Safety Assessment of GMO-derived Foods

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Abstract

In some respects genetically modified organisms (GMOs) can no longer be considered a novelty in plant breeding. In some countries the larger part of the production of specific crops is in fact GMO production. Examples are the production of soya bean and maize in the USA. In general, these crops have incorporated genes that code for resistance to either herbicide or insects, or both. There are, however, other interesting developments, both with relation to the techniques used to create GMOs as well as to the introduced traits. This review provides an overview of recent developments in the broad area of biotechnology. Furthermore, regulatory aspects are discussed in a global perspective, with a focus on the situation in the European Union. In general, the basic approach to the food and feed safety assessment of GMOs is the same in different parts of the world; there may, however, be differences in the detailed procedures as applied in different countries. The general aspects of the safety assessment strategies are described and explained. Also, an overview is provided on research developments in the area of the food and feed safety assessment strategies, especially on the application of the so-called 'omics'-technologies (transcriptomics, proteomics, and metabolomics) as a non-targeted approach in the comparative safety assessment of GMOs. It is concluded that it may in the future be necessary to adapt current national and international guidelines for the food and feed safety assessment of GMOs to accommodate the products of these novel developments having the potential to produce much more profound changes in the metabolism of crop plants than in today's GMOs.

Riassunto

Per certi versi gli organismi geneticamente modificati (OGM) non possono essere più considerati una novità nel miglioramento genetico delle piante. In alcuni paesi la maggior parte della produzione derivante da colture specifiche è rappresentata dalla produzione degli OGM. Un esempio in tal senso sono la soia ed il mais negli USA. In generale queste colture hanno incorporato geni che codificano per la resitenza agli erbicidi o agli insetti, o ad entrambi. Ci sono comunque altri interessanti

sviluppi, sia in relazione alle tecniche usate per creare gli OGM che in relazione ai geni introdotti. Questo capitolo si propone di fornire una panoramica sui recenti sviluppi nel vasto campo delle biotecnologie. Inoltre verranno discussi gli aspetti regolamentativi in una prospettiva globale, con particolare riguardo alla situazione dell'Unione Europea. In generale si può affermare che l'approccio di base per la valutazione della sicurezza di alimenti e mangimi è la stessa nelle diverse parti del mondo; ci possono comunque essere delle differenze nei dettagli delle procedure applicate nei diversi paesi. Saranno quindi descritti e spiegati gli aspetti generali delle strategie di valutazione della sicurezza. Inoltre verrà fornita una panoramica sugli sviluppi della ricerca nel campo delle strategie di valutazione della sicurezza di alimenti e mangimi, specialmente sull'applicazione della cosiddetta tecnologia "omica" (trascrittomica, proteomica e metabolomica) come approccio "non-target" nella valutazione non comparativa della sicurezza degli OGM. In conclusione, in futuro potrebbe essere necessario adattare le attuali linee guida nazionali ed internazionali sulla valutazione della sicurezza di alimenti e mangimi derivanti da OGM per adeguarle ai prodotti derivanti da questi nuovi sviluppi che potrebbero comportare cambiamenti più profondi nel metabolismo delle colture rispetto agli OGM attuali.

1. INTRODUCTION

In recent years, both the area of production of genetically modified organisms (GMOs), specifically genetically modified (GM) crop plants, has steadily increased, along with the range of GM varieties available on the world market. In this review, an overview is provided on the recent developments in the field of GM plants demonstrating that the diversity of GM plants will further expand. These developments include new molecular approaches that enable homologous recombination in plants, up to the use of synthetic biology to create new plant varieties with novel traits that have been developed on the basis of the state-of-the-art knowledge on the plant's genome and related metabolic networks. It is clear that these developments may have consequences for the safety assessment of GM plants and their derived products. This review describes current approaches for the safety assessment of GM crop plants and derived products, as well as research developments in the area of the food safety assessment strategies that may lead to (further) improved strategies in this area, especially for the next generation of novel plant varieties.

2. NEW DEVELOPMENTS IN BIOTECHNOLOGY

Genetic modification, similar to classical breeding, aims to alter metabolic processes in order to render the resulting plant varieties more favourable characteristics in terms of, for instance, agronomic, nutritional and/or processing quality. The first generation of GMOs was characterised by the use of genetic constructs that generally consisted of a single gene that either expresses a new protein or enhances/reduces the expression of genes already present in the pathway, flanked by regulatory elements and often an additional marker gene to monitor successful transformation events. The introduced trait was usually, and still is, either herbicide or insect resistance or another trait that is primarily of interest to plant producers, such as virus resistance (Zhang *et al.*, 2009), drought tolerance (Khan *et al.*, 2009), and salt tolerance (Zhu *et al.*, 2007). Engineered resistance against viruses is mainly mediated by: the expression of coat proteins and/or replication enzymes; iRNA; or insect (vector) suppression. Fungal (and bacterial) resistance is mainly based on Resistance gene (R-gene) products, genes coding for PR (Pathogenesis-related) proteins, microbial antimicrobial proteins, defence signalling genes (ethylene/jasmonic acid, salicylic acid), and on Reactive Oxygen Species (ROS)-inducing genes like glucose oxidase. The use of abiotic stress-tolerant crops, including crops tolerant of increases in heat, cold, water, drought and salt, can be two-fold. First, these crops may ensure that harvests of crops in existing agricultural systems will become less prone to losses due to adverse environmental conditions, including heat waves, dry spells, frost, flooding and soil salination. This may also be important from the view of global climate change, if the average climatic conditions are to change, but also if the frequency of extreme weather events is to rise. Second, the use of stress-tolerant crops may allow for the cultivation of specific crops in areas where this has hitherto not been possible, thereby expanding the arable area and the potential for agricultural production (extensification). In both ways, stress-tolerant crops can be envisaged to contribute to increased food security. It is also noted that certain modifications can render crops tolerant to multiple kinds of stress, given that the adverse effects of these stressors act through common mechanisms, including increased oxidative damage to plant cells caused by ROS. Various strategies for achieving drought tolerance in plants are described in the review by Gosal *et al.* (2009), whilst these strategies also commonly protect the plants against other kinds of stress, including salt and water stress, as well as heat and cold. One of

these strategies includes the elevation of the levels of osmolytes, which can be achieved by elevating the levels of the different metabolites in the plant (Gosal *et al.*, 2009).

At this moment, the introduction of more than one trait is often achieved by crossing individual single-gene GM lines, resulting in so-called stacked gene varieties. Table 1 shows that many of the GM varieties recently introduced into the European Union (EU) are such stacked gene varieties.

Table 1. Stacked GMO varieties that are currently authorised for food and/or feed uses in the EU (EC, 2010)

Cotton	MON15985 x MON1445 MON531 x MON1445
Maize	DAS1507 x NK603 MON863 x NK603 MON863 x MON810 NK603 x MON810 DAS59122 x NK603 MON863 x MON810 x NK603
Oilseed rape	MS8 x RF3

At the same time, developments are ongoing to generate new plant varieties with simultaneous insertion of multiple genes. The most well-known example is the so-called 'golden rice' variety (Hoa *et al.*, 2003). In this case, a gene cassette was introduced into the rice genome that contained multiple genes. Other recently developed 'multi-gene strategies' include the targeting of chloroplasts. Quesada-Vargas *et al.* (2005) show that chloroplasts can process multi-genic sequences via the chloroplast genome without significant intervention of chloroplast regulatory systems. These systems are, however, still in the experimental phase and in practice the expression of multiple genes still forms one of the major technical challenges for further extension of the potential of gene technology in plant breeding strategies (Halpin, 2005). A solution to this problem may be the use of mini-chromosome vectors. In a paper by Carlson *et al.* (2007), mini-chromosomes were assembled *in vitro* and subsequently introduced

in maize. When delivered into embryogenic tissue, the marker gene that was included appeared to be stable through four generations (Carlson *et al.*, 2007). This method may be useful for stacking genes in cereals and other plant species. Another solution for the delivery of large DNA sequences may be the use of Plant Artificial Chromosomes (PACs). Similar to mini-chromosomes, these PACs may overcome position effects and size restrictions, enabling the transfer of complete metabolic pathways. It has already been established that telomeres, tandem motifs of highly conserved sequences found at the end of chromosomes, play a role in the stability and protection of chromosomes. On the basis of this knowledge the stability of PACs has been enhanced (Moeller and Wang, 2008).

So far most GM plants are transgenic plants, i.e. plants that have incorporated genes that are derived from other species that could not have been incorporated into the target plant genome by normal crossing procedures. In recent years, however, cisgenic GM plants have also been developed, i.e. plants that have been genetically modified with a gene from a sexually-compatible plant. The advantage of using recombinant DNA techniques in the case of cisgenic plants is that the gene of interest can be transferred without the so-called 'linkage drag' of deleterious genes that may be associated with the desired trait in the source organism (Schouten *et al.*, 2006). It is not yet clear whether this approach will be applied often in the future: one of the main obstacles for applying cisgenesis may actually be the fact that the resulting plant is a GM plant and will have to comply with regulations laid out for GM plants and products thereof. At the same time, a similar traditional crossing with this 'linkage drag' does not fall under the current definition of a GMO and will not be assessed as such. The (regulatory) consequences of these new developments in plant breeding are currently under debate as part of the review of European regulations.

Another development in the application of recombinant DNA techniques is the use of homologous recombination. Homologous recombination is a common phenomenon in plants and serves as a DNA repair mechanism, but in the future it may also be applied as a means for directing genetic alterations (Terada *et al.*, 2007). It provides plant breeders with the possibility to direct the integration of genetic constructs into the plant genome, contrary to the current random integration of the introduced genetic elements in GMOs. It has, however, proven difficult to achieve homologous recombination in plants. It has furthermore been shown

that the genomic position of the gene to be altered to a large extent determines the number of successful homologous recombination events (Swoboda *et al.*, 1994; Filkowski *et al.*, 2004). This effect may in practice reduce the general applicability of the approach in plant breeding schemes. On the other hand, new innovative approaches may come to aid in this respect: Nanto *et al.* (2009) describe a site-directed integration (SDI) system for *Agrobacterium*-mediated transformation in tobacco that allows integration of a single copy of a desired gene into a predefined target locus by recombinase-mediated cassette exchange (RMCE).

In this respect, the use of Zinc Finger Nucleases (ZFNs) to drive site-directed DNA integration into GM and native gene loci is another recent development (Zeevi *et al.*, 2008). Both homologous recombination and ZFNs can be used for site-specific insertion as well as site-specific mutagenesis. Zinc fingers are highly specific DNA-binding motifs. Together with a non-specific nuclease domain, they deliver a double-stranded break at a targeted site in the genome (Moeller and Wang, 2008). ZFNs were used to target GM reporter loci in tobacco following homology-directed repair (Cai *et al.*, 2009). Experiments involving green fluorescent protein gene fragments and a *pat* selectable marker gene reconstituted a fully functional gene in both cases. The ability to apply a DNA double strand break at specific genomic locations in host cells substantially increases the frequency of targeted integration by up to 10% (Cai *et al.*, 2009). The method has been described in more detail by Tovkach *et al.* (2009) where genome editing in plant cells was further elaborated. Specific mutations in the acetolactate synthase genes ALS *SuRA* and *SuRB* are known to confer resistance to imidazolinone and sulphonylurea herbicides. Mutations in the SuR loci of tobacco were introduced by Townsend *et al.* (2009) using designed ZFNs. The relatively high transformation / mutation frequency (exceeding 2%) indicated that the ZFN method is mature enough to efficiently make targeted mutations in plant genomes.

Finally, a development that goes even one step further is the upcoming field of synthetic biology. Synthetic biology is a new form of biotechnology, where the modification of existing, natural forms of life gradually transforms into the targeted engineering of new, synthetic forms of life (de Vriend *et al.*, 2007). What is new in synthetic biology is the emphasis on systems behaviour, designing DNA sequences exhibiting pre-described physiological responses (Kaznessis, 2007). Tools for synthetic biology

include recombinant-DNA techniques, pathway analysis and modification, genome-scale mathematical-modelling and *in silico* simulation, hypothesis and experimental tests, etc. (Fu, 2006).

An important characteristic of the so-called second generation of GM plants is that the plant products have advantages for the consumer rather than for the plant breeder and/or food or feed producer. Previous reviews (e.g. Robinson, 2002; Chassy *et al.*, 2004) provide an overview of developments in gene technological applications in plant breeding. An interesting development is the production of low-allergen (or even allergen-free) crop varieties. Examples are low-allergen wheat varieties (Schmidt, 2005) and hypo-allergen apples (Gao *et al.*, 2005; Hoffmann-Sommergruber, 2005), but also efforts are reported to achieve a reduction of the cyanogen content in cassava (Siritunga and Sayre, 2007) by enhancing cyanogen detoxification and cyanide volatilisation during processing.

At the same time, an increasing number of different applications of novel plant varieties for industrial products can be envisaged (McKeon, 2003). This includes altered composition of plants with respect to oils, starch, fibre, protein, and also includes plants that may produce specific chemicals, natural polymers, pharmaceuticals, decontamination agents, or fuels. The website of the International Service for the Acquisition of Agri-biotech Applications (ISAAA; www.isaaa.org) provides an up-to-date overview of commercialised GM varieties worldwide.

This brief overview of developments in the area of biotechnology shows that in the near future a more diverse range of biotechnology-derived products may move towards the market. Some GM plants will show large similarity with traditionally-bred plants, such as in the case of specific applications of cisgenesis, while other GM plants may harbour completely new metabolic routes or networks. It is clear that these latter developments may have important consequences for the safety and nutritional assessment of the resulting plants and products derived thereof.

3. REGULATORY ASPECTS OF GMOs: A GLOBAL PERSPECTIVE

In most nations, the production, use, and marketing of GMOs are bound by legal requirements, implying that GMOs can only be deployed if they have gained regulatory approval. The safety evaluation of the pertinent

GMO under consideration is an important part of the regulatory procedure. Companies or other institutions that want to introduce a GMO therefore have to submit data on the safety of this GMO to the competent authorities. This is commonly done in the form of a "safety dossier" containing a vast quantity of data and information on different aspects of the GMO and its safety. As discussed below, international harmonisation has been achieved on how to carry out the regulatory safety assessment and which data are needed for these assessments. Despite this international consensus on the safety of GMOs *per se*, the legislative measures and procedures pertaining to GMOs may vary widely between jurisdictions. Below, the specific safety requirements for the food use of GMOs in various jurisdictions of interest are highlighted.

3.1. International Treaties and Guidelines

Before discussing regulations at the national and regional level, it is worthwhile to take note of the various international treaties, standards, protocols, and guidelines that GMOs have to comply with in member nations.

With regard to food safety, the Codex Alimentarius commission (www.codexalimentarius.net), established through a joint effort on food safety and quality by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), is the main international reference point for food safety standards. Most nations are members of Codex Alimentarius, and it has developed numerous standards, protocols, codes of conduct, principles and guidance on issues pertaining to specific food safety hazards, such as veterinary residues and pesticides, and products (e.g. dairy products, animal feeds, and vegetable oils). Under the international treaty of the World Trade Organization on the application of sanitary and phytosanitary measures (SPS Agreement; www.wto.org/english/tratop_E/sps_e/spsagr_e.htm), Codex Alimentarius documents serve as the reference in disputes between nations over the safety and quality of internationally-traded foods.

Codex Alimentarius also established an international task force, "the Codex *Ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnology (TFFDB)", dedicated to the safety of foods derived through modern biotechnology, i.e. recombinant DNA techniques, which therefore include GM foods. The TFFDB has prepared various documents (available

at www.fao.org/ag/agn/agns/biotechnology_codex_en.asp), which have subsequently been adopted by the Codex Alimentarius commission, including general principles of risk analysis of foods derived through modern biotechnology, incorporating risk assessment, risk management, and risk communication, which were first published in 2003. Further guidelines for the safety assessment of foods derived from GM plants, micro-organisms, and animals have been adopted, in addition to those that have been drafted on food derived from nutritionally-improved GM crops, and on the safety of traces of GM products derived from GM crops that have been evaluated positively for safety elsewhere and that are present in imported food commodities (follow links at www.fao.org/ag/agn/agns/biotechnology_expert_en.asp).

In addition, the Cartagena Protocol on Biosafety (the "Protocol"; www.cbd.int/biosafety/protocol.shtml), which is part of the international Convention on Biological Diversity (www.cbd.int/convention/convention.shtml), prescribes the actions to be taken by national governments for the international movement of "living modified organisms" (LMOs). Currently, 159 nations have ratified, approved, accepted or acceded to the Protocol. Commodities containing GMOs and intended for direct use as human food or animal feed or processing (LMO-FFP) are also considered by the Protocol. If a Party to the Protocol approves a GMO for food and/or feed use, it must report this to the other Parties within 15 days after its approval. This reporting must be done through the Biosafety Clearing House (BCH, http://bch.cbd.int/), which keeps records of the internationally-traded GMOs notified, including the required information outlined in Annex II of the Protocol (e.g. contact details of the applicant and authority responsible for the decision, a description of the LMO as well as the gene donor and the recipient organism, the risk assessment, and suggested methods for the safe handling, storage, transport and use). In addition, documentation accompanying internationally-traded commodities containing LMO-FFPs should clearly identify that the shipment "may contain" LMOs and are not intended for intentional introduction into the environment, as well as a contact point for further information.

3.2. European Union

The European Union currently comprises 27 member nations, i.e. the majority of the European nations. The EU has various legal documents in place that have to be implemented by each Member State, including

legal texts on GMOs. This unified legislation across Member States has the purpose of ensuring a communitarian market, in which national laws cannot impede cross-boundary trade between Member States. There are different types of legal documents that can be adopted at the EU-level, including regulations and directives. A regulation is a legal text that has to be transposed literally and with immediate effect into Member States legislation, whilst a directive may be complemented with additional national provisions.

In the EU, GMOs are regulated, not by a single regulation, but by multiple legal texts covering a wide range of different aspects and applications of GMOs. It is worth noting that these legal texts focus on GMOs because of the process through which they have been obtained, i.e. genetic modification, instead of the outcome of this process. For example, Directive 90/219/EEC pertains to the contained use of GMOs, such as GM micro-organisms in fermentors within industrial facilities. Interestingly, an amendment to this directive by Directive 98/81/EC exempts "self-cloned" micro-organisms containing introduced DNA from the same or closely related and safe micro-organisms from its scope. This exemption has not been made for the "cisgenic" GM plants discussed above.

Directive 2001/18/EC pertains to the environmental introduction of GMOs, including the cultivation of GM plants for field trials and commercial purposes, as well as the import and processing of GMOs. This directive also provides a legal definition of GMOs, which is wider than the products of recombinant DNA techniques covered by the Codex Alimentarius guidelines, including, amongst others, fusions of non-crossable organisms obtained through protoplast fusion and the introduction of genetic material through micro-injection.

Regulation 1829/2003/EC pertains to the use of GMOs as food and feed, including all kinds of food and feed products, such as food ingredients, food additives, food supplements, feed additives, amongst many others. Such food and feed can derive from both imported commodities and GMOs produced within the EU, such as cultivated crops. In many cases, this will require an additional approval for the environmental release of the same GMO under Directive 2001/18/EC. No separate approvals are allowed for GM food or GM feed only, taking into account the possibility of accidental introduction of either one into the other's manufacture chain.

With regard to the safety assessments of GMOs carried out under these regulations, the European Food Safety Authority (EFSA; www.efsa.europa.eu/) plays a central role. This centralised and independent institution has the task to provide the European Commission, other EU authorities, and EU Member States with scientific advice on food safety, as set out in the "general food law" Regulation 178/2002/EC. The EFSA Scientific Panel on GMOs is composed of individual members selected on the basis of their expertise, and is supported by EFSA staff. This panel prepares scientific opinions based on the safety assessment of the application dossiers provided by the applicants, i.e. usually companies producing GMOs, such as seeds of GM crops. The Panel has published various guidance documents that aim to assist applicants in preparing such safety dossiers (found at www.efsa.europa.eu/en/gmo/gmoguidance.htm). Whilst being in line with the aforementioned Codex Alimentarius guidelines, these guidance documents are more extensive and can also serve as a useful reference for the various safety issues surrounding GMOs. In the case of applications for environmental releases of GMOs under Directive 2001/18/EC, a Member State can act as rapporteur for that particular GMO, possibly drafting an initial assessment. During the assessment procedures for environmental release, food, and feed, other Member State authorities have the possibility to submit their comments, which will be considered by the Panel. During the assessment of the dossier data, requests may arise for additional details or clarification, which will be relayed to the applicant. When the Panel reaches a conclusion, an opinion will be prepared that will be published by EFSA (available at www.efsa.europa.eu/en/gmo/gmoscdocs.htm) and sent to the European Commission. The Commission, in turn, will draft a regulatory decision to be considered by other EU institutions, including a regulatory committee and a council of ministers, both with representations from Member States.

It should be noted that approvals of GM food and feed under these regulations do not exempt the applicant from any other requirements for the particular class of food and feed components. For example, a food additive approved as GM food still requires a separate approval as a food additive under the pertinent EU regulations. The same holds true for approvals under other legal texts pertaining to GMOs, such as for the environmental release of a GM crop, which still may need a variety registration as a new crop. It should also be noted that herbicides that can be applied specifically to GM crops, including the assessment of the

potential toxicity of the herbicide and the derived metabolites formed in the GM plant, are covered by separate general legislation on pesticides, as in many other jurisdictions outside the EU.

In addition to the general labelling provisions for GMOs as set out in Regulation 1829/2003/EC, Regulation 1830/2003/EC provides more details on the issues of labelling and traceability. Labelling of specific food additives and flavourings is mandated by Regulation 50/2000/EC. The labelling of GMOs required by European Community regulations does not relate to safety issues *per se*, but aims to ensure that consumers have the freedom to chose between GM and non-GM food products. Interestingly, Regulation 1829/2003/EC requires that all food and feed products derived from GMOs be labelled, including products that do not contain any traces of transgenic DNA or proteins, such as highly refined vegetable oils. In order to be able to enforce this requirement, a documentary system for the traceability of GM foods and feeds is required by Regulation 1830/2003/EC. In each step of the food and feed chains, except for the final sales to the retail consumer, manufacturers, processors and other handlers of food have to keep records containing documents provided by their suppliers on the content of GMOs within the supplied products.

3.3 Non-European Jurisdictions

As noted above, EU regulations are process-oriented, in that the technology through which the product has been obtained determines whether it should be evaluated for safety and approved by regulatory authorities. The legislation in various other jurisdictions is similar to that in the EU in this respect. For example, Australia and New Zealand jointly regulate food safety including GMOs. Safety assessments of GM foods are carried out by Food Standards Australia New Zealand (FSANZ) and detailed reports published on its website (www.foodstandards.gov.au/consumerinformation/gmfoods/gmcurrentapplication1030.cfm). They also require labelling of GMO-containing foods, except for highly-refined products. For environmental releases, national regulations in each country apply, as well as, for example, any pesticide built into a GM plant, which falls under national pesticide regulations (Kleter and Kuiper, 2006).

The regulations in the USA and Canada are more product-focused, i.e. on the changes caused by the genetic modification in foods. GM foods fall under the American Federal Food Drugs and Cosmetics Act and the

regulatory oversight of the Food and Drug Administration (FDA), which has a voluntary consultation procedure in place for parties intending to introduce a GM food (see completed consultations at www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=bioListing). Any pesticides introduced into GM crops, such as endotoxins from *Bacillus thuringiensis* (*Bt*), should be evaluated as such by the Environmental Protection Agency (EPA; see registrations at www.epa.gov/oppbppd1/biopesticides/pips/pip_list.htm), whilst the potential of a GM crop to become a pest is considered by the Animal and Plant Health Inspection Service of the United States Department of Agriculture (APHIS; view petitions for non-regulated status at www.aphis.usda.gov/brs/not_reg.html). In Canada, both GM and substantially altered non-GM crops, including crops obtained through mutation breeding, are considered "plants with novel traits" and are thus assessed for their safety. Novel foods and feeds are also assessed for their safety prior to marketing in Canada under the Food and Drug Act and Feeds Act. In both the USA and Canada, there are no labelling requirements for GM foods (Kleter and Kuiper, 2006).

4. GENERAL ASPECTS OF GMO FOOD SAFETY ASSESSMENT

Although there are clear differences between the regulations pertaining to the authorisation of GMOs in different parts of the world, there is a remarkable consensus in the basic approach for the food and feed safety evaluation of GMOs and products derived thereof (Kok *et al.*, 2008). This basic approach consists of a comparative safety assessment of the novel GM crop with comparators that are already on the market. The comparators are crops that already have a so-called "history of safe use". To date, as a rule, the comparators are traditionally-bred, non-GM crop varieties, but it can be speculated that in the near future that comparators may also be GM crop varieties that have been on the market for a considerable amount of time and have thus also obtained a history of safe use. In the comparative safety assessment, the GM crop and the comparators are assessed in both phenotypic as well as analytical terms, with the aim to identify differences between the two (types of) crops. Subsequent safety assessment steps will then focus on any differences that have been identified, to determine whether these detected differences have any (unintended) toxicological and/or nutritional consequences. In practice, if differences have been identified, the subsequent steps of the food and feed safety assessment procedure is decided on a case-by-case basis, depending on the nature of identified difference(s).

Basic information that will be part of a safety dossier in all parts of the world is data on the molecular characteristics of the GM plant with respect to the inserted trait(s), such as data on all detectable inserts, both complete and partial, and the derived phenotype. In some jurisdictions, such as the EU, information must also be provided on the insertion site, i.e. the organisation of the inserted genetic material at the insertion site, as well as sequence information at both the 5' and 3' ends of the genetic insert and into the plant genome (EFSA, 2006). On the basis of the latter information, a GMO-specific identification method must also be supplied by the applicant. In addition, data on gene expression will need to be provided. In specific cases, if relevant, this may be extended to different developmental stages of the plant or to different plant parts. If molecular characterisation has identified the possibility of a fusion protein, it will need to be shown whether this protein is actually expressed in the plant parts to be marketed. Finally, molecular characterisation will have to show the genetic stability of the inserted sequence in subsequent generations.

The second approach to detect potential unintended effects of the genetic modification, besides the molecular characterisation, is the compositional analysis of the GM plant and a close comparator. The compositional analysis should comprise all key nutrients and anti-nutrients, including natural toxins, of the specific crop under investigation. To this end, the Organisation for Economic Co-operation and Development (OECD) has formulated Consensus Documents (www.oecd.org/document/9/0,3343,en_2649_34391_1812041_1_1_1_1,00.htm) for many of the major crops, describing the crop and the relevant constituents in relation to its food and feed safety. In general, this comparative analysis is done in a two-tiered approach. In the first step, the comparison is made between the GM plant and the direct comparator. If significant differences are detected, the biological relevance is assessed in the second step, by comparing the observed values with data compiled in specific databases, such as the International Life Sciences Institute (ILSI) crop composition database (www.cropcomposition.org), or documented in the scientific literature. For the final safety assessment, it is furthermore necessary to have an estimate for the intake of the GM crop as food or feed on the basis of available consumption data of the crop in general and for specific consumer groups.

In the final step of the food and feed safety assessment, all this information is compiled and assessed for its potential toxicological and nutritional relevance.

Important aspects of this overall assessment is the safety assessment of the newly-expressed protein(s), usually on the basis of a sequence comparison of the introduced protein(s) with known toxic proteins, and both *in vitro* as well as *in vivo* experimental data. If there are new constituents besides proteins, for instance as shown by the compositional analysis, these will also have to be assessed. This will be done primarily on the basis of known characteristics of the constituents, but if this generates insufficient information it may need to be supplemented by focused toxicity testing. In specific cases it may be necessary to test the whole GM plant or derived food/feed products in a toxicological assessment. In general, this will be a semi-chronic toxicity study with rodents. There are no detailed protocols on how these studies should be performed in the case of complex plant materials, however in Europe, EFSA has published a report on animal feeding trials with complex plant products (EFSA, 2008). The report concludes, amongst other things, that it is not recommended to perform animal feed trials for whole GM-plant-derived food or feed when equivalence with conventional food or feed has been established, and any further indications that unintended effects of the genetic modification are lacking. Moreover, the report recommends developing guidelines for conducting safety and nutritional trials in animals. The toxicological assessment will also include potential allergenicity of the newly-introduced proteins and the entire plant product. For assessing the potential allergenicity of the newly-expressed proteins, a "weight-of-evidence" approach is followed in which the outcomes of multiple studies are combined in order to reach a conclusion on the likelihood of the protein being an allergen or not. These studies may include: (1) the collection of data on allergies linked with the gene donor; (2) a comparison of the amino acid sequence of the newly-expressed protein to sequences of known allergenic proteins, using bioinformatics; (3) an assessment of the sensitivity of the new protein to degradation by protein-degrading enzymes; and, if applicable, (4) cross-reactivity with allergen-linked immunoglobulin E (IgE) antibodies from sera from patients that are allergic to a particular organism which either contains an allergenic protein similar to the newly-expressed protein, or which is the gene donor for the genetic modification. The nutritional assessment will assess the nutrient composition of the GM plant product(s) in combination with the biological efficacy of the individual components and the dietary intake of the entire product. In specific cases, where relevant questions can not be answered by the nutritional assessment, this may lead to the necessity of a post-marketing monitoring programme to obtain further data for the nutritional evaluation of the GM plant and its products.

5. RESEARCH DEVELOPMENTS

Within the SAFOTEST project funded by the EU, research was conducted with regard to the use of transcriptomics for the discovery and utilisation of biomarkers for the early detection of toxic symptoms in experimental animals. To this end, rat cDNA microarrays were prepared with probes for 3,000 rat genes. In addition, the response of cells to purified transgenic proteins was tested in *in vitro* cytotoxicity tests, in addition to the respective GMO (in this case, GM rice) being tested in animal studies in the same project. The outcomes of the 90-day feeding studies with three GM rice lines have been published. Based on the outcomes, however, the investigators conclude that further development of this methodology is still needed in order to enable a meaningful interpretation of the outcomes of the *in vitro* studies. For example, *in vitro* assay conditions should be further developed and standardised, and sufficient background data about the variability in gene expression should be generated (Knudsen and Poulsen, 2007).

5.1. Profiling Methods

As discussed elsewhere in this document, the molecular characterisation and compositional analysis of a plant usually includes the analysis of pre-defined parameters. These parameters are, for example, the expression of a particular modified gene or protein, as well as the level of a given chemical compound in a given tissue. Any identified changes may then be further investigated for their significance to the safety of the given plant.

Up to now, this "targeted" approach towards the characterisation of novel plants and their comparators has worked well for the comparative safety assessment of genetically modified plants (Kok and Kuiper, 2003). In fact, the aforementioned OECD Task Force's consensus documents on the compositional analysis of key biochemical and chemical compounds of novel crop varieties aim to contribute to the international harmonisation of this targeted approach. The targeted analysis recommended by these consensus documents includes macronutrients, micronutrients, anti-nutrients, and toxins. It is expected that future generations of GM crops will have undergone more complex modifications, such as changed or introduced metabolic pathways, leading, for example, to increased levels of nutritionally-interesting compounds. It can be envisaged that these complex modifications may not only lead to the intended effects

of the genetic modification, but also to unintended effects. Factors that may contribute to this include, for example, the alteration of levels of intermediate compounds that are at the crossing points between different metabolic pathways, broad substrate specificity of introduced enzymes, or the activation of latent enzymes or pathways.

In conventional breeding, selection on the basis of phenotype has been the conventional way to select out plants with unintended and undesirable effects. In a numbers of cases, conventionally-bred crops are also tested for changes at the metabolite level, such as for the level of glycoalkaloids in potatoes and erucic acid and glucosinolates in oilseed rape. Currently-employed targeted analytical techniques detect any changes in known nutritionally and toxicologically important compounds that may originate from such unintended effects. However, these targeted techniques will fail to detect changes in other metabolites that are not measured. Various efforts have therefore been made towards developing and establishing non-targeted approaches for the detection of unintended effects in novel varieties of crops. An important EU project in this area has been "Safefoods" (www.safefoods.nl) which has investigated the potential of non-targeted approaches, including the use of technically advanced "profiling" methods. With these holistic methods, profiles, such as a chromatogram or spectrum, are made of the components of a crop, without the necessity of identifying all compounds analysed. These profiles can then be compared between the novel plant and its conventional comparator, in order to identify differences, e.g. changes in the presence or intensity of peaks or signals. These changes can then be traced back to their origins, i.e. the compounds responsible for the observed change, after which the safety implications of these changes can be addressed. These methods and their potential applicability in the safety assessment of GMOs will be discussed in the following sections.

Profiling methods can be employed for the analysis of a crop at various levels of biochemical/ molecular organisation:

- Transcription, i.e. the measurement of mRNA derived from active genes. The collection of expressed genes referred to as the "transcriptome" and the methods to analyse it as "transcriptomics".
- Protein expression, i.e. the proteins translated from the expressed mRNA, which together constitute the "proteome" of a given organism, which can be measured by "proteomics".

- Metabolites, i.e. the chemical components derived from the actions of enzymes, transport proteins etc. Taken together, these compounds constitute the "metabolome" and its analysis is called "metabolomics".

It should be emphasised that these techniques will probably be employed as a supplement, rather than a substitute, to the targeted analytical approach. The application of these methods in the safety assessment of GM crops are discussed in more detail in previous reviews (e.g. Kuiper *et al.*, 2003; Chassy *et al.*, 2004; Kok *et al.*, 2008).

5.2. Transcriptomics

Various methods are available for simultaneously analysing the expression of large numbers of genes. A method that has been developed in the last decades is that of DNA microarray analysis. This type of analysis is based on the propensity of polynucleotides, such as RNA and DNA, to "hybridise", *i.e.* to form a double-stranded complex, with other, complementary polynucleotides. If a probe consists of a DNA molecule with a specific nucleotide sequence, hybridisation of a sample to this probe indicates the presence in the sample of a polynucleotide with a sequence that is complementary to the probe. Using such a probe, the presence of a specific gene in a sample can be established. Furthermore, in the case of transcriptome analysis, the signal intensity after hybridisation is proportional to the level of expression of that gene.

In the early years of the technique, cDNA microarrays dominated the market. These usually consisted of a collection of PCR products spotted on a glass slide. Probes were typically several hundreds of basepairs long and usually amplified from a cDNA library with universal primers. Typical sizes were microarrays with several thousand cDNA probes on them, or "spots" as they are frequently named due to the process used to deposit the DNA molecules on the glass microscope slides. However, in recent years, more and more genomes of species, including plants, have been fully sequenced. This has allowed the design of microarrays consisting of single-stranded oligonucleotides (oligos) representing the entire mRNA population (or transcriptome) of an organism. The length and sequence characteristics of each oligo can be designed to be the same, so hybridisation conditions are very similar for all spots. This has greatly enhanced the reproducibility of the microarray technique. Also, advanced manufacturing techniques have contributed to both greater reliability and more spots on an individual

microarray. Initially, academic efforts dominated the microarray market, while today two companies do (Agilent and Affymetrix), although some microarrays are produced through a license or another form of cooperation with one or more academic institutions. Affymetrix sells microarrays currently containing up to 6.5 million probes, while Agilent manages to synthesise 244,000 probes per microarray. Both companies offer complete or near complete genome arrays for several species, including plants, such as *Arabidopsis*, barley, cotton, maize, rice, tomato, wheat (both companies), *Brassica*, tobacco (only Agilent), citrus, *Medicago*, soya bean, sugercane and grape (only Affymetrix). Both companies require dedicated equipment, and microarrays from one company cannot be analysed with equipment from the other, while the Agilent microarrays are compatible with most in house microarrays. Plant genome microarrays are also available through academic institutions such as the Maize Oligonucleotide Array Project, a cooperation of the Institute of Genomic Research (TIGR), the universities of Wisconsin and Arizona and the National Science Foundation (NSF) of the USA, the NSF rice oligonucleotide array project, which involves TIGR, NSF, the University of California at Davis and Iowa State University, or for tomato arrays, the University of Arizona.

In the case of microarrays, the mRNA molecules or transcriptome, *i.e.* the "messengers" derived from expressed genes, are isolated from a biological sample, such as plant tissues. Through an enzymatic reverse transcription reaction, complementary DNA copies (cDNA) are made of these isolated mRNA molecules, which have the advantage of being more stable than mRNA. In addition, labels, such as fluorescent molecules, may also be attached to the cDNA during the reverse transcription phase, which allows for their detection upon hybridisation to the microarray. As the cDNA sample is added to the microarray, cDNA molecules with complementary sequences bind to the probes. After washing, the spots containing bound probes can be visualised under a microscope thanks to the fluorescent molecule attached to the binding cDNA, i.e. fluorescent spots will become visible.

As an alternative to whole genome microarrays, it may be useful for the purpose of the safety assessment to consider especially those genes that are of nutritional or toxicological relevance, such as genes that are involved in the biosynthesis of important nutrients and anti-nutrients. Various studies have already shown the applicability of microarray analysis

for studying the effects of stress conditions on the expression of genes in plants, sometimes revealing the unexpected activation of certain pathways (Destefano-Beltran *et al.,* 2006; O'Rourke *et al.,* 2007; Qin *et al.*, 2008; Aprile *et al.,* 2009; Ergen *et al.,* 2009). It is conceivable that this principle can also be applied to the studies of potential changes in gene expression in novel crop varieties.

The amount of data generated from microarray analysis requires the availability and use of appropriate tools for data processing and statistics. Commonly used techniques include, for example, "clustering", i.e. the grouping together of genes that show similar gene expression patterns, and multivariate analysis, such as Principal Component Analysis (PCA). For example, it may be found that a cluster of genes behaves differently in a GM crop as compared to its conventional counterpart. The identity of these genes may then provide an avenue for further investigations. Importantly, the conditions under which the microarray data have been obtained and processed may need to be standardised in order to promote their comparability with data from other experiments. Initiatives to create general, accessible databases for microarray data of a standardised format are currently ongoing, such as MIAME ("Minimum Information about a Microarray Experiment"; Brazma *et al.*, 2001; http://www.mged.org/Workgroups/MIAME/miame.html).

In addition, in order to put the results into perspective, the observed differences should be compared to background data on the influences of environmental conditions, developmental stage, diurnal variation, variety etc., since these factors may also have a profound effect on gene expression. For the routine analysis of novel crops with cDNA microarrays for regulatory safety assessment, method validation and standardisation are still needed. However, this technique may already be useful in the developmental phase of a novel crop variety, in order to identify those genes that may need further scrutiny in the further development. At present, a number of studies have already looked into the transcriptome analysis of GM crops. Baudo *et al.* (2006) used transcriptomics to analyse GM and conventional wheat varieties. They primarily investigated the bandwidth of natural variation and found the natural variation in gene expression patterns in conventionally-bred wheat varieties to be much larger than the variation between different GM lines. Another study on the bandwidth of natural variation as a basis for using transcriptomics as part of safety assessment protocols was performed

in tomatoes (Kok *et al.*, 2008). Batista *et al.* (2008) compared an irradiated stable mutant rice line and a GM rice line with their respective parent lines, using transcriptomics. They found that transcriptome changes were more frequent in mutagenised plants, compared to GM plants. Similarly, Cheng *et al.* (2008) found differences in gene expression with whole genome soya bean microarrays to be more frequent and more pronounced between conventional lines than between GM and conventional lines. However, they also found changes in cysteine protease inhibitor expression levels as a potential unintended effect in GM soya beans, although they also state that this could still fall within natural variation had more conventional soya bean lines been included in the study.

5.3. Proteomics

After transcription to mRNA, translation of the coding sequences located on the mRNA molecules into proteins is the following step in gene expression. It should be borne in mind that the levels of mRNA may not be linearly related to the levels of proteins, since the level of translation is regulated differently for different proteins. These factors include, for example, sequences on the mRNA molecule that influence translation (*e.g.* "enhancers"), as well as the binding of regulatory proteins influencing the translation process.

To display the proteins present within a biological sample, a commonly-used method is two-dimensional gel electrophoresis. During gel electrophoresis, protein molecules move through the pores of a gel under the influence of an electric field, the speed and direction of their movement depending upon the electric charge of the protein. The type of separation in each dimension in two-dimensional electrophoresis is different, i.e. the first is based on the iso-electric point (the pH at which the protein has no net charge), while the second is based on the molecular size of the protein (with smaller proteins moving faster, after denaturation and formation of complexes with charged detergent molecules that provide an evenly-distributed charge density to the protein). After separation, proteins in the gel can be visualised by staining, such as by silver or Coomassie Brilliant Blue. Stained spots containing the proteins of interest can be excised, eluted from the gel, and further characterised by proteolytic digestion, and analysis of the resulting peptide fragments by mass spectrometry.

A limitation of the current methodology is that the number of proteins that can be analysed in one assay is limited as compared to the total number of

proteins that could in theory be present within a sample. In addition, it is not amenable to automation for high-throughput analysis. New developments in the area of protein analysis include the use of microarrays of antibodies or ligands (*e.g.* enzyme substrates, binding proteins) that bind to specific proteins within a sample, as well as other high-throughput technologies using sensitive detection methodologies, such as surface plasmon resonance (reviewed by Tomizaki *et al.*, 2005), and directed mass spectrometry (Schmidt *et al.,* 2009; Vermeulen *et al.,* 2009).

5.4. Metabolomics

The next level after proteins are the "metabolites", chemical compounds present within a biological sample, such as those formed by the activity of enzymes or transported into cells by transport proteins. This type of analysis is closest to the compositional analysis described in the various consensus documents by the OECD task force on the key compositional parameters of novel crops varieties.

Various methods are described in the literature for metabolite profiling of crop plant varieties. In many articles they include the coupling of a chromatographic separation method to a universal detection method. For example, methods used are gas chromatography coupled to mass spectrometry (GC-MS), liquid chromatography coupled to ultraviolet diode-array detection (LC-UV DAD), and liquid chromatography coupled to nuclear magnetic resonance (LC-NMR). The choice for each of these methods may be based on the properties of the compounds to be analysed, as well as the appropriateness of the method to separate and detect these compounds. For example, GC-MS may be useful for the rapid analysis of small molecules. However, given the high temperatures under which compounds are separated during gas chromatography, liquid chromatography would be more appropriate for heat-labile compounds. The chromatograms and the spectra of separated fractions may provide the "profiles" that are used for comparison of the novel crop variety with another. Should differences be observed within these profiles, the compounds causing the different peaks or signals may then be further identified. For example, this may be done by comparison with databanks of spectra of known compounds, which are currently being developed.

Differences that are thus identified between a novel crop variety and its counterparts may not necessarily constitute a hazard *per se*. For example, Noteborn *et al.* (2000) applied LC-NMR to the analysis of GM tomatoes

exhibiting insecticidal (transgenic Cry1Ab protein) or prolonged ripening (suppressed polygalacturonidase) traits. By subtracting NMR spectra of the GM and control tomatoes from one another, the authors observed that the levels of glutamic acid and citric acid had been modified in the longer-ripening variety. However, based on a comparison with other non-GM varieties, the authors considered these changes to be due to background variability (Noteborn *et al.*, 2000). Similar studies have been performed by others (Catchpole *et al.*, 2005; Colquhoun *et al.*, 2006) that show that the possibilities of metabolomics are increasing, but still need standardisation and validation. Progress has been made in this area with regard to the analysis of GM crops within various scientific projects. In addition, efforts are underway for the standardised reporting of data derived from plant metabolomic studies.

5.5. Linkages Between the Various Methodologies

In addition, the use of these technologies at different levels may provide for indications of the "cross-talk" between these levels, i.e. expressed genes, proteins, and metabolites. For example, Tohge *et al.* (2005) applied transcriptomics and metabolomics to the model plant *Arabidopsis thaliana* that had been genetically modified such that a transcription factor was activated, leading to elevated levels of antioxidant flavonoids. The authors thus found that the increased formation of certain types of flavonoids could be linked to the activation of particular biosynthetic genes (glycosyltransferases).

Of the methods described above, transcriptomics and metabolomics are in an advanced stage of development, and especially for metabolomics, experiments with GM crops have shown the applicability of this approach. Besides the methods discussed here, other "omic" techniques are also in development, such as "glycomics" and "lipomics", focusing on the carbohydrate and lipid fraction, respectively. The use of these profiling methods allows for the indiscriminate analysis of all components present within a biological system, and also for possible changes caused by genetic modification, as a supplement to the existing targeted methods of analysis. Before these profiling methods can be used on a routine basis for molecular characterisation as part of the safety assessment of novel crop varieties, standardisation and validation of these methods is needed, as is the establishment of databases with background data on the natural variability of the crops tested.

6. FUTURE PERSPECTIVE

This overview of developments shows that the diversity of GM crop plants is likely to increase in the years to come. Current (inter)national regulations and guidance documents for the food and feed safety assessment of GMOs may need to be adapted in time to accommodate the new developments that may entail much more profound changes in the plant's metabolism. New profiling techniques, such as the "omics" technologies, may be further developed to assess effects of the modification on the plant's physiology in the light of what is known of the natural variation in current commercial crop plant varieties that have a history of safe use. The focus of the safety assessment can then be on those differences that fall outside of natural variation and may indeed have toxicological and/or nutritional implications.

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