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Safety assessment of biotechnology used in animal production, including genetically modified (GM) feed and GM animals – a review*

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Since the beginning of the large-scale commercial cultivation of genetically modified (GM) crops in the mid-nineties, it has continuously increased. This has occurred in particular in non-European countries from which these crops may be exported as commodities to Europe and other markets. Before genetically modified organisms (GMO) are allowed onto the market as animal feed and/or food, they have to undergo a regulatory safety assessment as required by the law in many nations, including that of European Union (EU) nations under EU regulations. This safety assessment is based on an internationally harmonized approach of comparative safety assessment, in which the differences identified during the extensive comparison between a GMO and a conventional counterpart serve as basis for a further safety assessment. The GMOs that have been notified for regulatory approval and assessed for their safety as feed and food in the EU have so far been derived from crops and microorganisms. It is expected that in the near future, also several genetically modified (GM) animals may initially reach the market outside the EU. International activities to harmonize the safety assessment of GM animals have already started and have resulted in the issuance of specific guidelines by Codex alimentarius. Moreover, PEGASUS, an EU-funded project, will consider the perceptions, advantages and disadvantages of GM animals, including perspectives from the social- and life-sciences.

KEY WORDS: biotechnology / genetic modification / animal feed / food-producing animals / feed safety / food safety / risk assessment / comparative approach / international harmonization

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In the mid 1990s, the first genetically modified (GM) crops were released for large-scale cultivation. Since then, the area covered worldwide with these crops has continuously been increasing, reaching 125 millions of hectares in 2008 [James 2008]. These GM crops are not homogenously disseminated over the world, though, with some countries taking a large share in the global area, such as the USA (50%), Argentina (17%), Brazil (13%), Canada (6%), India (6%), China (3%), Paraguay (2%), and South Africa (1%). Conversely, the cultivation of GM crops in Europe, for example, has so far remained relatively limited, predominantly being constituted by GM maize being grown in Spain and Portugal, and, to a lesser extent, in other EU countries. The main GM crops are commodity crops, such as soybean, maize, canola (oilseed rape), cotton, whose products or by-products also are common ingredients of composite feeds for food-producing animals. Moreover, most of these crops have been genetically modified so as to incorporate either one or both of two agronomically important new traits into these crops, namely herbicide resistance and insect resistance [James 2008].

With regard to herbicide-resistant crops, herbicides are weed-killing compounds that aid crop cultivation by eliminating weeds that could compete with the crop for resources or whose seeds might otherwise contaminate the crop harvest. Because herbicides may also be toxic to the crop itself, the herbicides, when applied after emergence of the crop seedlings in the field, often have to be applied in a directed way so as to avoid application onto the crop plants themselves. Another form of weed management is mechanical weeding, such as with a plow. Herbicide resistance facilitates weed management in that it allows for over-the-top application of the herbicide to which the crop plant has been rendered resistant, allowing for broadcast sprays on these crops and also for flexible timing of spraying. The most widely grown GM crop is herbicide-resistant soy that has been rendered resistant against the broad-spectrum herbicide glyphosate.

Insect resistance usually has been achieved through the introduction, via genetic modification, of insecticidal proteins that naturally occur in the crystal-like parasporal inclusions of the soil bacterium *Bacillus thuringiensis* (Bt). These insecticidal proteins are also commonly referred to as "Bt proteins" or "Cry proteins," the latter referring to the crystal-like inclusions in which they naturally occur. At the time crops expressing Cry proteins were introduced, preparations of *B. thuringiensis* had for decades already been – and continued to be – used as biological pesticides, such as in organic agriculture and forestry. Whilst Cry proteins may be toxic to insects, they are not known to be toxic to humans and food-producing animals. Particular Cry proteins are toxic to specific insect species, such as the Cry1Ab protein, which has been introduced into insect-resistant GM maize, being toxic to larvae of lepidopteran pests, such as the European corn borer, that consume the tissue of maize plants. The incorporated Cry proteins, which are expressed at low levels in the tissues of the GM plant, may therefore help obviating the use of externally applied pesticides.

Whilst these crops are not grown in each nation to the same extent, the harvested crops and derived products may be traded internationally. It can therefore be envisaged that GM crops, if locally approved for usage, can be used as ingredients for animal feed in importing nations, such as in the European Union (EU).

Before GM crops and other genetically modified organisms (GMOs) are allowed onto the market, they have to be notified for regulatory approval. Part of the approval process will be the regulatory safety assessment of the GMO. Until present, the GMOs that have been notified for regulatory approval as food and/or feed in the EU, which fall within the scope of Regulation (EC) 1829/2003, consisted of either GM crops (and derived products) or products derived from GM bacteria (for example, microbial biomass). Another development is the use of GM animals for food production. Whilst various GM animals are used for non-food applications outside the EU, such as GM goats producing milk containing biological medicines, no food-producing GM animal is known to be on the market yet. Some experimental food-producing GM animals are known to be in an advanced stage of development and may therefore reach non-EU markets in the near future (for example, rapidly growing GM salmon and tilapia). The various issues surrounding the safety assessment of GM animal feeds and GM animals will be discussed in further detail in the following sections.

Safety assessment of GMOs

Years before the first GM crops came to the market, international organizations such as the Food and Agriculture Organization (FAO), World Health Organization (WHO), and the Organization for Economic Cooperation and Development (OECD), had been promoting the building of international consensus on how to carry out the assessment of the safety of GMOs. This led to the formulation of the comparative safety assessment approach, which later became enshrined in the guidelines for the safety assessment of foods derived from GM crops and from GM microorganisms in 2003 [Codex alimentarius, 2003ab]. Whilst these guidelines do not specifically address the assessment of GM feeds, the assessment of the latter will, in many cases, be very similar to that of foods.

Central to the comparative safety assessment approach is the comparison of the GMO with a conventional counterpart with a history of safe use, aiming to identify the differences between the GMO and the counterpart, which could then be further assessed for their safety. For GM crops, for example, this comparison entails an extensive chemical analysis of key macronutrients, micronutrients, antinutrients and toxins, besides the phenotypic and agronomic characteristics of the crop grown in locations representative of the circumstances of commercial cultivation. The conventional counterpart of the GMO should be preferably as genetically similar as possible and grown under the same environmental conditions in order to ensure that the differences that are thus observed are related to the genetic modification (and not to differences in background genotypes). Also a molecular characterization of the newly inserted

elements, including the "foreign" DNA inserted into the host organism's genetic material, is a common part of the safety assessment. It is therefore not an absolute safety assessment of the GM product, which may be difficult to perform anyway as feed and food products usually are complex mixtures of substances, each with different properties, but a relative safety assessment. Moreover, most conventional foods/feeds that are on the market have not really been tested for their safety in animals but are known to be safe based on long-standing experience with the pertinent feed/food.

Given the many different types of modifications that are possible and given also the wide range of food/feed-producing organisms, it can be envisaged that no standard protocol can exist for the performance of the comparative assessment with regard to the parameters that have to be assessed, but that this should be done on a case-by-case basis. Based on the outcomes of the comparison of the GMO *versus* its counterpart, a decision can be taken on which elements the safety assessment should be focusing further. In the dossiers with safety data that accompany the notifications for regulatory approval, a number of issues are commonly addressed, which are discussed below. More detailed information on the comparative approach can also be found in various scholarly reviews [*e.g.,* Kok *et al.* 2008] and in the guidance that has been published by the European Food Safety Authority (EFSA) [EFSA, 2006].

A description of the DNA that has been used to modify the host organism

This includes the source of the elements of the new genes and their elements (for example, promoters, coding region, terminators), the DNA that has been used for the genetic modification (for example, the structure of a DNA plasmid), and the technique used for the genetic modification (for example, *Agrobacterium-tumefaciens*-mediated genetic modification).

A description of the recipient (host) organism

This may include a description of the host organism's occurrence in nature, its applications in food and feed, history of safe use, typical crop cultivation and food/feed production practices, and trade and consumption data.

Molecular characterization of the newly inserted DNA

The "foreign" DNA that has been newly inserted into the host organism's genetic material, including the structure of the DNA, such as the presence of genes, location of the insert, structure of the insert, and expression of newly introduced genes, are described. This also covers sequence analysis of the insertion site, which also allows for the analysis of any potential rearrangements of the host's DNA at the insertion site, as well as the potential formation of new "fusion genes."

Comparative analysis of the compositional, agronomic and phenotypic characteristics

In the comparison of the compositional characteristics of the GMO versus a conventional counterpart, as explained above, the parameters to be analyzed have to be

chosen on a case-by-case basis. Interestingly, the OECD's Task Force on the Safety of Novel Foods and Feeds has developed consensus documents on the key compositional parameters, including macronutrients, micronutrients (vitamins, minerals), anti-nutrients, toxins, and secondary compounds for the analysis of new crop varieties. The crops for which these documents have been developed include alfalfa (and other forage legumes), barley, bread wheat, canola, cotton, maize, potato, rice, soybean, sugar beet, sunflower, and tomato [OECD, 2009]. Also the phenotypic and agronomic characteristics may vary from one crop species to another, and typically include parameters related to plant morphology, development, and reproduction, as well as yield and susceptibility to abiotic and biotic stressors. The latter kind of characteristics are usually also considered during conventional plant breeding.

Potential toxicity of the GM feed/food

The potential toxicity of any newly introduced substances (including gene products, such as newly expressed proteins, and chemical compounds) is commonly considered. The known toxic properties of the source of the transgene may provide indications of potential toxicity of the newly introduced substances. If the substance is a protein, its amino acid sequence can be compared to the sequences of known protein/peptide toxins using bioinformatics for similarity searches. In addition, the newly expressed protein may be tested *in vitro* for its resistance to proteolytic enzymes, which may provide an indication of the likelihood that it or any derived fragment will maintain its integrity under the conditions of the gastrointestinal tract. The latter may provide insight into whether it is likely or not that the newly expressed protein or derived fragments would be able to exert an adverse effect following consumption of the GM feed/food containing this protein. It may also be considered to conduct toxicity experiments in laboratory animals, such as repeated-dose oral toxicity studies with the purified protein. Because the newly expressed protein may only be present in very low amounts in the GMO, such as in GM crop tissues, the purification of sufficient quantities of the transgenic protein from these animals may be cumbersome. Therefore, the use of analogues of the newly expressed proteins produced by GM bacteria modified with a gene for the same protein is allowed provided that the analogue is demonstrated to be equivalent to the newly expressed protein, for which usually a range of techniques are used, including, for example, gel electrophoresis, immunoblotting, glycosylation analysis, mass spectrometry, N-terminal sequencing, and functional characteristics. The toxicity of the whole food may in some cases also be considered for testing, if it addresses specific outstanding safety issues that have emerged during the assessment. As pointed out above, however, feeds/foods are complex mixtures and cannot be tested like a purified chemical substance. Issues that have to be considered when testing whole feeds/foods, for example, are nutritional balance, palatability, and bulkiness, which may account for the limited dose ranges that are possible with whole products compared to pure chemical compounds. These

issues may therefore limit the ability of general toxicity testing to detect minor changes in the GMO *versus* its comparator.

Potential allergenicity of the GM feed/food

The potential allergenicity, *i.e.* the ability to act as an allergen, of newly expressed proteins is commonly considered. Allergens are substances that are able to elicit allergy, which is a hypersensitive immune reaction involving, for example, acute reactions, such as nausea, formation of skin hives, and a drop in blood pressure. Some foods are known to contain allergens, all of which are proteins, whilst most of the proteins in foods are not known to be allergens. For a new protein in a GM feed/food it is therefore required that its potential allergenicity be assessed. Similar to the assessment of potential toxicity of proteins (see above), this will entail the consideration of the history of allergenicity of the source of the foreign gene, the bioinformatics-supported comparison of the new protein's sequence with the sequences of allergens, and the testing of the protein's resistance to proteolytic enzymes *in vitro*. Further testing with sera from allergy patients may be warranted if positive indications are found for the potential allergenicity of a newly expressed protein, such as the potential cross-reactivity in allergy patients that are allergic to an allergen showing similarity to the new protein in bioinformatics-supported sequence comparisons. Because no test is completely predictive of allergenicity of a protein, Codex alimentarius recommends a "weight-of-evidence" approach, in which the outcomes of the various above-mentioned tests are being considered together [Codex alimentarius, 2003a].

If the host organism itself is known to be associated with allergic reactions, such as in the case of allergenic foods (for example, soybean), also the potential impact of the genetic modification on the intrinsic allergen profile of the host may be considered. For example, various immunoassays can help determining whether the levels of intrinsic allergens have been changed in the GMO versus its conventional counterpart.

Nutritional characteristics

If the nutritional characteristics of the GMO have been altered as compared to its counterpart, for example with regard to the levels and/or bioavailability of certain nutrients, the nutritional characteristics of the GMO should be tested in feeding trials with target animals. A commonly performed study is performance testing in rapidly growing broiler chicken, although this would not have been imperative in many cases given the lack of alterations in nutritional properties of the GMO versus its counterpart. The chicken is considered an attractive and sensitive model as it reaches its adult size within six weeks so that any major nutritional alterations will likely be detected. Measured parameters include feed intake, body weight, and mortality during the experiment, whilst carcass characteristics, including carcass weight and carcass part weights, are usually measured *post mortem*. Other models that are used, for example, are performance studies in lactating dairy cows, cattle beef, and swine.

Horizontal gene transfer

Horizontal gene transfer is a kind of natural genetic modification, in which genes are exchanged between different types of organisms. Bacteria have various mechanisms through which they can exchange genes, such as through conjugation or phage transmission. Yet the only envisaged mechanism by which GM crops could transfer genes to bacteria is through release of DNA after degradation of the plant cell, followed by uptake of that DNA and incorporation into the bacterial genetic material. Such spontaneous transfer, if it occurs, will probably be a very rare event, as it has not been able to be demonstrated scientifically despite many studies. Particularly for newly introduced antibiotic resistance marker genes (ARMGs), the issue of potential horizontal gene transfer is considered. These ARMGs are used in order to facilitate the laboratory phase of GMO development but do not serve any purpose in the final GMO. Items that are commonly addressed in the assessment of horizontal gene transfer are the likelihood of transfer, the kind of antibiotic to which resistance has been introduced, and the background presence of the same resistance in nature.

Unintended effects

Besides the intended effects of a genetic modification (for example, herbicide resistance), it can be envisaged that this modification may also cause unintended effects. Such unintended effects can be either predictable or unpredictable. Prediction of the unintended effects can be based on, for example, a disruption of an intrinsic gene with known function by the insertion of DNA, or the known substrate specificity of an introduced enzyme, catalyzing additional reactions besides the reaction of interest. Unintended effects can become visible as differences in the GMO versus its counterpart during the extensive analysis of their compositional, phenotypic, and agronomic characteristics. For the current generation of GM crops with relatively simple modifications that do not affect metabolic routes, for example, the likelihood of unintended effects can be considered relatively low. For future generations of GM crops with relatively complicated modifications, such as nutritionally improved GM crops containing whole new introduced metabolic routes, the likelihood of such unintended effects obviously increases. Various research projects that have recently been concluded or that are still ongoing aimed at developing supplementary tools for the current comparative assessment of compositional characteristics. These new tools are "profiling" methods that consider the ensemble of many substances at a given level of cellular organization, such as the genome, transcriptome, proteome, or metabolome, without having to know the identity of each substance analyzed on beforehand. Differences in the profiles between the GMO and its counterpart are thus identified and traced back to the substance that is at the cause of the difference. An example is the "profiling" of extracts of a long-ripening GM tomato and conventional tomato with the use of liquid chromatography followed by off-line nuclear magnetic resonance. The researchers were thus able to determine that the levels of glutamic acid were increased, and those of citric acid decreased, in the GM tomato. This finding,

however, did not raise concerns, given the magnitude of the change compared to the levels in other commercial varieties [Noteborn *et al*. 2000]. The utility of profiling techniques for the analysis of crops produced using different agricultural practices (GMO, conventional, organic) has been explored in various recent EU projects, such as the EU-funded SAFE FOODS project (http://www.safefoods.nl).

Pesticide residues

It can be envisaged that pesticide usage on GM crops may alter as a result of the genetic modification, such as in herbicide-resistant crops. With regard to herbicide-resistant crops, the altered use of the target herbicide in the GM crop as compared to that in a conventional crop with regard to timing and spraying method, as well as the altered metabolism of the herbicide may also lead to an altered nature and levels of residues. These considerations are taken into account when assessing the safety of the application of the pesticides on the GM crop, for which regulatory approval will be needed as well. This is usually done under parallel legislation, *i.e.* under pesticide regulations instead of GMO regulations, in many countries.

GM animals

As noted in the introduction above, whilst the GM products that are currently on the market consist of – or are derived from – GM crops and micro-organisms, various experimental GM food-producing animals are in an advanced stage of development. With regard to the safety issues surrounding the food use of GM animals, Codex alimentarius recently published guidelines for the conduct of safety assessment of these animals [Codex alimentarius, 2008]. Whilst this safety assessment, in general terms, follows the same approach as for GM plants and microorganisms, there are a number of issues that are specifically highlighted for GM animals. For example, the health status of the GM animals themselves may already provide indications for potential biological effects of the newly introduced DNA and derived products. In addition, the potential altered tendency of the GM animal to accumulate certain toxic substances or zoonotic pathogens that could be transmitted to the consumer have to be considered.

A number of other specific issues surrounding GM animal safety assessment have been identified by various authors. Kleter and Kuiper [2002], for example, note that, whilst food-producing animals generally do not produce anti-nutrients or toxins, some exceptions should be taken into account, such as the thiaminase enzyme in raw fish. Another consideration is the impact that protein hormones expressed by GM animals may have on other animals if the first are to be processed into feed for the latter. If retroviruses are used for genetic modification, potential recombination with wild-type viruses, leading to formation of new viruses, should be considered [Kleter and Kuiper 2002].

The EU-funded PEGASUS project ("Public Perception of Genetically modified Animals – Science, Utility, and Society") will run from Mid 2009 until Mid 2012. It will collate the vast scientific data on public perceptions towards GM animals as well as construe scenarios of GM animals that may enter the market in the near future (most likely outside the EU). These scenarios will then be further explored from the perspectives of both the social- and life- sciences, the latter also including potential safety issues linked to GM animals. Through a participatory approach, which actively involves stakeholders, the ethical and policy-related issues will be explored, as well as the opinion of a "citizens' jury" solicited. An important goal of the project is to formulate policy recommendations for EU policy makers in order to be prepared for future developments in GM animal technology.

Conclusions

Whilst the regulatory procedures for the approval for the marketing GMOs may differ across nations, their safety assessment is carried out according to the internationally harmonized approach of comparative safety assessment. The GMOs that are currently on the market for feed and food use are GM crops and GM micro-organisms. It can be envisaged, though, that GM animals may enter the market in some parts of the world in the near future. Various proactive initiatives consider the specific issues surrounding the safety of GM animals.

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Ocena bezpieczeństwa związanego z wykorzystaniem biotechnologii w produkcji zwierzęcej z uwzględnieniem genetycznie modyfikowanej (GM) paszy i GM zwierząt – praca przeglądowa

S t r e s z c z e n i e

Od momentu wprowadzenia na szeroką skalę upraw genetycznie modyfikowanych roślin w połowie lat dziewięćdziesiątych, obserwuje się ciągły wzrost ich zasięgu. Zjawisko to ma miejsce zwłaszcza w krajach pozaeuropejskich, z których produkty GM eksportowane są do Europy i na inne rynki. Zanim genetycznie modyfikowane organizmy uzyskają certyfikat na dany rynek jako pasza dla zwierząt lub produkt spożywczy, muszą zostać poddane ocenie bezpieczeństwa stosownie do wymogów prawnych danego kraju, w tym stosownie do regulacji Unii Europejskiej, obejmującej wiele krajów. Ocena bezpieczeństwa opiera się o międzynarodowo synchronizowaną metodykę, podstawę której stanowi porównanie produktów GM z konwencjonalnymi, wyznaczającej kolejne etapy oceny. Genetycznie modyfikowane organizmy, jakie do tej pory były obiektem oceny prawnej i oceny bezpieczeństwa, zarówno jako pasza jak i produkt spożywczy, obejmują rośliny i mikroorganizmy. Oczekuje się, że w niedalekiej przyszłości również genetycznie modyfikowane zwierzęta pojawią się na różnych rynkach, początkowo nieeuropejskich. Podjęto już międzynarodowe wysiłki zmierzające do ujednolicenia metodyki oceny bezpieczeństwa takiego produktu, które zaowocowały już wydaniem stosownych rekomendacji w *Codex alimentarius*. Ponadto projekt PEGASUS, finansowany przez Unię Europejską, będzie rozpatrywał postrzeganie zwierząt genetycznie modyfikowanych, korzyści i niekorzyści związane z ich wykorzystaniem oraz perspektywy rozwoju z punktu widzenia nauk socjologicznych i przyrodniczych.