Chapter 9

Genetic Engineering and Omitted Health Research: Still No Answers to Ageing Questions

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Introduction

Some of the most crucial scientific questions concerning the health effects of genetic engineering (GE) and genetically modified organisms (GMOs) were raised up to twenty years ago.[1] Most of them have still not been answered at all, or have found unsatisfactory answers. We believe, as Mayer and Stirling[2] said, 'in the end it is often the case that those who choose the questions determine the answers'. Will another twenty years pass before societies realize the urgent need for public funding of genuinely independent risk- and hazard-related research? The time for such investment is now, so that a new scientific culture with working hypotheses rooted in the Precautionary Principle (PP)[3] can discover other, possibly even more important questions of safety.

In this chapter we will mainly confine ourselves to putative health hazards related to GM plants used as food or feed, with some brief notes on GM vaccines as well as the novel RNAi- and nanobio-technologies. Our focus is not because we do not recognize the paramount, indirect threats to public health posed by social, cultural, ethical, and economic issues, as well as the complexities posed by the relevant legal and regulatory environments, but for reasons of space. In the specific context of food or feed safety assessment, 'hazard' may be defined as a biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect. The hypothetical hazards of whole GM foods, i.e. those hazards that have been realized so far, fall into a few broad categories.

First, there are those either related to the random and inaccurate integration of transgenes into recipient plant genomes, with uncertainty with regard to direct or indirect effects of the polypeptide product of the transgene, or uncertainty with regard to DNA types and circumstances promoting uptake and organ establishment of foreign DNA from mammalian gastro-intestinal tracts.[4] Second, there are those that might come from the purposeful production of potential hazards, such as allergens or powerful pharmaceutical products.

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[1]See for instance: Freese, W. and Schubert, D. (2004). Safety testing and regulation of genetically engineered foods. Biotechnology and Genetic Engineering Reviews 21: 299-324, or Pusztai, A. (2002). Can science give us the tools for recognizing possible public health risks for GM food? Nutrition and Health 16: 73-84.
[2]Mayer, S. and Stirling, A. (2004). GM crops: good or bad? EMBO Reports 5: 1021-1024.
[3]Myhr, A.I. and Traavik, T. (2002). The precautionary principle: scientific uncertainty and omitted research in the context of GMO use and release. Journal of Agricultural and Environmental Ethics 15: 73-86.
[4]For a recent, authoritative review see: The Royal Society of Canada (2001). Elements of Precaution: Recommendations for the regulation of food biotechnology in Canada. An expert panel report on the future of food biotechnology prepared by the Royal Society of Canada for Health Canada, Canadian Food Inspection Agency and Environment Canada (ISBN 0-920064-71-x), www.rsc.ca/foodbiotechnology/index/EN.html

A number of scientific concerns have been raised in connection with public and animal health. In the following sections we will discuss, in some detail, a few of these. Some of them have been thoroughly discussed in excellent, recent reviews.[5]

Our contribution is based on 'gene ecology', *a new, cross-disciplinary scientific field aimed at providing holistic knowledge based on the Precautionary Principle.*[6] Some of the concerns we raise will also be relevant for environmental risk assessments of GMOs, due to the fact that the processes discussed can take place in large ecosystems as well as in the ecosystems at the scale of the human being.

Do we know whether any GM food/feed is safe for consumption?

For a composite material such as food/feed, reductionist approaches testing single components *in vitro* are highly unsatisfactory and cannot clarify important safety issues. In spite of the obvious need, very few studies designed to investigate putative effects of GM nucleic acids or food/feed on potential animal or human consumers have been published in peer-reviewed journals.[7] A consensus has emerged that the effects observed in some published studies[8] must be experimentally followed up. To date, this has not been done.

Most of the animal feeding studies conducted so far have been designed exclusively to reveal husbandry production differences between GMOs and their unmodified counterparts. Studies designed to reveal physiological or pathological effects are extremely few, and they demonstrate a quite worrisome trend[9]: Studies performed by the GM plant producers find no problems, while studies from independent research groups often reveal effects that should have merited immediate follow-up, confirmation and extension. Such follow-up studies have not been performed. There are two main factors accounting for this situation: The lack of funds for independent research, and the reluctance of producers to deliver GM materials for analysis.[10]

Can we rely on the transgenic DNA sequences given by GM food/feed producers?

If the transgenic DNA sequences given in the notifications differ from the inserted sequences found in the GM plants, the risk assessments made prior to approval of the GM plants for marketing do not necessarily cover the potential risks associated with the GM plants.
The most thoroughly studied transgenic events are:

- Bt-transgenic maize Mon810
- Bt- and glufosinate-transgenic maize Bt176
- Glyphosate-transgenic maize GA21
- Glufosinate-transgenic maize T25 (Liberty Link)
- Glyphosate-transgenic soybean GTS 40-3-2.

[5]See Footnote 1, and e.g. Pusztai, A., Bardocz S. and Ewen S.W.B. (2003). Genetically modified foods: potential human health effects. Pp. 347-371, in Food Safety: Contaminants and Toxins, edited by JPF D'Mello. CAB International.

[6] For further information see the homepages of GENOK-Norwegian Institute of Gene Ecology, www.genok.org and INBI-Centre for Integrated Research in Biosafety, www.inbi.canterbury.ac.nz

[7]Domingo, J.L. (2000). Health Risks of GM Foods: Many opinions but few data. Science 288: 1748-1749.

[8]E.g. Fares and El-Sayed (1998). Fine structural changes in the ileum of mice fed on endotoxin-treated potatoes and transgenic potatoes. Natural Toxins 6(6): 219-233; Ewen and Pusztai (1999). Effect of diets containing genetically modified potatoes expressing Galanthus nivalis lectin on rat small intestine. The Lancet, Vol. 354, 16 October 1999.

[9]Pryme, I.F. and Lembcke, R. (2003). In vivo studies on possible health consequences of genetically modified food and feed – with particular regard to ingredients consisting of genetically modified plant materials. Nutr Health 17(1): 1-8.

[10]For documentation and further reading see Footnotes 1 and 2 and references therein.

Even amongst the most thoroughly studied and some of the oldest commercial GM plants, recent independent work has revealed that rearrangements occur in transgene inserts and the nature of the rearrangements varies. Deletions (Mon810, GA21, Bt176), recombination (T25, GTS 40-3-2, Bt176), tandem or inverted repeats (T25, GA21, Bt176), as well as rearranged transgenic fragments scattered through the genome (Mon810) have been reported.[11]

The transgenic modification techniques are prone to introduce such rearrangements because exogenous DNA transfer in plants elicits a 'wound' response, which activates nucleases and DNA repair enzymes. This may result in either degradation of the incoming DNA, or insertion of rearranged copies into the plant DNA.[12] In addition, the nature of the DNA constructs used to make transgenic plants may influence the rearrangement tendencies for a given transgenic event. Some genetic elements in the constructs may act as 'hotspots' and elicit recombination at high frequencies.[13]

While it was earlier assumed that integration of transgenic constructs took place at random locations in the recipient plant genome, it has now become apparent that integration sites are often concentrated in or near elements such as retrotransposons (T25, Mon810, GA21) and repeated sequences (Bt11 maize),[14] and this poses additional risks. Firstly, by introducing a new promoter or new enhancer motifs, transgenic insertions into, or close to, such elements may lead to altered spatial and temporal expression patterns of plant genes located close to and even far from, the insert. Secondly, a strong retrotransposon LTR promoter may upregulate the transgene expression level. Thirdly, defective retrotransposons may start 'jumping' under the influence of transacting factors recruited by the insert.[15] All these events may have unpredictable effects on the long-term genetic stability of the GMOs, as well as on their nutritional value, allergenicity and toxicant contents. These putative processes represent areas of omitted research with regard to health effects of GMOs.

[11]Hernandez et al. (2003). A specific real-time quantitative PCR detection system for event MON810 in maize YieldGuard based on the 3'-transgene integration sequence. Transgenic Research 12: 179-189; Holck et al. (2002). 5'-Nuclease PCR for quantitative event-specific detection of the genetically modified MON810 MaisGard maize. Eur Food Res Technol 214: 449-453; Collonnier et al. (2003). Characterization of commercial GMO-inserts: A source of useful material to study genome fluidity?; Windels et al. (2001). Characterisation of the Roundup Ready soybean insert. Eur Food Res Technol 213: 107-112; Rönning et al. (2003). Event specific real-time quantitative PCR for genetically modified Bt11 maize (Zea Mays). Eur Food Res Technol 216: 347-354.

[12]Takano et al. (1997). The structures of integration sites in transgenic rice. The Plant Journal 11(3): 353-361; Collonnier et al. (2003). Characterization of commercial GMO-inserts: A source of useful material to study genome fluidity? In addition to cellular mechanisms controlling the transgene integration, subsequent selection procedures of the GE material may introduce further genomic reorganisations (Hernandez et al. (2003). A specific real-time quantitative PCR detection system for event MON810 in maize YieldGuard based on the 3'-transgene integration sequence. Transgenic Research 12: 179-189).

[13]This is the case for the 35S CaMV promoter that is present in most GEPs marketed so far, and also for the Ti plasmid of Agrobacterium tumefasciens and the nos terminator (Kohli et al. (1999). Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology mediated recombination. The Plant Journal 17(6): 591-601; Collonnier et al. (2003). Characterization of commercial GMO-inserts: A source of useful material to study genome fluidity? Hot spots may lead to tandem transgene repeats with interspersed plant DNA sequences in a single genetic locus. Presence of several inserts may also result from multimerisation in the plasmid before transformation or from multiple insertions.

[14]Rönning et al. (2003). Event specific real-time quantitative PCR for genetically modified Bt11 maize (Zea Mays). Eur Food Res Technol 216: 347-354.

[15]Jank and Haslberger (2000). Recombinant DNA insertions into plant retrotransposons. Trends in Biotechnology 18: 326.

Are transgenic DNA and proteins taken up from the mammalian GIT (gastro-intestinal tract)?

If DNA and proteins from GMOs persist in, and are taken up from the mammalian GIT, this could theoretically (as will be explained further) ultimately lead to development of chronic disease conditions. The fate and consequences of DNA persistence and uptake is, however, not extensively studied, and therefore represents yet another area of uncertainty connected to GM plants.

It has generally been claimed that DNA and proteins are effectively degraded in mammalian GITs. This has been based on assumptions that have never been systematically examined.[16] A restricted number of recent publications have shown that foreign DNA and also proteins may escape degradation, persist in the GIT and even be taken up from the intestines and transported by the blood to internal organs in biologically meaningful versions.[17] These findings should not have come as such a surprise, since scientific articles from the 1990s[18] strongly indicated that this was an area of omitted research, as stated by a number of reports.[19]

Briefly summarized, there is evidence that relatively long fragments of DNA survive for extended periods after ingestion. DNA may be detected in the faeces, the intestinal wall, peripheral white blood cells, liver, spleen, and kidney, and the foreign DNA may be found integrated in the recipient genome. When pregnant animals are fed foreign DNA, fragments may be traced to small cell clusters in foetuses and newborns. The state of GIT filling, and the feed composition may influence DNA persistence and uptake. Complexing of DNA with proteins or other macromolecules may protect against degradation.

So far, only two published reports have investigated the fate of foreign/transgenic DNA in humans.[20] The consequences of DNA persistence and uptake thus represent yet another area of

[16]Palka-Santani et al. (2003). The gastrointestinal tract as the portal of entry for foreign macromolecules: fate of DNA and proteins. Mol Gen Genomics 270: 201-215.

[17]Schubbert et al. (1994). Ingested foreign (phage M13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice. Mol Gen Genet. 242(5): 495-504; Schubbert et al. (1997). Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. Proc Natl Acad Sci USA 94(3): 961-6; Schubbert et al. (1998) On the fate of orally ingested foreign DNA in mice: chromosomal association and placental transmission to the fetus. Mol Gen Genet. 259(6): 569-76; Hohlweg and Doerfler (2001). On the fate of plants or other foreign genes upon the uptake in food or after intramuscular injection in mice. Mol Genet Genomics 265: 225-233; Palka-Santani et al. (2003). The gastrointestinal tract as the portal of entry for foreign macromolecules: fate of DNA and proteins. Mol Gen Genomics 270: 201-215; Einspanier et al. (2001). The fate of forage plant DNA in farm animals; a collaborative case-study investigating cattle and chicken fed recombinant plant material. Eur Food Res Technol 212: 129-134; Klotz et al. (2002). Degradation and possible carry over of feed DNA monitored in pigs and poultry. Eur Food Res Technol 214: 271-275; Forsman et al. (2003). Uptake of amplifiable fragments of retrotransposon DNA from the human alimentary tract. Mol Gen Genomics 270: 362-368; Chen et al. (2004). Transfection of mEpo gene to intestinal epithelium in vivo mediated by oral delivery of chitosan-DNA nanoparticles. World Journal of Gastroenterology 10(1): 112-116; Phipps et al. (2003). Detection of transgenic and endogenous plant DNA in rumen fluid, duodenal digesta, milk, blood, and feces of lactating dairy cows. J Dairy Sci. 86(12): 4070-8.

[18]Wolff et al. (1990). Direct gene transfer into mouse muscle in vivo. Science 247: 1465; Jones et al. (1997). Oral delivery of poly(lactide-co-glycolide) encapsulated vaccines. Behring Inst Mitt. Feb (98): 220-8.

[19]E.g. a number of articles cited in Traavik, T. (1999). An orphan in science. Research Report for DN No. 1999-6, www.naturforvaltning.no/archive/attachments/01/05/Vacci006.pdf

[20]Forsman et al. (2003). Uptake of amplifiable fragments of retrotransposon DNA from the human alimentary tract. Mol Gen Genomics 270: 362-368; Netherwood et al. (2004). Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. Nat Biotechnol 22(2): 204-209. In the former study, volunteers were fed rabbit meat. Rabbit retrotransposon sequences (RERV-H) were detected in the blood stream and in peripheral white blood cells for a considerable length of time after ingestion. In the latter study volunteers were fed epsps-transgenic (glyphosate-tolerant) soy as burgers and soy-milk. The transgenic DNA was detected in the small intestinal contents and bacteria. The volunteers were ileostomists, i.e. individuals in which the terminal ileum is resected and digesta are diverted from the body via a syoma to a colostomy bag.

omitted research. Extrapolating from a number of experiments in mammalian cell cultures and in experimental animals, it is conceivable that in some instances insertion of foreign DNA may lead to alterations in the methylation and transcription patterns of the recipient cell genome, resulting in unpredictable levels of gene expression levels and products. Furthermore, even small inserts may result in a 'destabilization' process, the end-point of which may be malignant cancer cells.[21] The BSE/new variant Creutzfeld-Jacob's Disease epidemics caused by prion proteins painfully illustrated the phenomenon of protein persistence, uptake and biological effects. Two recent publications indicate that this phenomenon may be more general that realized.[22] A hallmark of prion diseases and a number of other debilitating, degenerative diseases, e.g. Alzheimer's and Huntington's diseases, is deposition of 'amyloid fibrils'. Recent studies indicate that any protein can adopt a confirmation known as 'amyloid'[23] upon exposure to appropriate environmental conditions. Whether such conditions are more likely when proteins are expressed in different species and at very different concentrations, as is often the case for GM food/feed that are already in the marketplace, is unknown.

The consequences of protein persistence and uptake will vary with the given situation. Generally speaking, there is a possibility that toxic, immunogenic/allergenic or carcinogenic molecules may gain entry to the organism via cells in the gastrointestinal walls. The persistence of the Bt toxin Cry1Ab in faeces means a potential for spread on fields through manure. The ecological effects, e.g. on insect larvae and earthworms,[24] are presently a matter of sheer speculation.

Have the protein contents of GM food been altered in unpredictable ways?

Transgenes or upregulated plant genes may give rise to toxicants, anti-nutrients, allergens, and, putatively, also carcinogenic or co-carcinogenic substances. The concentration of a given transgenic protein may vary according to the location(s) in the recipient host cell genome of inserted GM construct DNA, and to environmental factors influencing the activity of the transgenic regulatory elements, e.g. the 35S CaMV promoter. The biological effects of a given transgenic protein, e.g. the Cry1Ab Bt toxin or the α-amylase inhibitor from beans when expressed in peas,[25] may be unpredictably influenced by post-translational modifications, alternative splicing,[26] alternative start codons for transcription, chimeric reading frames resulting

[21]E.g. Misteli, T. (2004). Spatial positioning: a new dimension in genome function. Cell 119: 153-156; Deininger, P.L. et al. (2003). Mobile elements and mammalian genome evolution. Curr Opin Genet Develop 13: 651-658; Costello, J.F. and Plass, C. (2001). Methylation matters. J Med Genet 38: 285-303; Gatza, M.L. et al. (2005). Impact of transforming viruses on cellular mutagenesis, genome stability, and cellular transformation. Environmental and Molecular Mutagenesis 45(2-3): 304-325.

[22]The first (Palka-Santani et al. (2003). The gastrointestinal tract as the portal of entry for foreign macromolecules: fate of DNA and proteins. Mol Gen Genomics 270: 201-215), based on feeding of gluthathione-S-transferase to mice, demonstrated undegraded protein in stomach/small intestinal contents, and trace amounts in kidney extracts, 30 minutes or more after feeding. Very significantly, incubation with stomach contents of control mice resulted in faster degradation than in feeding experiments. The second study concerned cattle fed cry1ab-transgenic maize Bt176 (Einspanier et al. (2001). The fate of forage plant DNA in farm animals; a collaborative case study investigating cattle and chicken fed recombinant plant material. Eur Food Res Technol 212: 129-134). Cry1Ab protein was detected in all parts of the GIT, and it was still detectable in the faeces.

[23]Demonstrated in a series of recent articles, e.g. Bucciantini et al. (2004). Prefibrillar amyloid protein aggregates share common features of cytotoxicity. J. Biol Chem 279: 31374-31382; Kayed et al. (2003). Common structure of soluble amyloid oligomers implies common mechanisms of pathogenesis. Science 300: 486-489.

[24]Zwahlen et al. (2003). Effects of transgenic Bt corn litter on the earthworm Lumbricus terrestris. Molecular Ecology 12: 1077-1086.

[25]Prescott, V.E., Campbell, P.M., Moore, A., Mattes, J., Rothenberg, M.E., Foster, P.S., Higgins, T.J.V. and Hogan, S.P. (2005). Transgenic expression of bean alpha-amylase inhibitor in peas results in altered structure and immunogenicity. J Agric Food Chem 53: 9023-9030.

[26]Rang, A., Linke, B. and Jansen, B. (2005). Detection of RNA variants transcribed from the transgene in Roundup Ready soybean. Eur Food Res Technol 220: 438-443.

from integration into the reading frame of a plant gene, and complex formation with endogenous plant proteins.

The influence of foreign DNA insertion on endogenous plant gene expression patterns may vary with local environmental factors, the actual insertion site(s), the number and stability of the inserts, transgenic promoter effects, methylation patterns of the insert(s), and post-transformational mutations in the transgenic protein coding as well as in regulatory sequences. Even a single nucleotide change may affect the properties of a protein, or it may create a new transcription factor binding motif. Detailed studies of these phenomena under authentic conditions are lacking, and hence we are confronted with yet another area of omitted research.

Could GM food/feed cause allergies?

One of the major health concerns related to GM plants is that the transgenic product itself, e.g. a Bt toxin, changed expression of endogenous plant genes, or chemical reactions that occur during the cooking of novel foods, may result in exposure to *allergenic* compounds. The risk assessment of allergens often follows an *allergenicity decision tree.*[27] These 'trees' are based on *in vitro* tests comparing a limited number of structures, usually only one, of the transgenic protein with known allergens. Hence, these comparisons are made in the hope that the protein isolated for the test matches all proteins produced from the same gene in the GM plant. In fact, this is unlikely because allergenicity tests are usually carried out with bacteria-, not *in planta*-produced versions of the transgenic protein. Glycosylation invariably takes place in plants, but not in bacteria, so this form of post-translational modification of both the transgenic protein and endogenous proteins would not be tested. Allergenic characteristics of proteins, and also their resistance to degradation in the organism, can be affected by glycosylation. Other protein modifications may also take place, adding to the unpredictability of transgenic products.[28]

Another important question related to allergenicity is whether post marketing surveillance can provide useful information about allergens in GM foods. For a number of reasons, this is not likely to happen.[29] Treatment of allergy is symptomatic, whatever the cause may be. The allergic case is often isolated, and the potential allergen is rarely identified. The number of allergy-related medical visits is not tabulated. Even repeated visits due to well-known allergens are not counted as part of any established surveillance system. Thus, during the October 2000 Starlink episode, it proved very difficult to evaluate Starlink (containing Bt toxin Cry9C) as a human allergen.[30] An additional reason for this was that the ELISA tests, used by FDA, that found no anti-Cry9C antibodies in suspected human cases, were dubious because bacterial, recombinant antigens were used instead of the Cry9C maize versions that the individuals had been exposed to.

Case: Bt toxins in Bt-transgenic GM plants

It is very important to be aware of the fact that the Bt toxins expressed in GM plants have never been carefully analysed, and accordingly, their characteristics and properties are not known. What is clear from the starting point, however, is that they are vastly different from the bacterial *Bacillus thuringiensis* protoxins, used in organic and traditional farming and forestry for

[27]Bernstein et al. (2003). Clinical and laboratory investigation of allergy to genetically modified foods. Environ Health Perspect 111: 1114-1121.
[28]Schubert, D. (2002). A different perspective on GM food. Nat Biotechnol 20: 969; Submissions on A549 High Lysine Corn LY038 http://www.inbi.canterbury.ac.nz/ly038.shtml
[29]Bernstein et al. (2003). Clinical and laboratory investigation of allergy to genetically modified foods. Environ Health Perspect 111: 1114-1121.
[30]Bucchini, L. and Goldman, L.R. (2002). Starlink corn: a risk analysis. Environ Health Perspect 110: 5-13.

decennia.[31] The difference is evident already at the gene level, since the versions found in GMOs are engineered to produce active Bt toxins. By extrapolation, these have a number of potentially unwanted biological characteristics, ranging from solubilization of the protein under natural conditions and effects on insect and mammalian cells, to persistence and non-target effects in the environment.[32] In addition, the post-translational modifications that may influence conformations, cellular targets and biological effects of GM plant-expressed Bt toxins are unknown, and hence we once more identify an area of omitted research.

During the last few years a number of observations that may be perceived as 'early warnings' of potential health and environmental risks have appeared in the literature.[33] Most of them have, however, not been followed up by extended studies.

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[31]Stotzky, G. (2002). Release, persistence, and biological activity in soil of insecticidal proteins from Bacillus thuringiensis. Pp. 187-222 in: Deborah K. Letourneau and Beth E. Burrows: Genetically Engineered Organisms. Assessing Environmental and Human Health Effects. CRC Press LLC (ISBN 0-8493-0439-3).

[32]Andow, D.A. (2002). Resisting resistance to Bt-corn. Pp. 99-124 in: Deborah K. Letourneau and Beth E. Burrows: Genetically Engineered Organisms. Assessing Environmental and Human Health Effects. CRC Press LLC (ISBN 0-8493-0439-3).

[33]Human and monkey cells exposed to Bt-toxins from the extra- or intra-cellular environment are killed or functionally disabled (Taybali and Seligy (2000). Human cell exposure assays of Bacillus thuringiensis commercial insecticides: Production of Bacillus cereus-like cytolytic effects from outgrowth of spores. Environ Health Perspect online, 18 August 2000; Tsuda et al. (2003). Cytotoxic activity of Bacillus thuringiensis Cry proteins on mammalian cells transferred with cadherine-like Cry receptor gene of Bombyx mori (silkworm). Biochem J 369: 697-703; Namba et al. (2003). The cytotoxicity of Bacillus thuringiensis subsp. coreanensis A 1519 strain against the human leukemic T cell. Biochimica et Biophysica Acta 1622: 29-35). Influenza A infections in mice were changed from silent to lethal encounters by co-exposing the animals to Bt-toxin (Hernandez et al. (2000). Super-infection by Bacillus thuringiensis H34 or 3a3b can lead to death in mice infected with the influenza A virus. FEMS Immunology and Med Microbiol 209: 177-181). Farm workers exposed to Bt spores developed IgG and IgE antibodies to Bt-toxin (Cry1Ab) (Taylor et al. (2001). Will genetically modified foods be allergenic? Journal of Allergy and Clinical Immunology, May 2001, 765-771). The Bt-toxin Cry1Ac was found to have very strong direct and indirect immunological effects in rodents (Vazquez et al. (2000). Characterization of the mucosal and systemic immune response induced by Cry1Ac protein from Bacillus thuringiensis HD 73 in mice. Brazilian Journal of Medical and Biological Research 33: 147-155; Moreno-Fierros et al. (2000). Intranasal, rectal and intraperitoneal immunization with protoxin Cry1Ac from Bacillus thuringiensis induces compartmentalized serum, intestinal, vaginal and pulmonary immune response in Balb/c mice. Microbes and Infection 2: 885-890; Moreno-Fierros et al. (2002). Slight influence of the oestrous cycle stage on the mucosal and systemic specific antibody response induced after vaginal and intraperitoneal immunization with protoxin CryA1c from Bacillus thuringiensis in mice. ELSEVIER Life Sciences 71: 2667-2680). Earthworms exposed to Bt toxin Cry1Ab experience weight loss (Zwahlen et al. (2003). Effects of transgenic Bt corn litter on the earthworm Lumbricus terrestris. Molecular Ecology 12: 1077-1086). Cattle fed the Bt176 maize variety demonstrated undegraded Cry1Ab through the whole alimentary tract, and the intact toxin was shed in faeces (Einspanier et al. (2004). Tracing residual recombinant feed molecules during digestion and rumen bacterial diversity in cattle fed transgene maize. Eur Food Res Technol 218: 269-273). Cry1Ab is much more resistant to degradation under field soil conditions than earlier assumed (Zwahlen et al. (2003). Degradation of the Cry1Ab protein within transgenic Bacillus thuringiensis corn tissue in the field. Mol Ecol 12: 765-775). Potentially IgE-binding epitopes have been identified in two Bt-toxins (Kleter and Peijnenburg (2002). Screening of transgenic proteins expressed in transgenic food crops for the presence of short amino acid sequences identical to potential IgE-binding linear epitopes of allergens. BMC Structural Biology 2:8), and it should be added that many IgE-binding epitopes are conformationally not linearly determined. Finally, it is a matter of concern that Bt-toxins have lectin characteristics (Akao et al. (2001). Specificity of lectin activity of Bacillus thuringiensis parasporal inclusion proteins. J Basic Microbiol. 41(1): 3-6). Lectins are notorious for finding receptors on mammalian cells. This may lead to internalization and intracellular effects of the toxins. Occupational exposure to novel proteins, and potential allergic sensitization, has had little study, but could be of public health significance. An amazing number of foods have been proven to evoke allergic reactions by inhalation (Bernstein et al. (2003). Clinical and laboratory investigation of allergy to genetically modified foods. Genetically Modified Foods, Mini-Monograph, Volume 111, No. 8, June 2003). In this connection the findings of serum IgG/IgE antibodies to B. thuringiensis spore extracts (Bernstein et al. (1999). Immune responses in farm workers after exposure to Bacillus thuringiensis pesticides. Environmental Health Perspectives 107(7): 575-582), in exposed farm workers should be given further attention. Inhalant exposure to Bt-toxin containing GMP materials may take place through pollen in rural settlements and also through dust in workplaces where foods are handled or processed.

Case: Transgenic, glyphosate-tolerant (Roundup Ready) GM plants

Glyphosate kills plants by inhibiting the enzyme 5-enolpyruvoyl-shikimate-3-phosphate synthase (EPSPS) necessary for production of important amino acids. Some microorganisms have a version of EPSPS that is resistant to glyphosate inhibition. The transgene, *cp4 epsps*, used in genetically modified crops was isolated from an *Agrobacterium* strain. The whole idea is the combined use of the GM plant and the herbicide. Recent studies indicate that in some cases such GM plants are associated with greater usage of glyphosate than the conventional counterparts.[34] A very restricted number of experimental studies have been devoted to health or environmental effects of the GM plants or the herbicide itself. Some of these may be considered 'early warnings' of potential health and environmental risks, and they should be rapidly followed up to confirm and extend the findings.[35] Consequently, this is yet another area of omitted research.

Is the 35S CaMV promoter inactive in mammalian cells?

Cauliflower mosaic virus (CaMV) is a DNA containing para-retrovirus replicating by means of reverse transcription. One of the viral promoters, called 35S, is a general, strong plant promoter. It has been used to secure expression of the transgenes in most of the GMOs commercialized so far. Industry proponents have claimed unconditionally that the 35S is an exclusive plant promoter, and hence cannot, even theoretically, represent a food/feed safety issue.[36]

[34]Benbrook, C. Impacts of genetically engineered crops on pesticide use in the United States: The first eight years. Biotech InfoNet Paper No. 6, November 2003. www.biotech-info.net/technicalpaper6.html

[35]Mice fed GE soybean demonstrated significant morphological changes in their liver cells (Malatesta et al. (2002). Ultrastructural morphometrical and immunocytochemical analysis of hepatocyte nuclei from mice fed on genetically modified soy bean. Cell Structure and Function 27: 173-180). The data suggested that epsps-transgenic soybean intake was influencing liver cell nuclear features in both young and adult mice, but the mechanisms responsible for the alterations could not be identified by the experimental design of these studies. Treatment with glyphosate (Roundup) is an integrated part of the epsps-transgenic GMP application. A number of recent publications indicate unwanted effects of glyphosate on aquatic (Solomon & Thompson (2003). Ecological risk assessment for aquatic organisms from over-water uses of glyphosate. J Toxicol Environ Health B Crit Rev. 6(3): 289-324) and terrestric (Ono et al. (2002). Inhibition of Paracoccidioides brasiliensis by pesticides: is this a partial explanation for the difficulty in isolating this fungus from the soil? Med Mycol 40(5): 493-9; Blackburn and Boutin (2003). Subtle effects of herbicide use in the context of genetically modified crops: A case study with glyphosate (Roundup). Ecotoxicol 12: 271-285) organisms and ecosystems. Recent studies in animals and cell cultures point directly to health effects in humans as well as rodents and fish. Female rats fed glyphosate during pregnancy demonstrated increased foetal mortality and malformations of the skeleton (Dallegrave et al. (2003). The teracogenic potential of the herbicide glyphosate Roundup in Wistar rats. Toxicology letters 142: 45-52). Nile Tilapia (Oreochromis niloticus) fed sublethal concentrations of Roundup exhibited a number of histopathological changes in various organs (Jiraungkoorskul et al. (2003). Biochemical and histopathological effects of glyphosate herbicide on Nile tilapia. Environ Toxicol 18(4): 260-7). A study of Roundup effects on the first cell divisions of sea urchins (Marc et al. (2002). Pesticide Roundup provokes cell division dysfunction at the level of CDK1/Cyklin B activation. Chem Res Toxicol 15: 326-331) is of particular interest to human health. The experiments demonstrated cell division dysfunctions at the level of CDK1/Cyclin B activation. Considering the universality among species of the CDK1/Cyclin B cell regulator, these results question the safety of glyphosate and Roundup on human health. In another study (Axelrod et al. (2003). The effect of acute pesticide exposure on neuroblastoma cells chronically exposed to diazinon. Toxicoloy 185: 67-78) it was demonstrated a negative effect of glyphosate, as well as a number of other organophosphate pesticides, on nerve-cell differentiation. Surprisingly, in human placental cells, Roundup is always more toxic than its active ingredient. The effects of glyphosate and Roundup were tested at lower non-toxic concentrations on aromatase, the enzyme responsible for estrogen synthesis (Richard, S. et al. (2005). Differential effects of glyphosate and Roundup on human placental cells, Environ. Health Perspect. 113: 716-720). The glyphosate-based herbicide disrupts aromatase activity and mRNA levels and interacts with the active site of the purified enzyme, but the effects of glyphosate are facilitated by the Roundup formulation. The authors conclude that endocrine and toxic effects of Roundup, not just glyphosate, can be observed in mammals. They suggest that the presence of Roundup adjuvants enhances glyphosate bioavailability and/or bioaccumulation.

[36]E.g. Gasson, M. and Burke, D. (2001). Scientific perspectives on regulating the safety of genetically modified foods. Nat Rev Genet 2: 217-222.

In addition to studies in yeast[37] and in *Schizosaccharomyces pombe*,[38] there are published studies indicating that the 35S CaMV promoter *might* have potential for transcriptional activation in mammalian systems.[39] The final proof has become available during the last couple of years. First, 35S promoter activity was demonstrated in human fibroblast cell cultures,[40] thereafter in hamster cells,[41] and very recently a research group led by Terje Traavik (co-author of this chapter) has demonstrated substantial 35S promoter activity in human enterocyte-like cell cultures.[42] Such cells line the surface of human intestines. However, no published studies have investigated 35S CaMV activity *in vivo*, and this is therefore yet another area of omitted research.

Could the use of antibiotic resistance marker genes (e.g. nptII) present health hazards?

The antibiotic kanamycin is used extensively in crop genetic engineering as a selectable marker, *inter alia* in GM oilseed rape event lines such as MS1Bn x RF1Bn and Topas 19/2.
A selectable marker is a gene inserted into a cell or organism to allow the modified form to be selectively amplified while unmodified organisms are eliminated. In crop genetic engineering, the selectable marker is used in the laboratory to identify cells or embryos that carry the genetic modifications that the engineer wishes to commercialize. The selection gene is used once briefly in the laboratory, but thereafter the genetically modified crop has the unused marker gene in each and every one of its cells.

[37]Hirt, H. et al. (1990). Evolutionary conservation of transcriptional machinery between yeast and plants as shown by the efficient expression from the CaMV 35S promoter and 35S terminator. Curr Genet 17: 473-9.

[38]Gmunder and Kohli (1989). Cauliflower mosaic virus promoters direct efficient expression of a bacterial G418 resistance gene in Schizosaccharomyces pombe. Mol Gen Genet 220(1): 95-101; Probjecky et al. (1990). Expression of the beta-glucuronidase gene under the control of the CaMV 35s promoter in Schizosaccharomyces pombe. Mol Gen Genet 220(2): 314-6.

[39]The promoter initiates transcription in rabbit reticulocyte lysate (Ryabova and Hohn (2000). Ribosome shunting in the cauliflower mosaic virus 35S RNA leader is a special case of reinitiation of translation functioning in plant and animal systems. Genes & Development 14: 817-829) and in Xenopus oocytes (Ballas et al. (1989). Efficient functioning of plant promoters and Poly(A) sites in Xenopus oocytes. Nucleic Acids Research 17(19): 7891-7903). In the latter studies it was found that circular, supercoiled 35S CaMV driven expression plasmids were more active than linear forms. The CaMV genome carries structural and functional resemblance to mammalian Retroviridae and to Hepadnaviridae, which contains the human hepatitis B virus (HBV). A 19 bp palindromic sequence, including the TATA box of the 35S CaMV promoter, may act as a recombination hotspot in plants (Kohli et al. (1999). Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology mediated recombination. Plant Journal 17(6): 591-601), and it is unknown whether this is also the case in mammalian cells. In a recent review article (Ho et al. (2000). Hazardous CaMV? Nat Biotechnol 18(4): 363) it was hypothesized that the 35S CaMV promoter might represent health hazards to human and animal consumers of transgenic plant materials. Against this it was argued that humans and mammals are continuously being exposed to CaMV particles through infected plant materials. This is true enough, but it is then forgotten that there are documented examples of animal species being resistant to intact viruses, but highly susceptible to infection by DNA from the same virus (Refs: Rekvig et al. (1992). Antibodies to eukaryotic, including autologous, native DNA are produced during BK virus infection, but not after immunization with non-infectious BK DNA. Scand J Immunol 36(3): 487-95; Zhao et al. (1996). Infectivity of chimeric human T-cell leukaemia virus type I molecular clones assessed by naked DNA inoculation. Procedures of National Academy of Sciences USA 93: 6653-6658; reviews: Traavik, T. (1999). An orphan in science. Research Report for DN No. 1999-6; Ho et al. (2000). Hazardous CaMV promoter? Nat Biotechnol 18(4): 363).

[40]Vlasak, J., Smahel, M., Pavlik, A., Pavingerova, D., and Briza, J. (2003). Comparison of hCMV immediate early and CaMV 35S promoters in both plant and human cells, J Biotechnol 103: 197-202.

[41]Tepfer, M., Gaubert, S., Leroux-Coyau, M., Prince, S., and Houdebine, LM. (2004). Transient expression in mammalian cells of transgenes transcribed from the Cauliflower mosaic virus 35S promoter. Environ Biosafety Res 3: 91-97.

[42]Myhre, M.R., Fenton, K.A., Eggert, J., Nielsen, K.M. and Traavik, T. (2006). The 35S CaMV plant virus promoter is active in human enterocyte-like cells. Eur Food Res Technol 222: 185–193.

There are multiple well-known mechanisms for cross-resistance to antibiotics of a particular type.[43] Kanamycin is a member of the family aminoglycoside antibiotics. There are approximately 17 different classes of aminoglycoside-modifying enzymes. Some of these inactivate up to four different aminoglycosides. Cross-resistance between kanamycin and other aminoglycosides, e.g. gentamycin and tobramycin, was found to vary markedly between isolates.[44] All of the antibiotics mentioned are used to treat human diseases.
In spite of the belief of many genetic engineers that kanamycin is no longer employed in medical applications, there is evidence that the antibiotic is used extensively for some applications.[45]

Concluding remarks: Where do we go from here?

We have discussed in some detail a handful of selected, unanswered risk questions related to the first generation of transgenic GMOs. There are many more risk issues. Among them are issues of Horizontal Gene Transfer (HGT),[46] the new generations of multitransgenic GMOs for pharmaceutical and industrial purposes,[47] safety questions related to GM vaccines,[48] the new nanobiotechnology approaches,[49] and the applications of small double-stranded (ds)RNAs (which can cause RNAi) for a number of medical purposes.[50] Furthermore, we have the 'questions not yet asked', and we have the problem of whether available methods and regulatory frameworks will be able to pick up and manage the conceived risks once they become reality.
In recent publications it has been demonstrated that the presently used sampling and detection methods may fail to detect GM materials in food and feed.[51] In another article it was demonstrated that HGT events, that potentially carry very serious public health consequences, would not be detected in time for any meaningful preventive actions.[52] In addition, it has been shown that the dsRNA techniques are not as 'surgically targeted' as initially indicated.[53]

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[43]Heinemann, J.A., Ankenbauer, R.G., and Amábile-Cuevas, C.F. (2000). Do antibiotics maintain antibiotic resistance? Drug Discov Today 5: 195-204.

[44]The aminoglycoside antibiotic neomycin was found to cross react with kanamycin B in inhibiting RNase P ribozyme 16s ribosomal RNA and tRNA maturation (Mikkelsen et al. (1999). Inhibition of RNase P RNA cleavage by aminoglycosides. Proc Natl Acad Sci USA 96: 6155-6160).

[45]Kanamycin is used prior to endoscopy of colon and rectum (Ishikawa et al. (1999). Prevention of infectious complications subsequent to endoscopic treatment of the colon and rectum. J Infect Chemother 5: 86-90) and to treat ocular infections (Hehl et al. (1999). Improved penetration of aminoglycosides and fluorozuinolones into the aqueous humour of patients by means of Acuvue contact lenses. Eur J Clin Pharmacol. 55(4): 317-23). It is used in blunt trauma emergency treatment (Yelon et al. (1996). Efficacy of an intraperitoneal antibiotic to reduce the incidence of infection in the trauma patient: a prospective, randomized study. J Am Coll Surg 182(6): 509-14), and has been found to be effective against E coli 0157 without causing release of verotoxin (Ito et al. (1997). Evaluation of antibiotics used for enterohemorrhagic Escherichia coli O157 enteritis-effect of various antibiotics on extracellular release of verotoxin. Kansenshogaku Zasshi 71(2): 130-5).

[46]Heinemann, J.A. and Billington, C. (2004). How do genomes emerge from genes? Horizontal gene transfers can lead to critical differences between species when those genes begin reproducing vertically. ASM News 70: 464-471.

[47]Twyman, R.M. et al. (2003). Molecular pharming in plants: host systems and expression technology. Trends in Biotechnology 21: 570-578.

[48]Traavik, T. (2002).Environmental risks of genetically engineered vaccines. In: DK Letourneau and BE Burrows (eds): Genetically Engineered Organisms: Assessing Environmental and Health Effects. CRC Books, La Boca, Florida (ISBN 0849304393).

[49]Mazzola, L. (2003). Commercializing nanotechnology. Nat Biotechnol 21: 1137-1143; Colvin, V. L. (2003). The potential environmental impact of engineered nanomaterials. Nat Biotechnol 21: 1166-1170.

[50]Hannon, G.J. and Rossi, J.J. (2004). Unlocking the potential of the human genome with RNA interference. Nature 431: 371-378.

[51]Heinemann J.A., Sparrow A.D. and Traavik T. (2004). Is confidence in the monitoring of GM foods justified? Trend Biotechnol 22: 331-336.

[52]Heinemann J.A. and Traavik, T. (2004). Problems in monitoring horizontal gene transfer in field trials of transgenic plants. Nat Biotechnol 22: 331-336; Heinemann J.A. and Traavik T. (2004). Monitoring horizontal gene transfer. Reply. Nat Biotechnol 22: 1349-1350.

[53]E.g. Jackson, A.L. et al. (2003). Expression profiling reveals off-target gene regulation by RNAi. Nat Biotechnol 21: 635-637, and a number of other recent articles.

We are therefore left with a high number of risk issues lacking answers, adding up to a vast area of omitted research, and this falls together in time with a strong tendency towards corporate take-over of publicly funded research institutions and scientists.[54]
We must, as citizens and professionals, join together to reverse the present situation. Publicly funded, independent research grants need to become a hot political issue. This would be the most efficient remedy for chronically unanswered questions and the corporate take-over of science. In conclusion, we once more quote Mayer and Stirling:[55] 'Deciding on the questions to be asked and the comparisons to be made has to be an inclusive process and not the provenance of experts alone'. Then again, whom should society rely on for answers and advice should the time come when all science resource persons work directly or indirectly for the GM producers?

[54]Mayer, S. and Stirling, A. (2004). GM crops: good or bad? EMBO Reports 5: 1021-1024; Martin, B. (1999), in Science and Technology Policy Year Book. Washington DC, USA: American Association for the Advancement of Science, www.aaas.org/spp/yearbook/chap15.htm; Graff GD et al. (2003). The public-private structure of intellectual ownership in agricultural biotechnology. Nat Biotechnol 21: 989-995; Heinemann, J.A. and Goven, J. The social context of drug discovery and safety testing. In Multiple Drug Resistant Bacteria (C.F. Amábile-Cuevas, ed., second edition). Horizon Scientific Press, in press.
[55]Mayer, S and Stirling, A. (2004). GM crops: good or bad? EMBO Reports 5: 1021-1024.