Genetic Engineering (GE) and Omitted Health Research: Still No Answers to Ageing Questions.

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Introduction.

Some of the most crucial scientific questions concerning health effects of GE and GEOs (genetically engineered organisms) 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 the present article we will mainly confine ourselves to putative health hazards related to GE plants (GEPs) used as food or feed, with some brief notes on GE vaccines as well as the novel si RNA- and nanobio-technologies. This does not mean that we do not recognize the paramount, indirect threats to public health posed by social, cultural, ethical, economic and legal issues.

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. They are either related to

[1] See for instance: Freese, W. and Schubert, D. Safety testing and regulation of genetically engineered foods. Biotechnology and Genetic Engineering Reviews 21: 299-324, 2004, or Pusztai, A. Can science give us the tools for recognizing possible public health risks for GM food. Nutrition and Health 16: 73-84, 2002
[2] Mayer, S. and Stirling A. GM crops: good or bad. EMBO Reports 5: 1021-1024, 2004
[3] Myhr AI and Traavik, T. The precautionary principle: scientific uncertainty and omitted research in the context of GMO use and release. JAGE (Journal of Agricultural and Environmental Ethics) 15: 73-86, 2002

the random and inaccurate integration of transgenes into recipient plant genomes, 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].

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

Our contribution is based on "gene ecology"; a new, cross-disciplinary scientific field intended to provide holistic knowledge based on the precautionary principle[6].
Some of the concerns we raise will also be relevant for environmental risk assessments of GEOs due to the fact that the processes discussed can take place in an ecosystem at large as well as in the ecosystems represented by mammalian organisms.

Do we know that any GE food/feed is safe for consumption?

For a composite material like food/feed, reductionistic approaches testing single components *in vitro* are highly unsatisfactory and cannot by definition clarify important safety issues. In spite of the obvious need, very few studies designed to investigate putative effects of GE 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 this day, this has not been done.

Most of the animal feeding studies performed so far have been designed exclusively to reveal husbandry production differences between GEOs 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 industry find no problems, while studies from independent research groups often reveal effects that should have merited immediate follow-up, confirmation and extension. Such

[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 at the request of Health Canada, Canadian Food Inspection Agency and Environment Canada (ISBN 0-920064-71-x), www.rsc.ca/foodbiotechnology/index/EN.html

[5] See footnote 1, and e.g. Pusztai A, Bardosz S and Ewen SWB.Genetically modified foods: potential human health effects, pp. 347-371, in Food Safety: Contaminants and Toxins, edited by JPF D'Mello. CAB International, 2003.

[6] For further information: See the homepages of GENOK-Norwegian Institute of Gene Ecology, www.genok.org, and NZIGE-New Zealand Institute of Gene Ecology, www.nzige.canterbury.ac.nz

[7] Jose L. Domingo (2000). "Health Risks of GM Foods: Many Options but Few Data". Science, vol 288 Issue 5472, 1748-1749, 9 June 2000

[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, Vol. 6, Issue 6, pages 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 and Lembcke, 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. 2003;17(1):1-8.

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 GE materials for analysis[10].

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

If the transgenic DNA sequences given in the notifications differ from the inserted sequences found in the GEPs, the risk assessments made prior to approval of the GEPs for marketing do not necessarily cover the potential risks associated with the GEPs.

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

Even amongst the most thoroughly studied and some of the oldest commercial GEPs, recent independent work has revealed that the nature of the rearrangements vary, and deletions (Mon810, GA21, Bt176), recombinations (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 recombinations at high frequencies[13].

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[10] For documentation and further reading: see footnotes 1,2 and references therein.

[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, 2003; Holck et al., 2002. "5'-Nuclease PCR for quantitative event-specific detection of the genetically modified MON810 MaisGard maize". Eur Food Res Technol (2002) 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 (2001) 213: 107-112; Rönning et al., 2003. "Event specific real-time quantitative PCR for genetically modified Bt11 maize" (Zea Mays). Eur Food Res Technol (2003) 216: 347-354.

[12] Takano et al., 1997. "The structures of integration sites in transgenic rice". The Plant Journal 1997, 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, 2003

[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 comfirms the predominance of microhomoloy mediated recombination". The Plant Journal (1999)

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 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 GEOs, 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 GEOs.

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

If DNA and proteins from GEOs persist in, and are taken up from mammalian GIT, this could theoretically, as will be further explained below, 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 uncertainties connected to GEPs.

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 demonstrated that foreign DNA and also proteins may escape degradation, to persist in the GIT and even to 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

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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 quantative PCR for genetically modified Bt11 maize" (Zea Mays). Eur Food Res Technol (2003) 216: 347-354.

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

[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 (2003) 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. 1994 Mar;242(5):495-504, Schubbert et al., 1997, 1998; 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 (2001) 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 (2003) 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

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 summarised, the present conception of DNA persistence and uptake includes long fragments of ingested DNA. 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 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 so-called "destabilisation" process, the end-point of which may be malignant cancer cells[21].

The BSE/new variant Creutzfeld-Jacob's Disease epidemics caused by the prion proteins painfully illustrated the phenomenon of protein persistence, uptake and biological effects.

(2001) 212:129-134; Klotz et al., 2002. "Degradation and possible carry over of feed DNA monitored in pigs and poultry". Eur Food Res Technol (2002) 214:271-275; Forsman et al., 2003. "Uptake of amplifiable fragments of retrotranspon DNA from the human alimentary tract". Mol Gen Genomics (2003). 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 2004, 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. 2003 Dec;86(12):4070-8

[18] Wolff et al., 1990. "Direct Gene Transfer into Mouse muscle in vivo". Science New Series Vol 247, No. 4948, page 1465; Jones et al., 1997. " Oral delivery of poly(lactide-co-glycolide) encapsulated vaccines". Behring Inst Mitt. 1997 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 retrotranspon DNA from the human alimentary tract". Mol Gen Genomics (2003). 270:362-368;Netherwood et al., 2004. "Assessing the survival of transgenic plant DNA in the human gastrointestinal tract". Nature Biotechnology , Volume 22, No. 2, February 2004. 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.

[21] E.g. Misteli T. Spatial positioning: a new dimension in genome function. Cell 119: 153-156, 2004; Deininger PL et al. Mobile elements and mammalian genome evolution. Current Opinions in Genetics and Development 13: 651-658, 2003; Costello JF and Plass C. Methylation matters. J Med Genet 38: 285-303, 2001; Gatza ML et al. Impact of transforming viruses on cellular mutagenesis, genome stability, and cellular transformation. Environmental and Molecular Mutagenesis 45: 000-000, 2005, published online in Wiley Interscience (www.interscience.wiley.com)

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, i.e. 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 that is the case for GE food/feed that is already in the marketplace is unknown.

The consequences of protein persistence and uptake will vary with the given situation. Generally spoken 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 the fields through manure. The ecological effects, e.g. on insect larvae and earthworms[24], are at the moment an issue of shear speculation.

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

Transgenes or upregulated plant genes may give rise to toxicants, anti-nutritients, 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 GE 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, may be unpredictably influenced by posttranslational modifications, alternative splicings, alternative start codons for transcription, chimeric reading frames resulting 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.

[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 (2003) 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. And, 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 (2001) 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. Prefibrillar amyloid protein aggregates share common features of cytotoxicity. J. Biol. Chem. 279: 31374-31382, 2004, Kayed et al. Common structure of soluble amyloid oligomers implies common mechanisms of pathogenesis. Science 300: 486-489, 2003

[24] Zwahlen C. *et al.,* 2003

May GE food/feed give allergies?

One of the major health concerns related to GEPs is that the transgenic product itself, e.g. a Bt toxin, or changed expression of endogenous plant genes may result in *allergenic* compounds. The risk assessment of allergens often follows an *allergenicity decision tree*[25]. 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 hopeful that the protein isolated for the test matches all proteins produced from the same gene in the GEP. But 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[26].

Another important question related to allergenicity is whether post marketing surveillance can provide useful information about allergens in GE foods. For a number of reasons this is not likely to happen[27]. 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[28]. 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 GEPs

It is very important to be aware of the fact that the Bt-toxins expressed in GEPs 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 decennia[29]. The difference is evident already at the gene level, since the versions found in GEOs are engineered to produce active Bt toxins. By extrapolation these have a number of potentially unwanted biological characteristics, ranging from

[25] 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

[26] Schubert, D. A different perspective on GM food. Nature Biotechnology 20: 969, 2002

[27] 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

[28] Bucchini and Goldman, 2002

[29] Stotzky, 2000

solubilization of the protein under natural conditions and effects on insect and mammalian cells, to persistence and non-target effects in the environment[30]. In addition, the posttranslational modifications that may influence conformations, cellular targets and biological effects of GEP-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 conceived of as "early warnings" of potential health and environmental risks, have appeared in the literature[31]. Most of them have, however, not been followed up by extended studies.

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[30] Andow, 2002

[31] 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". Environmental Health Perspectives 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)" Biochemical Journal (2003) 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 (2003) 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 Medical Microbiology 29 (2000), 177-181**.** Farmworkers 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., 1999. "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 (2000) 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, 2000, 885-890; Moreno-Fierros et al, 2002. "Slight influence of the estrous 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 (2002) 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 Echology (2003) 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 (2004) 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". Molecular Ecology (2003) 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, 12 December 2002, page 1, 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. 2001;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 Volume 107, No. 7, July 1999), 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) GEPs

These GEPs have an inserted transgene, *cp4 epsps,* coding for an enzyme that degrades the herbicide glyphosate. The whole idea is of course the combined use of the GEP and the herbicide. Recent studies indicate that in some cases such GEPs are associated with greater usage of glyphosate than the conventional counterparts[32]. A very restricted number of experimental studies have been devoted to health or environmental effects of the GEPs 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[33]. Consequently: 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 (Poogin et al., 2001). 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 GEOs 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[34].

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[32] 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

[33] 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 (2002). The data suggested that *epsps*-transgenic soybean intake was influencing livercell 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 and Thompson 2003. " Ecological risk assessment for aquatic organisms from over-water uses of glyphosate". J Toxicol Environ Health B Crit Rev. 2003 May-Jun;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. 2002 Oct;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)". Ecotoxicology, 12, 271-285, 2003) 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 (2003), 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. 2003 Aug;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. 2002, 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 (2003) 67-78) it was demonstrated a negative effect of glyphosate, as well as a number of other organophosphate pesticides, on nerve-cell differentiation.

[34] 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[35] and in *Schizosaccharomyces pombe*[36], there are published studies indicating that the 35S CaMV promoter *might* have potential for transcriptional activation in mammalian systems[37]. And the final proofs have been made available during the last couple of years. First, 35S promoter activity was demonstrated in human fibroblast cell cultures[38], thereafter in hamster cells[39], and very recently one of us (TT) has demonstrated substantial 35S promoter activity in human enterocyte-like cell cultures[40]. Such cells are lining up the surface of human intestines. However, no published studies have investigated 35S CaMV activity *in vivo*, and this is hence an obvious area of omitted research.

May 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 GE oilseed rape event lines like MS1Bn x RF1Bn and Topas 19/2.

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[35] Hirt et al., 1990

[36] Gmunder and Kohli, 1989. " Cauliflower mosaic virus promoters direct efficient expression of a bacterial G418 resistance gene in Schizosaccharomyces pombe". Mol Gen Genet. 1989 Dec;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. 1990 Jan;220(2):314-6.

[37] 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 (2000)) and in Xenopus oocytes (Ballas et al., 1989. "Efficient functioning of plant promoters and Poly(A) sites in Xenopus oosytes". Nucleic Acids Research Vol 17 Issue 19 7891-7903 1989). 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 comfirms the predominance of microhomoloy mediated recombination". The Plant Journal (1999) 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 promoter?". Nature Biotechnology volume 18, April 2000) 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. 1992 Sep;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, Vol. 93, pp. 6653-6658, June 1996, Medical Sciences; reviews: Traavik, 1999a "An Orphan in science". Research Report for DN No. 1999-6; Ho et al., 2000. "Hazardous CaMV promoter?". Nature Biotechnology volume 18, April 2000).

[38] 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.

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

[40] Marit R. Myhre , Kristin A. Fenton , Julia Eggert , Kaare M. Nielsen and Terje Traavik. The 35S CaMV plant virus promoter is active in human enterocyte-like cells. Submitted for publication, 2005.

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 (GM) crop has the unused marker gene in each and every one of its cells.

There are multiple well-known mechanisms for cross-resistance to antibiotics of a particular type[41]. 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[42]. All of the antibiotics mentioned are used to treat human diseases.

Along with cross-resistance to aminoglycoside antibiotics, pathogenic bacteria frequently develop multiple drug resistance transmitted on a single plasmid[43]. Pathogenic bacteria do acquire plasmids with multiple antibiotic resistance genes in areas where the antibiotics are used extensively. Such incidents illustrate the potential health effects of HGT. Multiple resistance genes on a single plasmid can simultaneously adapt a bacterium to several unrelated antibiotics. One antibiotic at a time is all that is necessary to maintain the plasmid.

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[44].

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

[42] 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". National Academy of Sciences, USA, Vol. 96, page 6155-6160, May 1999

[43] For example, the cholera pathogen *Vibrio cholerae*, first isolated from India, Bangladesh and Thailand (Yamamoto et al., 1995. "Emergence of tetracycline resistance due to a multiple drug resistance plasmid in Vibrio cholerae O139". FEMS Immunology and Medical Microbiology 11 (1995) 131-136**)** was found to have a plasmid resistant to tetracycline, ampicillin, chloramphenicol, kanamycin, gentamycin, sulphaethoazole and trimethoprim; Heinemann, J. A., Ankenbauer, R. G., and Amábile-Cuevas, C. F. (2000). Do antibiotics maintain antibiotic resistance? Drug Discov Today 5, 195-204.

[44] 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 1999, 5:86-90 (Exhibit NOR-28)) 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. 1999 Jun;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. 1996 Jun;182(6):509-14.), and has been found to be effective against *E coli 0157* without causing release of verotoxin (Ito et al., 1997)

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 GEOs. There are many more risk issues. Among them are issues of Horizontal Gene Transfer (HGT)[45], the new generations of multitransgenic GEOs for pharmaceutical and industrial purposes[46], safety questions related to GE vaccines[47], the new nanobiotechnology approaches[48] and the applications of small inhibitory (si) RNAs for a number of medical purposes[49]. 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 GE materials in food and feed[50]. 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[51]. And it has been illustrated that the siRNA techniques are not as "surgically targeted" as initially indicated[52].

We are 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[53].

We must as citizens and professionals join together to reverse the present situation. Publicly funded, independent research grants must become a hot political issue. That would be the most efficient remedy for lacking answers and corporate take-over of science. And finishing off, we

[45] Heinemann, JA and Billington C. 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, 2004.
[46] Twyman, RM et al. Molecular pharming in plants: host systems and expression technology. Trends in Biotechnology 21: 570-578, 2003
[47] Traavik T. 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, 2002 (ISBN 0849304393).
[48] Mazzola, L. Commercializing nanotechnology. Nature Biotechnology 21: 1137-1143, 2003; Colvin, V. L. (2003). The potential environmental impact of engineered nanomaterials. Nat Biotechnol 21, 1166-1170.

[49] Hannon, GJ and Rossi, JJ. Unlocking the potential of the human genome with RNA interference. Nature 431: 371-378, 2004
[50] Heinemann JA, Sparrow AD and Traavik T. Is confidence in the monitoring of GE foods justified? Trends in Biotechnology 22: 331-336, 2004
[51] Heinemann JA and Traavik, T. Problems in monitoring horizontal gene transfer in field trials of transgenic plants. Nature Biotechnology 22: 331-336, 2004; Heinemann JA, Traavik T. 2004. Monitoring horizontal gene transfer.Reply. Nature Biotechnology 22: 1349-1350, 2004
[52] e.g Jackson, AL et al. Expression profiling reveals off-target gene regulation by RNAi. Nature Biotechnology 21: 635-637, 2003, and a number of other recent articles.
[53] Mayer S and Stirling A. GM crops: good or bad? EMBO Reports 5: 1021-1024, 2004; 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. The public-private structure of intellectual ownership in agricultural biotechnology. Nature Biotechnology 21: 989-995, 2003

once more quote Mayer and Stirling[54]: "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". But then again, whom should the society rely on for answers and advice when the time comes that all science resource persons work directly or indirectly for the GE producers?

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[54] Mayer S and Stirling A. GM crops: good or bad? EMBO Reports 5: 1021-1024, 2004

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