

CONTRIBUTIONS FROM SCIENTIFIC RESEARCH TO THE RISK ASSESSMENT OF GMOs

21ST OCTOBER 2010

- 10.00 **Welcome**
J. Peeters | Scientific Institute of Public Health (WIV-ISP) | General Director of WIV-ISP
P. Herman | Scientific Institute of Public Health (WIV-ISP) | Head of Unit of Biosafety and Biotechnology

SESSION 1: SCIENTIFIC CONTRIBUTIONS TO RISK ASSESSMENT OF GMOs CHAIR: D. Breyer | Scientific Institute of Public Health (WIV-ISP)

- 10.30 **The GMO Regulatory framework and the methodology for the risk assessment**
K. Pauwels | Scientific Institute of Public Health (WIV-ISP)
- 10.50 **Research, science and expertise - Why and how to interact**
P. Baret | (1) Belgian Biosafety Advisory Council – (2) Université de Louvain (BE)
- 11.15 **The role of the scientific community in the GMO risk assessment in Belgium**
D. Reheul | (1) Belgian Biosafety Advisory Council – (2) Universiteit Gent (BE)
- 11.40 **The High Council for biotechnologies in France - the scientific community at the heart of risk assessment of GMOs**
C. Golstein | Haut conseil des biotechnologies (FR)
- 12.05 **COGEM and the scientific community - description of a partnership**
B. Zoeteman | Dutch Commission on Genetic Modification (NL)
- 12.30 **LUNCH**

SESSION 2: GMMS, VECTOR FOR GENE DELIVERY CHAIR: M. Goossens | Scientific Institute of Public Health (WIV-ISP)

- 14.00 **Influence of the design and mode of delivery of AAV vectors on risk assessment**
L. Tenenbaum | Lausanne Hospital University Hospital (CH)
- 14.30 **Effect of administration route on biodistribution and shedding of HAdV-5 and AAV-2**
H. Hermesen | Centre for Biological Medicines and Medical Technology (BMT)
National Institute for Public Health and the Environment (RIVM) (NL)
- 15.00 **The potential of GM *Lactococcus lactis* in therapeutic applications**
P. Rottiers | ActoGeniX (BE)
- 15.30 **Coffee break**

SESSION 3: EPIGENETIC EFFECTS CHAIR: K. Pauwels | Scientific Institute of Public Health (WIV-ISP)

- 16.00 **Epigenetic modulation of gene activity as new challenge for GMO risk assessments**
J-P. Nap | (1) Hanze University Groningen (2) Plant Research International (NL)
- 16.30 **Complex trait dynamics following epigenomic perturbation**
F. Johannes | Groningen Bioinformatics Centre, University of Groningen (NL)
- 17.00 **Reverse breeding approach: simplifying inheritance patterns**
E. Wijnker | Wageningen University, WU (NL)
- 17.30 **COCKTAIL**



22ND OCTOBER 2010

9.00 Coffee

SESSION 4: DESIGN OF FIELD STUDIES CONTRIBUTING TO ENVIRONMENTAL RISK ASSESSMENT OF GM PLANTS
CHAIR: A. De Schrijver | Scientific Institute of Public Health (WIV-ISP)

9.30 **Problem formulation and hypothesis testing in ecological research and environmental risk assessment**
A. Raybould | Syngenta, Jealott's Hill International Research Centre (UK)

10.00 **Field studies for environmental risk assessment of GM plants: pros and cons**
S. Renckens | Syngenta (BE)

10.30 **Quantitative aspects of field studies on risk assessment of GM plants**
J. Perry | Consultant Biometrician & Ecologist (UK)

11.00 Coffee break

SESSION 4: DESIGN OF FIELD STUDIES CONTRIBUTING TO ENVIRONMENTAL RISK ASSESSMENT OF GM PLANTS (CONTINUED)
CHAIR: A. De Schrijver | Scientific Institute of Public Health (WIV-ISP)

11.30 **3-year field study with MON810 and MON88017: direct effects on non-target organisms**
S. Rauschen | RWTH Aachen University, Institute for Biology III (DE)

12.00 **Non-target effects of herbicide tolerant maize on herbivore and predatory arthropods**
R. Albajes | University of Lleida, Centre UdL-IRTA (ES)

12.30 LUNCH

SESSION 5: OMICS
CHAIR: P. Herman | Scientific Institute of Public Health (WIV-ISP)

14.00 **Comparative safety assessment of plant-derived foods ; possibilities for transcriptomics**
J. van Dijk | RIKILT Institute of Food Safety (NL)

14.30 **Mutagenesis vs. transgenesis: What's beyond the phenotype?**
R. Batista | National Institute of Health (PT)

15.00 **Potential contributions of metabolite profiling to the safety assessment of crops**
T. Frank | Technische Universität München (DE)

15.30 **Field release of transgenic pathogen-resistant barley: Transcriptome and metabolome profiling studies on transgenic crops**
G. Langen | Research Centre for BioSystems, Land Use and Nutrition
Justus-Liebig-Universität Gießen (DE)

CLOSING REMARKS

16.00 **D. Breyer** | Scientific Institute of Public Health (WIV-ISP)

16.30 Guided visit of the Royal Belgian Institute of Natural Sciences



The GMO regulatory framework and the methodology for risk assessment

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A number of parallel evolutions, such as the emergence of DNA recombinant technologies, the use of genetically modified organisms (GMOs) in medicinal area and agrifood and the biological risk classification of pathogenic micro-organisms, has led to the development of guidelines^{1,2} that at an international level, have been used as a reference for the development of recommendations with respect to the use of GMO in laboratories or for their use for commercial exploitation or release into the environment. These guidelines introduced two important concepts that are still considered as one of the keystones of GMO regulation in Europe: namely the case-by-case approach and the step-by-step approach.

The EU regulatory framework, which has been set-up with the establishment of two Directives^{3,4}, is also based on the safety for human health and environment, the precautionary principle and the process of risk assessment and risk management. In parallel, regulations specifically related to certain types of products, such as those for the use of GMOs as food and feed and those for the application of GMOs medicinal products for human or veterinary use, have been developed. Today, the GMO regulatory framework involves different administrative bodies representing different institutional levels. In Belgium, a single common science-based biosafety advisory system was established allowing coordinated assessment of all regulatory-related aspects of the use of GMOs and pathogens.

Key to the GMO regulatory framework in Europe is the risk assessment of GMOs that aims at identifying and evaluating potential adverse effects of GMOs on safety for human health and the environment, including protection of biodiversity. It is a methodology that consists in a structured process involving a defined number of steps and that is performed in a scientifically sound and transparent manner. Whereas the methodology for risk assessment of GMOs is based on the risk classification of the organisms and the assignment of a containment level to the contained use activity, the risk assessment of GMO for deliberate release is driven by the comparative safety assessment between the GMO to be assessed and an appropriate comparator. During the presentation, some issues encountered during the risk assessment of GMOs will be highlighted.

(1) National Institutes of Health, Donald S. Fredrickson: Guidelines for Research Involving Recombinant DNA Molecules. June 1976

(2) Recombinant DNA Safety Considerations : Safety considerations relating to the use of organisms obtained through DNA

(3) Directive 90/219/CEE on the contained use of genetically modified micro-organisms, OJ L 117 of 08.05.1990, p 1, which was repealed in 2009 by Directive 2009/41/EC, OJ L 125, 21.05.2009, p.75)

(4) Directive 90/220/CEE on the deliberate release into the environment of genetically modified organisms, OJ L 117 of 08/05/1990, p.15. Directive 90/220/EEC was repealed in 2001 by Directive 2001/18/EC, OJ L 106, 17.04.2001, p.1.



The High Council for biotechnologies in France – the scientific community at the heart of risk assessment of GMOs.

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The High Council for biotechnologies (HCB) is a French independent body, set up in April 2009, in charge of advising the French Government on issues relating to biotechnologies, including genetically modified organisms (GMOs). In particular, HCB assesses the potential risks to the environment and public health associated with GMOs, as well as their social and economic impact.

HCB was created in accordance with the June 2008 French law relative to GMOs, following a broad consultation on the environment in France, the "Grenelle de l'Environnement". HCB takes on several mandates previously scattered among different organisations, including the Genetic Engineering Commission (CGG) and the Biomolecular Engineering Commission (CGB). The scope of HCB covers all fields of biotechnologies, involving microorganisms, plants or animals, for contained use or deliberate release, for research, development, education, or commercial purposes, in the sectors of agriculture, health, food and feed, energy and the environment.

HCB is original in the European landscape in that it consists of two independent committees: a scientific committee (SC) — composed of leading scientists of diverse expertises covering the scope of HCB —, and an economic, ethical and social committee (EESC) — composed of representatives of civil society and various stakeholders relevant to HCB's mandate. Both committees include members qualified in law, social science and economics. The SC and EESC provide case-by-case scientific opinions and recommendations, respectively, to the relevant competent authorities of the French Government.

One year on, HCB has evaluated over 400 dossiers of GMOs for contained use, and 13 dossiers relative to GMOs for deliberate release. Of those, six dossiers for cultivation of genetically modified plants were assessed as part of the European procedure for authorisation by the European Commission. Other dossiers included clinical trials for gene therapy, and dossiers on transversal issues, such as a multi-faceted reflection over the definition of "GM-free" production.

HCB works in close interaction with the scientific community. The scientific community is strongly represented at the heart of HCB activities in GMO risk assessment, since most members of the SC are themselves research scientists in activity. In addition, HCB frequently resorts to external experts from the scientific community at large, in order to complement its internal expertise to cover all the specific issues raised by application dossiers. Finally, HCB solicits the scientific community at large in the organisation of events on critical issues associated with GMO risk assessment. These events aim at providing HCB members with the state of the art in some of the transversal issues associated with the risk assessment of GMOs, directly from the best available experts. So far, we have organized three events, covering the issues related to herbicides and herbicide-tolerant plants, to the containment level of lentiviral vectors, and to the assessment of the toxicity and allergenicity of GMOs. The next event, planned for the 1st and 2^d December 2010, will focus on the new technologies of genetic modification. Unlike the previous ones, this event has turned into an international symposium, opened to guest scientists from the European community, notably to HCB homologues and members of the European Commission New Techniques working group. In this way, we hope that this HCB symposium will fuel the reflection on the topic of new technologies of genetic modification for as many of our homologues as possible in preparation for the consultation on a future adaptation of European legislation.



COGEM and the scientific community – Description of a partnership

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The context for scientific risk assessment of GMOs is continuously changing. A global study on publications in the written media by COGEM over the period 2005-2008 shows trends and differences between continents and nations. The technology and society driven positions are most different between North-America and Africa. The EU places itself between North-America and Asia. Industrial and medical biotechnology are more private enterprise oriented than agricultural biotechnology. All media messages on biotechnologies are strongly dominated by what is technically possible in stead of what is desirable from a societal point of view.

New risk assessment issues arise such as new gm-techniques requiring different legal responses and increasing societal resistance to monopolizing tendencies in the business sectors.

Advisory committees to governments on these issues, such as COGEM, have to represent the scientific community in a way that is independent and authoritative. More than in the past this requires attention for issues such as avoidance of conflicting interests of members, integrity in serving the higher purpose of the committee's mission, good procedures for minority opinions and transparency, including providing explicitly all used arguments and a periodic evaluation of the functioning of the committee.

The intermediate function of advisory committees between the scientific community and government institutions as well as the general public requires different roles. At increasing scientific uncertainty and societal debate the role of science shifts from developing standard rules, to case by case assessment or to informing the political authorities of all arguments used in the debate without drawing a final conclusion with advice. At the highest level of uncertainty and debate science's role of forging a consensus is very small. In the end politicians have to take their responsibility and choose in the midst of the highest uncertainty.



Influence of the design and mode of delivery of AAV vectors on risk assessment

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Recombinant AAV (rAAV) vectors are constructed by replacing the wild-type adeno-associated *rep* and *cap* genes with the transgene of interest and transcriptional elements while retaining the AAV non coding inverted terminal repeat (ITR) sequences. Replication and packaging of these vectors is accomplished by providing permissive cells with *rep*, *cap*, and the adenovirus helper gene functions. Originally, this was done by infecting cells with adenovirus before co-transfection with a *rep/cap*-expressing helper and vector plasmids.

In the first generation helper plasmids, due to the presence of sequences homologous to the vector plasmid, a high percentage of wild-type AAV virus, with a selective replication advantage, were generated and could not be separated from the recombinant AAV particles. The current protocols are using *rep* and *cap* sequences cloned in plasmids or baculoviruses which do not harbour any sequence homologous to the vector plasmid. In addition, the generation of replication-competent AAV viral particles is usually rendered impossible e.g. by using large heterologous expression promoters which make the potential recombinant too large to be encapsidated.

The first generation rAAV stocks were also contaminated with wild-type adenovirus, which, thanks to AAV remarkable stability, could be selectively heat inactivated at 56°C, nevertheless leaving AAV preparations heavily contaminated with highly-immunogenic adenoviral proteins. Later on, adenoviral helper functions have been identified as E1a, E1b, E2a, E4 and VA-RNA and could be provided by plasmid transfection in the absence of virus.

Similarly, the downstream purification methods have been gradually improved, beginning with CsCl density gradients and progressing through various column chromatography techniques.

However encapsidation being not an exact process, exogenous plasmidic or baculovirus DNA sequences might be present in a certain percentage of viral particles. This could create a potential hazard, for example if antibiotic-resistance sequences are present.

The first rAAV vectors were based on serotype 2 adeno-associated virus. However, other serotypes are continuously being discovered either in human biopsies or in non-human primates, cloned in plasmids, and used to derive pseudotyped rAAV vectors. Usually, a unique vector plasmid harbouring the AAV2 ITRs is encapsidated by using hybrid helper plasmids containing the *rep* coding sequence from AAV2 and the *cap* coding sequence from the desired serotype.

It will be shown that the serotype greatly influences the biodistribution of transgene expression. Examples of intravenous and intracerebral administration will be discussed.

In addition to the serotype, the promoter might also influence the biodistribution of transgene expression. Examples of attempts to target the liver and specific brain regions will be discussed.

In the brain, axonal transport of viral particles and/or secreted proteins constitutes an additional challenge for the restriction of the diffusion of transgene products.

In addition, the presence of proliferative precursor cell types in some brain regions and their migration properties has to be taken into account in the evaluation of the consequence of gene transfer into the central nervous system.

The titer of the viral preparation might also influence the relative transduction of different cell types.

Finally, rAAV vectors have been delivered to the brain through several modes of administration: intraparenchymal, intraventricular, intra-cisternal in the presence of mannitol to transiently open the blood-brain barrier, intramuscular followed by retrograde transport into motoneurons, intravenous using rAAV9 which is naturally able to cross the blood-brain barrier. All these delivery methods pose different and specific safety concerns which will be discussed.



Effect of administration route on biodistribution and shedding of HAdV-5 and AAV-2

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Gene therapy is a rapidly developing field in which recombinant nucleic acid sequences are introduced to individuals. Its therapeutic, prophylactic or diagnostic effect relates directly to the sequence it contains or to the product of genetic expression of this sequence. Recombinant adenoviral vectors (in particular HAdV-5 vectors) are frequently used in gene therapy. Knowledge on biodistribution and shedding is crucial in the risk assessment for the patient and the patient's environment.

This presentation presents an overview on biodistribution and shedding data of non-replicating viral vector HAdV-5, related to the used administration route. Based on these data, a qualitative model for the biodistribution and shedding of HAdV-5-based viral vectors is presented.

Biodistribution and shedding depend on the route of administration. Some routes lead to local biodistribution and no shedding or one shedding route only. Other routes lead to systemic biodistribution and to shedding *via* several excreta.

Shedding *via* semen and transport across the blood-brain barrier is not expected for HAdV-5. The presented qualitative model may help to determine the possible distribution in the body and may facilitate the risk assessment of shedding *via* the different excretion routes.



The potential of genetically modified *L. lactis* in therapeutic applications

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Food-grade bacteria have been consumed throughout history without associated pathologies and are, therefore, absolutely safe to ingest. Unexpectedly, *Lactococcus lactis* (*L. lactis*), known from cheese production, can be genetically engineered to constantly secrete satisfactory amounts of bioactive molecules derived from a viral, bacterial or eukaryotic background. Both of these features enabled the development of a new kind of topical delivery system: topical and active delivery of therapeutic molecules by genetically modified (GM) micro-organisms. The host organism's record inspired the development of applications that target gastrointestinal and immune diseases. Orally formulated *L. lactis* strains (ActoBiotics™), engineered to synthesize and secrete therapeutic peptides and proteins in the gastrointestinal tract, are already in advanced stages of preclinical and clinical development. Such novel therapeutic strains are textbook examples of GM micro-organisms. There are legitimate concerns with regard to the deliberate release of GM micro-organisms. On development of the clinical applications, therefore, we have engineered these bacteria in such a way that environmental containment is guaranteed. The essential gene *thyA*, encoding thymidylate synthase, has been exchanged for IL-10. This makes the GM strain critically dependent on thymidine. Lack of thymidine, for example, resulting from thymidine consumption by *thyA*-deficient strains—will irreversibly lead to induced “thymidine-less death.” This accomplishment has created the possibility of using this strategy for application in human medicine.

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Huibregtse IL, Marietta EV, Rashtak S, Koning F, Rottiers P, David CS, van Deventer SJ, Murray JA. Induction of antigen-specific tolerance by oral administration of *Lactococcus lactis* delivered immunodominant DQ8-restricted gliadin peptide in sensitized nonobese diabetic Abo Dq8 transgenic mice. *J Immunol* 2009;183(4):2390-2396

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Epigenetic modulation of gene activity as new challenge for GMO risk assessments (?)

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Epigenetics is here defined as the study of changes in gene function that are heritable without changes in DNA sequence. This field is strong in development and adds new layers of complexity to the regulation of gene expression on top of (~epi) DNA sequence-based mechanisms of gene regulation. As such, epigenetics broadens the baseline of known (or anticipated) natural variation that could help assessing the biosafety of GMO's. Epigenetic inheritance occurs between cells of a single organism as integral part of development, known as mitotic or somaclonal epigenetic inheritance. Changes are stable during mitosis and supposed to be reset fully during meiosis. It also occurs between generations of organisms and is then known as meiotic or transgenerational or gametic epigenetic inheritance. Modifications are supposed to be stable during both mitosis and meiosis, require modification of germ-line cells and may be stochastic in nature. Examples in mammals are debated, whereas in plants the occurrence of transgenerational epigenetics is amply demonstrated and supposed to help plants react to (adverse) environments. The cellular epigenetic machinery currently known involves three different mechanisms addressed by a variety of new technologies. These three parts of the epigenetic machinery are closely interconnected in what is seen as 'the epigenetic triad'. The field of epigenetics is generating its own vocabulary, risk assessment should be aware of, such as epiallele, epigenome, epimutation or epi(genetic) memory. Mechanisms are:

- *DNA (de)methylation*; occurs at symmetric as well as asymmetric sites. Possibly also hydroxyl-methylation may play a role. There is a direct or indirect relationship with gene silencing.
- *non-coding RNA*; various forms of small (and not-so-small) RNA are involved in gene regulation (miRNA, siRNA, RNAi etc.). Small RNA approaches are already used in plant GMO's.
- *chromatin modification*; the dynamics of various modifications of histone proteins, such as acetylation and methylation is thought to create a 'histone code' for gene regulation. In addition, chromatin itself has structural characteristics involved in accessibility for transcription.

In applications, the epigenome can be deliberately targeted (such as in RNAi), or epigenetic effects could be -or generate- an unintended effect in GM events. Epigenetics may be at the root of all pleiotropic and/or unintended effects, but current understanding and examples of epigenetic effects in GM plants seem to indicate that epigenetic regulation is local, subtle and broadens the spectrum of natural variability. Moreover, there are new approaches on the horizon, proposed to be called 'epigenetic engineering' (epiGM). A transient (GM?) vector (e.g. virus-based) may be used to make an epimodification in a plant genome that is present and stable long after the vector is gone. The resulting epimutation could be considered to fall within the realms of natural variation. EpiGM is not likely (yet?) to involve the introduction of new genes, but only affect the regulation of endogenous genes. EpiGM may be motivated by the wish to evade current regulation. It should be discussed and decided upon better what needs to be known prior to considering the incorporation of an epigenetic evaluation into current regulatory GM assessment.

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Assessment of the impact of transgenerational epigenetic variation on complex traits

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Understanding the role of epigenetic variation in complex trait inheritance is one of the major challenges at the horizon of quantitative genetics. To address this challenge experimentally two groups have recently constructed so-called Epigenetic Recombinant Inbred Lines (EpiRILs)^{1,2}. These populations were derived from two parents with identical DNA sequences but drastically divergent methylation profiles as a result of differential epigenomic perturbation. This system therefore provides a unique opportunity to study patterns of epigenetic inheritance against an isogenic background. Estimates suggest that epigenetic variation in these populations can account for up to 30% of the variation observed in complex traits, even eight generations later¹. However, many of the segregating epialleles display intriguing time-dependent characteristics. These non-Mendelian properties hint at a dynamic rather than a static epigenetic architecture. Here we incorporate these phenomena into a quantitative genetics framework³. We explicitly model the time-dependent behavior of epigenetic variation over generation time, and trace its phenotypic consequences at the level of the population. Theoretical arguments show that the simultaneous action of allele-specific expression, recombination and epiallelic stability are key parameters in this system.

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2. Reinders J, Wulff-Brandt BH, Mirouze M, Mari-Ordonez A, Dapp M *et al.* (2009) Compromised stability of DNA methylation and transposon immobilization in mosaic Arabidopsis epigenomes. *Genes & Development* 8:939--950.
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Reverse breeding approach : simplifying inheritance patterns

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A plant breeders' most important goal is the efficient creation of desirable allele combinations for the improvement of elite hybrids. New allele combinations are obtained through meiotic recombination, a process in which meiotic crossovers and chromosome segregation lead to gametes of unpredictable genetic make-up. This unpredictable genetic composition in certain cases works against breeders when desired allele combinations are lost from one generation to the other. *Reverse breeding* is a new plant breeding technique that was designed to effectively simplify chromosome inheritance by transiently suppressing meiotic crossovers. We show that suppression of crossover recombination indeed leads to offspring of highly predictable genetic composition. This opens doors to very interesting new breeding applications. For knocking down the crossover machinery there are various possible methods. We recently used an RNAi-construct targeted at a gene essential for crossover formation. Since crossover suppression is only required during one step in the breeding process (i.e. during gamete formation), and the transgene segregates in offspring, *Reverse breeding* generates breeding lines that are free of transgenes.



Problem formulation and hypothesis testing in ecological research and environmental risk assessment

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Ecological research and environmental risk assessment are similar in that they address interesting problems by formulating and testing hypotheses. They differ in the types of problems that are interesting, the characteristics of good hypotheses to solve those problems, and the methods for rigorous testing of hypotheses.

1. Types of problem

Scientific research is often regarded as objective because science involves the objective comparison of observations with predictions. Problem selection for research is objective in the sense that scientific problems arise from the failure of observations to match predictions; however, as there are infinite disagreements between observations and predictions, there is inevitably some subjectivity in problem selection based on the interests of the scientist and society. The problem in risk assessment is to characterise the probability that harmful effects will arise following a certain activity. Harm is an adverse change in something of value, and as value is subjective, the selection of problems for risk assessment must be explicitly subjective by defining what is to be regarded as a harmful effect. Attempts to eliminate subjectivity from problem selection for risk assessment are counter-productive because they lead to predictions about effects, not to assessment of risk.

2. Types of hypothesis

Hypotheses seek to be accurate and interesting. It is easy to formulate accurate hypotheses; for example, the hypothesis that predicts "oilseed rape and wild turnip will hybridise somewhere in Europe next year" is likely to be corroborated. The hypothesis is uninteresting, however, because it excludes so few possibilities. A more scientifically interesting hypothesis may predict that "there will be 30,000 hybrids in the UK and 15000 hybrids in France next year". The precision of this prediction makes it more interesting because if it is corroborated, the theory on which it is based will be shown to have great explanatory power. Scientific hypotheses therefore seek to be accurate and precise. The purpose of risk assessment is to help decision-making, not to increase scientific knowledge itself. If a decision can be made based on the probability that at least one oilseed rape x wild turnip hybrid will form in Europe next year, then characterisation of the number and location of the hybrids is unnecessary for the risk assessment; the extra detail is irrelevant. Hypotheses for risk assessment therefore seek to be accurate and relevant. Data requirements for risk assessment may become excessive if relevance and precision are confused.

3. Tests of hypotheses

Strong corroboration of research hypotheses in ecology often comes from field studies that are seeking to establish the ecological importance of an effect that has been detected in the laboratory; although the effect may be clear in the laboratory, it may have no ecological relevance owing to the influence of many other factors in the field. In ecological risk assessment for GM crops, often there is no adverse effect of, say, a *Bt* protein on non-target organisms in under laboratory conditions that maximise the ability to detect such effects. In these situations, field studies add little rigour to the risk assessment because they are less likely to detect effects than are field studies. Ecological research may need field studies to establish the ecological importance of effects detected in the laboratory; and the reasons why this is so mean that field studies are generally not needed for risk assessment when a condition necessary for harm to arise is not observed in the laboratory.

It is important to recognize the differences between environmental risk assessment and basic ecological research because confusing them can lead to ineffective risk assessment and missed opportunities to advance ecological theory. Uncertainty in regulatory decision-making about transgenic crops may be reduced more effectively by clarifying the purpose and structure of environmental risk assessments than by further research on the ecology of the crops.



Quantitative aspects of field studies of risk assessment of GM plants

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I discuss several quantitative aspects of the risk assessment of genetically-modified (GM) plants. Starting with risk assessment for food/feed safety, I discuss attempts to quantify the principle of substantial equivalence and to formalise the associated tests. The concept of equivalence testing has its roots in the biopharmaceutical literature. Equivalence tests will be used to supplement the traditional difference tests. These new tests will be employed in future field trials to assess the composition of GM plants. They differ from previous trials because they include reference varieties which are used to set equivalence limits required by the equivalence tests. I discuss the implications of these tests for risks to consumer and producer in the context of statistical power.

Equivalence testing also features in new guidance for the environmental risk assessment of GM plants, although not in this case employing reference varieties. Instead, equivalence limits will be set directly, using limits of concern that relate closely to environmental harm and protection goals.

Finally, I describe work to build a mathematical model to analyse the exposure of non-target Lepidoptera to *Bt* pollen from maize MON810. For the first time, bioassays in the laboratory that relate mortality to dose are combined with field measurements of maize pollen that relate dose to distance from *Bt* fields to obtain a simple relationship between mortality and distance. The resulting relationship is useful to supplement estimates of mortality rates in situations where field observations may be challenging.



3-year field studies with MON810 and MON88017: direct effects on non-target organisms

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Two 3-year field studies were carried out, assessing the environmental impact of the cultivation of the genetically engineered Bt-maize events MON810 (expressing Cry1Ab against the European corn borer) and MON88017 (expressing Cry3Bb1 against the Western corn rootworm). The genetically engineered lines were assessed in their impact on a diverse set of non-target organisms and compared to their near-isogenic parental lines, to conventional management practices for the control of the agricultural pests (i.e. insecticide applications), and to conventionally bred maize varieties.

The diversity of non-target organisms that were sampled and surveyed included important biological control agents (e.g. ladybirds, minute pirate bugs, ground beetles, and spiders), herbivores (e.g. plant- and leafhoppers, plant bugs), detritivores (e.g. fly larvae, soil mesofauna), and the community of soil microorganisms.

No consistent or reproducible negative impacts of the two maize events were found on beneficial arthropods (Toschki et al., 2007; Eckert et al., 2006; Rauschen et al., 2010), herbivores (Eckert et al., 2006; Rauschen et al., 2008, 2009), or on soil microorganisms (Baumgarte & Tebbe, 2005). If some influence was observed in the lab, it could never be substantiated in the field. The talk will give details on the two field experiments, highlight some of the most important results, and will give a short outlook on the efforts in the environmental biosafety research on the stacked maize MON89034 x MON88017 currently undertaken by a research consortium under a grant of the German Federal Ministry of Education and Research (BMBF).

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Non target effects of HT maize on herbivore and predatory arthropods

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Corn is a major crop in global agriculture, occupying an area of 158 million ha in 2009 (James, 2009). Insect pests and weeds are among the factors that reduce crop yield. Preventing losses caused by lepidopteran pests and improving weed control have been the objectives of the development of genetically modified corn, which was grown on 41.7 million ha in 2009, accounting for 26% of the global corn cultivated area (James, 2009). Genetically modified herbicide-tolerant (GMHT) corn allows postemergence spraying with broad spectrum herbicides and gives growers more flexibility to choose among several spraying times in comparison with the classical preemergence treatments.

Herbicide tolerance trait may affect non-target organisms through different pathways: (i) the effect of the trait, (ii) the effect of the herbicide, (iii) effects of the herbicide treatment on the food web. Several field trials carried out in Spain have not detected any significant effect of the transgenic trait on arthropod herbivores and natural enemies and there are very few reports on effects of broad spectrum herbicides on arthropods. On the contrary, modified weed management has been reported to affect arthropod abundance through the changes in weed abundance and composition (Albajes et al. 2009); these authors concluded that the classical statement that diversified vegetation leads to lower pest densities and higher natural enemies (see a review in Andow, 1991) is not as general as thought. Consequences of changes in weed management practices on biological control functions are difficult to predict as they are the result of complex interactions between plants, herbivores and natural enemies (Norris and Kogan, 2000).

In this presentation we report some results on a four-year field trial of comparing plots treated twice with glyphosate (2006-2009), with plots treated with a conventional preemergence herbicide treatment (2007-2009) and, during the first two years with untreated plots (2006-2007). Abundance or activity of arthropods on the crop plants and on the soil and of flying parasitoids were estimated by means of visual crop plant sampling, pitfall traps, and yellow sticky traps respectively.

As expected, glyphosate-treated plots differed from those with a preemergence treatment in weed abundance, particularly in gramineae but also in broad-leaf species. However, most significant differences were found only one year (2007). The most abundant arthropod herbivores on the crop plants were leafhoppers which densities were significantly higher in glyphosate-treated plots but these same plots also showed significantly higher numbers of generalist predators like *Orius* spp. and spiders on crop plants on the same years that there were significantly more weeds and leafhoppers. A significant correlation between leafhopper densities and *Orius* predators was found. It is hypothesized that the amount of leafhoppers on the crop plants is one of the main factors determining generalist predator abundance.

On the contrary, the prevalent group of soil-dwelling predators in the trial, carabid beetles, were significantly more active in conventionally-treated plots than in glyphosate plots. This could be presumably due to a higher activity in these plots of several species of decomposer Collembola, a common prey of predatory carabids, and to a higher availability of weed seeds for seed-predator carabids. No significant differences were found in number of catches in sticky traps. In these, mymarids were the most commonly trapped parasitoid family.

It is concluded that trophic interactions among arthropods may account for the most significant differences in predator abundance and activity in plots submitted to different weed management practices. Crop plant-dwelling predators and parasitoid activity are less sensitive to changes in weed abundance and composition in comparison with soil predator activity. A more thorough knowledge of economic thresholds of arthropod herbivores in maize and of relationships among the several functional groups of arthropods should allow taking benefit from the flexibility that HT maize gives to growers for managing weeds with broad spectrum herbicides.

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Comparative safety assessment of plant-derived foods: possibilities for transcriptomics.

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Today's food market is moving towards more complex products and methods of production. Plant biotechnology is moving towards insertion of multiple genes up to entire pathways, reaching the discipline of synthetic biology. Even when plants are used as biofactories for biofuels or pharmaceuticals, rest products may end up in the food and or feed chain. Biochemical analyses for food products have likewise experienced a tremendous development with the emergence of techniques as transcriptomics and proteomics and the application of older, chemical techniques in new areas (metabolomics). The potential of more complex methods to evaluate more complex foods will be discussed. Examples of the discriminative power of transcriptomics will be shown and the application towards food safety assessment will be discussed.



Mutagenesis vs. Transgenesis: What's beyond the phenotype?

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Genetically modified foods and their potential impact on human health continue to be a very hot topic that raises enormous discussion at political, economical and scientific levels. Much is said but not much is done in terms of scientific research. The controversy concerning genetically modified (GM) foods is in contrast with the pacifism regarding other foods that also suffered genetic modification but are not considered GM foods and so are not evaluated accordingly. These are for instance food products obtained from new cultivars raised from modern plant breeding techniques such as mutagenesis. Despite all the controversy we can resume it to a straightforward question: What is behind the phenotype of these foods? (i.e. what is the extent of global molecular modifications that occur through genetic modification?- either by genetic engineering or other modern plant breeding techniques). This work aimed to contribute to answer this crucial question. To achieve this goal we evaluated the extent of transcriptome modifications occurring during rice improvement using genetic engineering techniques and compare it to the ones obtained by mutation breeding. For that we have used oligonucleotide microarrays (GeneChip® Rice Genome Array) to analyse gene expression in 4 different pools of 4 types of rice plants and respective controls: (1) a gamma irradiated stable mutant (Estrela A mut); (2) the M1 generation of a 100Gy gamma irradiated plant; (3) a stable transgenic plant obtained for the production of an anti-cancer antibody (ScFv); (4) the T1 generation of a transgenic plant that we produced aiming for abiotic stress improvement (Nipponbare GM), and respective controls. We could undoubtedly observe that, despite the different type of genetic modification used, the improvement of a plant variety through the acquisition of a new desired trait, lead to an altered expression of untargeted genes. We could also observe that, as expected, the tested genetically stable samples (ScFv and Estrela A mut) are more tightly grouped together with their corresponding controls than non-stable ones. More, the tested mutagenized plants unambiguously showed higher transcriptome alterations than tested GM plants. We have also verified, in this study, that transcriptomic alterations caused by the induction of rice genetic modifications, either by modern plant breeding or by genetic engineering techniques, are closely related to plant defence responses. It seems that, even after several generations post-genetic modification, the plant still maintains the "memory" of that incident and responds accordingly.

We finally advise for the safety assessment of improved plant varieties to be carried out on a case-by-case basis and not to be restricted to foods obtained through genetic engineering.



Potential contributions of metabolite profiling to the safety assessment of crops

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In recent years unbiased metabolomics-based approaches have been developed providing tools that complement other untargeted techniques, such as transcriptomics or proteomics. Metabolite profiling can be considered as one of the most pragmatic approaches presently applied. It aspires to provide a comprehensive picture by extracting, detecting, identifying and quantifying a broad spectrum of the metabolites present in biological matrices. In addition to targeted analytical methods, metabolite profiling has been suggested to provide valuable information in plant-derived food analysis related to genotypic and phenotypic biodiversity and thus to improve the crop agronomic and nutritional characteristics. In recent years, metabolite profiling techniques have been applied to investigate the metabolic changes during crop development and growth and to assess the influence of genetic background and environmental effects on metabolite profiles.

The aim of the present study was to apply a capillary gas chromatography-based metabolite profiling methodology to the comparative investigation of different plant breeding systems. The impact of induced mutations and the potential effects of genetic engineering on the crop metabolite profiles were analyzed. The metabolic differences in mutated and genetically modified crops should be assessed in the light of the natural variability of the metabolites originating from environmental impacts, e.g. growing locations and seasons, and from variety-based influences.

Metabolite profiling of low phytic acid (*lpa*) rice and soybean mutants and genetically modified insect-resistant (Bt) and herbicide-tolerant (RR) maize lines was performed according to the following extraction and fractionation procedure: Lipids and polar compounds were consecutively extracted from the freeze-dried crop flour. The lipids were transesterified with methanol and subsequently separated by solid phase extraction into a fraction containing fatty acid methyl esters and hydrocarbons (fraction I) and a fraction containing minor lipids, e.g. sterols, free fatty acids and tocopherols (fraction II). Selective hydrolysis of silylated derivatives was applied to separate the polar extract into a fraction containing silylated sugars and sugar alcohols (fraction III) and a fraction containing acids, amino acids and amines (fraction IV). The four fractions obtained were analyzed by gas chromatography. The metabolite profiling data (peak heights and corresponding retention times) were standardized by means of *Chrompare*, a software tool developed for comparative analysis of metabolite profiling data (www.chrompare.com). The consolidated data were assessed via multivariate and univariate analytical methods.

Results obtained by the investigation of induced rice and soybean mutants demonstrated the usefulness of the applied metabolite profiling to assist in the elucidation of mutation events. For the crops analyzed, considerable amounts of the peaks detected were statistically significantly different between the wild-types and the *lpa* mutants grown in the same field trial. However, only a few of these differences could be consistently observed in all analyzed field trials, indicating a strong influence of the biological variability. Metabolites consistently shown to be significantly different between wild-types and *lpa* rice and *lpa* soybean mutants, respectively, were found to be closely related to the biogenetic pathways leading to phytic acid, allowing a prediction of potential mutation targets in the biosynthesis of phytic acid.

To assess the influence of genetic modification in the light of the natural variability, GM maize lines were grown together with their near isogenic lines at different locations in several consecutive years. Despite partly obvious differences between GM lines and isogenic maize determined for one location / year, no separations of the different maize lines were detectable when combining the metabolite profiling data obtained from the maize lines for all analyzed growing locations and years. This indicates that the environmental effects are considerably more pronounced than those from the genetic background.

The applied metabolite profiling approach demonstrated its applicability to the detection of changes in the metabolite phenotype induced by mutation breeding and genetic engineering. The combination of the described extraction and fractionation scheme with an efficient software enables the detection of potential unintended effects and thus provides the experimental platform to incorporate metabolite profiling as additional tool in the safety assessment of such crops. Investigation of crops grown at different locations / years allows consistent differences to be searched for in order to distinguish between natural variability and changes induced by different treatments such as genetic modification.



Field release of transgenic pathogen-resistant barley: Transcriptome and metabolome profiling studies on transgenic crops

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Wheat and barley roots are attacked by necrotrophic fungi such as the causal agent of root rot, *Rhizoctonia solani*. Transgenic barley plants engineered to express a codon optimized endochitinase of *Trichoderma harzianum* show enhanced resistance to this pathogen. On the other hand, obligate biotrophic arbuscular mycorrhiza (AM) fungi confer beneficial effects to their host plants, e.g. phosphate supply, resulting in higher yields. Like *Rhizoctonia* sp., cell walls of AM fungi contain chitin which might be affected by the activity of the engineered endochitinase as well, thereby resulting in reduced colonisation and less beneficial effects after mycorrhization. To test this possibility, we conducted field experiments with transgenic barley lines during three seasons and at two locations and analysed mycorrhization in genotypes ubiquitously expressing the optimized endochitinase in Golden Promise (Endochitinase Golden Promise, ChGP) and transgenic plants with seed-specific expression of (1,3-1, 4)- β -glucanase in Baronesse (Glucanase Baronesse, GluB) as well as the respective parental lines Golden Promise (GP) and Baronesse (B) using quantitative PCR. No significant alteration in root colonization by AM fungi was observed in all tested lines. In parallel, leaf transcriptome and metabolome analyses were conducted using microarrays, metabolite profiling with 72 metabolites and fingerprinting using mass spectrometry. In contrast to the transcriptome, metabolites were found to be altered in all genotypes in response to root colonization by AM. However, no significant difference between ChGP and GP could be observed. In contrast, 22 genes and 4 metabolites were differentially abundant when comparing GluB and B whereas more than 1,600 transcripts were found to be differentially regulated between varieties GP and B including almost all of the 22 genes differentially regulated in GluB compared to B. The glucanase transgene was transferred from GP to B by backcrossing. Our results from simple sequence repeat-marker analysis suggest that the distinctive alleles were inherited from GP. Thus, the effect of introduction of the transgenes had substantial lower impact on transcriptome than the environment (mycorrhization) or the effect of introgression of genes between varieties by classical breeding techniques.

Reference:

Kogel et al., Transcriptome and metabolome profiling of field-grown transgenic barley lack induced differences but show cultivar-specific variances.

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