segments during the process of integration. This affects the stability of the resulting transgene expression (if any) (McBurney et al., 2002). These genomic modifications are generally unstable overtime and the cell population tends to lose most of the foreign DNA fragments during the first week post-transfection (Würtele et al., 2003). These processes give rise to large barriers that need to be overcome in order for integration of dietary DNA fragments into the genomic DNA of the consumer to take place. This is well known to those involved in developing new strategies to increase the level and stability of transgene expression in either protein bio-manufacturing using mammalian cell factories or gene therapy strategies (Neville et al., 2017).

Despite intense efforts on this topic no study has reported the integration of transgenes from GM crops into the genomes of either animal or human consumers. It is nonetheless interesting to note that human genomes have acquired genes from multiple species through HGT over evolutionary timescales. A study evaluating the acquisition of new genes in 26 animal species (10 primates, 12 flies and four nematodes) by HGT found a total of 145 “foreign” genes in the human genome (17 previously-reported and 128 newly reported) (Crisp et al., 2015). For example, the human ABO group of genes is thought to have been acquired from bacteria to enhance mutualism between vertebrates and microorganisms (Brew et al., 2010). More surprisingly, the *FTO* gene implicated in human obesity was only found in marine algae and vertebrates (Robbens et al., 2008). A recent study also showed that cultivated sweet potato has incorporated a transfer DNA (T-DNA) sequence from *Agrobacterium* sp. in its genome (Kyndt et al., 2015). Although this finding may have implications for the debate on GM crop safety, the authors’ comparison to man-made transgenic crops is in our view farfetched. There are indeed many differences in the ways a GM

crop is produced, including the use of a plant tissue culture phase (Fonseca et al., 2015), compared to the natural incorporation of exogenous genes by HGT and their selection for fitness through evolutionary timescales described in the aforementioned study.

8. Conclusion

Global commercialization of GM food and feed has stimulated much debate over the fate of GM food-derived DNA, especially transgene sequences, in the body of the human or animal consumer and as to whether it poses any health risks. The aim of this review was to address concerns over the fate of dietary DNA and RNA in the human body.

Food DNA can survive harsh processing and digestive conditions with fragments up to a few hundred base pairs detectable in the gastrointestinal tract. We have also found compelling evidence for the presence of food-derived DNA in the bloodstream. Although there is sufficient evidence that small fragments of dietary DNA can enter the bloodstream, the presence of intact genes still remains an unanswered question. We find no evidence that transgenes in GM crop-derived foods have a greater propensity for uptake and integration than the host DNA of the plant food. We also found no evidence of plant food DNA function (that is, gene expression) following transfer to either the gut bacteria or somatic cells of the consumer, although this appears to be a poorly explored area.

With respect to plant food miRNAs, we identified evidence that these molecules can indeed survive digestion and enter the body of the consumer. However, to date we find that there is far less, but still compelling evidence, to suggest that these ingested siRNA molecules can target homologous sequences in the consumer and affect gene expression. Nevertheless, even based on the currently available evidence off-target effects from the ingestion of novel siRNAs present in foods derived from either GM crops or foliar insecticidal or anti-viral spray application, cannot be ignored and thus should form an integral part of the risk assessment of these products.

Despite abundant research on the fate of GM diet-based DNA, more detailed and long-term research should be undertaken. New studies can even be made at no extra cost given the large number of whole-genome or gut metagenome sequencing datasets, as well as RNA-sequencing datasets, which are published every year. These can be scrutinised for rare integration events using DNA sequence alignment algorithms. This should provide more definitive data as to whether HGT of transgenic material, including its integration and expression in the genome takes place. We envisage that this multi-dimensional review will enable questions regarding the fate of GM food derived DNA and gene regulatory RNA in the human body to be addressed.

Author's contribution

AMT and MAN designed and outlined the review. RM, MAN, and MNA wrote the manuscript. AMT, KSG and SHY reviewed and improved the manuscript. MNA and GC supervised the study.

Acknowledgment

This work was carried out with the support of “Cooperative Research Programme for Agriculture Science and Technology Development (Project No. PJ013726)” Rural Development Administration, Republic of Korea.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.030.

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