

Toxicological assessment

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1 Introduction

Genetically modified plants can be altered for agronomic traits, such as virus-, insect- or herbicide tolerance, and for quality traits, such as enhanced or altered nutritional properties. The genes introduced into the plant may result in the synthesis of new substances that are conventional components of plant foods such as proteins, fats, carbohydrates, or vitamins that are novel in the context of the genetically modified crop. The genetic modification can also result in the synthesis of active substances, which are toxic for adverse exogenous organisms (such as pest organisms). Moreover, as a result from the activity of enzymes generated by the expression of the introduced DNA, new substances may include metabolites of endogenous origin or arising from the use of xenobiotics. Finally, because of technical reasons, the inserted genetic material consists often, in addition to the gene(s)-of-interest, of a molecular marker (in many cases an antibiotic² or herbicide resistance marker) and border-DNA (non-coding sequences). At present, transformation methods used result in random integration of the sequences in the genome of the plant, potentially leading to a series of unexpected changes. It is, therefore, clear that requirements for the toxicological assessment of genetically plants may vary from one kind of modification to another. The toxicological evaluation of a genetically modified crop expressing a biological biocide should comply with the requirements for the evaluation of the original organism used as biological biocide, whereas the toxicological evaluation of a well known nutrient may pose much less problems.

Substances expressed by the insertion of the defined DNA sequences should be subjected to the toxicological evaluation as described in this document. In case additional components or altered levels of existing components are present as an unintended result of the genetic modification (e.g. by the disruption, modification or silencing of active genes or the activation of silent genes), the assessment of these substances should follow the same criteria as those intended by the genetic modification. To date, toxicity assessment is relying on the traditional validated methods starting from some required tests, followed by others requested on a case-by-case basis. Moreover, testing remains accessible to new supporting developing methods. Profiling technologies such as metabolomics, proteomics and transcriptomics are considered as emerging technologies to extend the breadth of comparative analyses and to identify the need for further risk assessment. Should new technologies be applied, the expectation is then that all approaches are properly validated and that statistical analyses have been performed to the highest standard.

² Antibiotic resistance marker genes in genetically modified organisms which may have adverse effects on human health and the environment, will have to be phased out according to Directive 2001/18/EC (EU, 2001).

If the new substance modifies the fate of xenobiotic substances, e.g., biocides applied to the plant, the advice of other scientific committees is requested competent within the framework of Directive 91/414/EEC concerning the placing of plant protection products on the market (EU, 1991). Similarly, if the new substance claims therapeutic properties, the advice of the scientific committee competent within the framework of Regulation (EEC) 2309/93 for the authorisation of medicinal products for human and veterinary use (EU, 1993), should be requested.

The assessment of toxicological effects on non-target organisms other than humans and animals (farm or pet) is beyond the scope of this document.

2 Comparative analysis (molecular characterisation and compositional analysis)

Following the comparative approach as mentioned in Chapter I, section 6, the degree of substantial equivalence of the genetically modified food or feed with its conventional counterpart will determine the extent of further toxicological analyses.

2.1 Information on expressed DNA-sequences

Information should be provided as described in Chapter II on the new traits expressed in the plant, as well as on the possible occurrence of additional expressed products as a consequence of unintended effects of the genetic modification.

2.2 Marker-DNA

The safety of marker genes should be assessed, as would be the case for any other expressed gene product. If evaluation of the information as mentioned in Chapter II suggests that the presence of the marker gene or gene product presents a risk to human or animal health, the marker gene or gene product should not be present in the genetically modified crop. Alternative transformation technologies that do not result in clinically relevant antibiotic resistance marker genes should be encouraged in the future development of genetically modified organisms.

2.3 Allergenicity

All newly expressed proteins should be assessed for potential allergenicity as mentioned in Chapter IV. It is recommended to consider the outcome of the allergenicity assessment along with the toxicological evaluation.

3 Toxicological assessment

3.1 Study of literature

The notifier should perform a comprehensive literature review, discussing the absence of toxicity to humans and animals of the new substances (proteins and non-proteins). This literature search has to be clearly referenced (e.g. search methods used).

A comprehensive literature review has also to be performed concerning the toxic potential of the donor organisms used.

3.2 Screening for structure-activity relationship

The homology between the new substance (proteins and non-proteins) and known toxic components has to be screened by e.g. comparing the sequence of a protein with known protein toxins, using databases, predicted 3-D-structure, and amino acids sequence in regions of the protein that are critical to toxicological properties. If the newly expressed substance is an enzyme, the characteristics and biological effects of that enzyme should be described and considered. Database consultation and the use of computer-based amino acid search programs should be clearly documented and verifiable.

3.3 Exposure assessment

An estimation of the intake of the new substance (protein and non-protein) has to be carried out:

- per unit plant
- per unit food compound

in order to derive a daily intake (DI).

This predicted exposure to the substance of interest has to be compared with the potential exposure to the same substance, produced by the donor organism.

3.4 Toxicological tests

3.4.1 Requirements

Protein and non-protein new substances

Toxicological studies should be conducted using internationally agreed state of the art protocols, such as OECD/EU protocols, and be carried out according to the principles of Good Laboratory Practice (GLP).

If the substance of interest to be used in the toxicology tests is produced through molecular biology techniques in another organism (e.g. bacteria) than the plant, it has to be proven that the test substance is structurally, biochemically and functionally equivalent to the substance produced in the plant. Factors that should be examined to establish equivalence are: post-translational modification, full length amino acid sequence, amino acid composition/sequence, molecular weight (using the most appropriate methods), functional characteristics (immunorecognition in a Western blot assay and similar bioactivity).

3.4.2 Metabolic / toxicokinetic studies

Protein new substances

An *in vitro* digestibility assay in simulated gastric and/or intestinal fluids is required. It is important to note that resistance to *in vitro* digestion is not a toxicity endpoint by itself, but simply an indication that the protein warrants closer examination and perhaps different types of testing. On a case-by-case basis, also an *ex vivo* gastric fluid test (e.g. pig, cattle, dog) or *in vivo* models may be required.

Non-protein new substances

For new non-protein substances (e.g. those exerting biocidal or pharmacological effects), toxicity should be assessed on a case-by-case basis, depending on the identity and biological function of the substance in the plant and dietary exposure, and according to the appropriate guidelines and the conventional toxicological approach (including metabolism studies, studies on toxicokinetics). Also the use of human relevant testing systems for metabolic profiling will be encouraged, although most of these have not yet been validated.

3.4.3 Acute toxicity

Acute oral toxicity of the new substance (protein and non-protein)

Acute oral toxicity testing in laboratory rodents is required to confirm the lack of toxicity suggested by the literature reviews performed. A single dose study may also generate useful data to describe the relationship of dose to systemic and/or local toxicity. Further, these data can be used to select doses for repeated dose toxicity studies.

The maximum hazard dose test is generally adequate to address substance toxicity. It should be performed with a single high dose according to Directive 96/54/EEC (EU, 1996). The animals should be observed for 14 days to ascertain that no adverse sign occurs, and should then be subjected to gross necropsy. In the observation period, incorporation of parameters such as changes in skin and fur, eyes and mucous membranes, but also respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern should be considered in the design of these studies.

As an alternative approach, mammalian toxicity can be tested with the purified substance. The dose level to be used in the test should be selected from one of the four fixed dose levels, namely 5, 50, 500 or 2000 mg/kg BW according to test method B1 bis of Directive 96/54/EEC (EU, 1996).

As the doses of the substance to be administered may be quite large, it is possible that the quantities required cannot be reasonably purified from the genetically modified crop, necessitating a production in an alternative organism (see 3.4.1).

3.4.4 Irritation tests

Protein and non-protein new substances

Dermal and eye irritation testing should be considered, as workers may be exposed to pollen and crop dust.

Non-protein new substances

Photosafety testing (photoirritation), conducted consistent with the appropriate guideline protocols, should be considered on a case-by-case basis.

3.4.5 Sensitisation

Protein and non-protein new substances

Sensitisation testing (as workers are exposed to pollen and crop dust) should be considered along with the allergenicity assessment as described in Chapter IV.

To assess the potential for immunological sensitisation, tests of interest are the Guinea Pig Maximisation test or newer tests such as the Local Lymph Node Assay according to the OECD guideline No. 429 (OECD, 2002). Promising developing methods, such as the Mouse Intra-Nasal Test (Robinson et al, 1996; Robinson et al,

1998) and the Brown Norway rat model (Penninks and Knippels, 2001), although not yet validated, should be considered as complementary information.

The use of the i.p. route of administration of the substance may provide a more direct way to assess systemic toxicity and is also an alternative approach to evaluate the sensitizing potential of the test substance (Dearman and Kimber, 2001).

There may be special instances where an inhalation exposure (worker exposure) should be considered, for example when a protein related to an aeroallergen is found to be expressed in an anemophilous plant, with copious wind-born pollen, or when a significant exposure to grain dust is anticipated.

Non-protein new substances

Photosafety testing (photoallergy), conducted consistent with the appropriate guideline protocols, should be considered on a case-by-case basis.

3.4.6 Genotoxicity

In general, genotoxicity testing, whether it is performed or not, should always be well motivated.

Protein new substances

In vitro mutagenicity tests (bacterial mutagenicity tests, chromosome aberration tests including cytogenicity tests in cultured mammalian cells) should be performed on a case-by-case basis depending on the identity and biological function of the substance in the plant and dietary exposure.

Non-protein new substances

In vitro mutagenicity testing (bacterial mutagenicity tests, chromosome aberration tests including cytogenicity tests in cultured mammalian cells) is obligatory, unless convincing evidence can be provided to deviate from standard procedures.

The use of in vitro toxicological profiling, such as general cyto- and genotoxicity testing and the use of eukaryotic and bacterial stress gene assays, may become an important part in evolving strategies for a tiered approach (Noteborn et al., 2000).

3.4.7 Repeated dose toxicity - Oral route

Protein new substances

The 28-day oral toxicity test should be performed as a minimum requirement with a diet that properly nourishes the test animal (rodent), yet contains sufficient amounts of the new protein. The highest dose level should be the maximal achievable without causing nutritional imbalance, while the lowest level used should be comparable to the anticipated human intake.

The repeated dose study should include a tier I immunotoxicity screen according to the modified OECD guideline No. 407 (OECD, 1995) to establish dose-response characteristics and provide an indication for a Tier II screen. In other words, additional targeted investigations should be conducted if the new protein is suspected to act on specific organs or tissues including the endocrine, reproduction, or nervous system.

Non-protein new substances

Non-protein new substances, biological available metabolites, stable degradation products, should be evaluated according to the traditional toxicological approach on a case-by-case basis as provided by Directive 91/414/EEC (EU, 1991) or Directive 89/107/EEC (EU, 1988). This implies the submission of information on a core set of studies and the consideration of whether any other type of study might also be appropriate.

3.5 Whole-food toxicology testing

In principle, whole food testing should allow to answer the question whether unintended adverse effects (secondary pleiotropic effects) have been introduced following the genetic modification.

Whole food testing should be performed on a case-by-case basis in the following situations: 1) a completely new gene and/or transgenic organism; 2) organisms extensively changed as a result of biotechnology (metabolic pathway engineering); 3) new substances as anti-nutrients; 4) new substances without a clear threshold (e.g. bacterial toxins); 5) products with predicted high intake levels of the new protein; 6) non-rapidly degradable proteins (e.g. protease inhibitors, lectins) or crop plants with profoundly altered compositions (e.g. low glutelin rice, golden rice); 7) transgenic plants inactivating herbicides producing metabolised degradation products, which might be present in the plant; 8) the presence or altered level of phytotoxins (e.g., alkaloids).

The food product should be tested in the appropriate test animal, over an appropriate time span. For foods, a 90-day feeding study in rodents should be performed. For feeds, it is recommended that the study is conducted with a fast growing livestock species such as broiler chickens. Special attention must be paid to the avoidance of problems of nutritional imbalance (see also Chapter V).

Complete end-points (including biochemical, haematological, histological end-points) according to the OECD-guidelines for toxicity testing in analogy with irradiated foods are requested.

The food product tested should be in a similar form to that which would be consumed by humans or animals (e.g. processed foods).

The plants used should be grown under conditions that represent normal practice for the crop plant (e.g. pesticide use in case of herbicide resistance).

4 Background documents and references

(Draft) Codex Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants, March 2002.

<http://codexalimentarius.net/biotech/en/DNAPlant.htm>

Dearman RJ, Kimber I (2001). Determination of protein allergenicity: studies in mice. *Toxicology Letters* 120, 181-186.

EMA (1997). Preclinical safety evaluation of biotechnology-derived pharmaceuticals. Step 4, Consensus guideline, 16 July 1997. ICH Harmonised Tripartite Guideline, CPMP/IHC/302/95.

EU (1988). Council Directive 89/107/EEC of 21 December 1988 on the approximation of the laws of the Member States concerning food additives

- authorized for use in foodstuffs intended for human consumption. Official Journal of the European Communities, L40: 27-33.
- EU (1991). Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market. Official Journal of the European Communities L230: 1-32.
- EU (1993). Regulation (EEC) No 2309/93 of 22 July 1993 laying down Community procedures for the authorization and supervision of medicinal products for human and veterinary use and establishing a European Agency for the Evaluation of Medicinal Products. Official Journal of the European Communities L 214: 1-21.
- EU (1996). Commission Directive 96/54/EC of 30 July 1996 adapting to technical progress for the twenty-second time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (Text with EEA relevance). Official Journal of the European Communities L 248: 1 - 230.
- EU (2001). Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal of the European Communities L106: 1-39.
- EU (2003). Guidance Document for the risk assessment of genetically modified plants and derived food and feed. 6-7 March 2003. Prepared for the Scientific Steering Committee by the Joint Working Group on Novel Foods and GMOs.
- FIFRA Scientific advisory Panel Meeting June 6-7 (2000). Sets of scientific issues being considered by the environmental protection agency regarding: Session II – Mammalian toxicity assessment guidelines for protein plant pesticides. <http://www.epa.gov/scipoly/sap/2000/june/finbtmamtox.pdf>
- Background document for the FIFRA scientific advisory panel on mammalian toxicity assessment guidelines for protein plant pesticides, June 7, 2000. <http://www.epa.gov/scipoly/sap/2000/june/mammalttox.pdf>
- Nordic Council of ministers, Nordic working group on food toxicology and risk evaluation (1998): safety assessment of novel food plants. TemaNord 1998:591.
- Noteborn H, Lommen A, van der Jagt R, Weseman J (2000). Chemical fingerprinting for the evaluation of unintended secondary metabolic changes in transgenic food crops. Journal of Biotechnology 77, 103-114.
- OECD (1993). Safety evaluation of foods derived by modern Biotechnology: concept and principles (Organisation for Economic Co-operation and Development, Paris).
- OECD (1995). OECD Guidelines for the Testing of Chemicals. Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents (adopted 27th July 1995).
- OECD (2001). OECD series on testing and assessment No. 24: Guidance document on acute oral toxicity testing (ENV/JM/MONO(2001)4).
- OECD (2002). OECD guidelines for testing of chemicals. Test No. 429: Skin Sensitisation: Local Lymph Node Assay (adopted 24th April 2002).
- Penninks AH, Knippels LMJ (2001). Determination of protein allergenicity: studies in rats. Toxicology Letters 120, 171-180.
- Robinson MK, Babcock LS, Horn PA, Kawabata TT (1996). Specific antibody responses to subtilisin Carlsberg (Alcalase) in mice: Development of an intranasal exposure model. Fundam. Appl. Toxicol. 34, 15-24.
- Robinson MK, Horn PA, Kawabata TT, Babcock LS, Fletcher ER, Sarlo K (1998). Use of the Mouse Intranasal Test (MINT) to determine the allergenic potency of detergent enzymes: Comparison to the Guinea Pig Intratracheal (GPIT) test. Toxicological Sciences 43, 39-46.
- VIB (2001). Safety of genetically engineered crops. VIB publication, March 2001.
- WHO (1995). Application of the principle of substantial equivalence to the safety evaluation of foods or food components from plants derived by modern

biotechnology, Report of a WHO Workshop. World Health Organisation, Food Safety Unit, Geneva.

WHO/FAO (2000). Topic 2: Application of substantial equivalence data collection and analysis, Report of a joint FAO/WHO expert consultation on foods derived from biotechnology. World Health Organisation, Food Safety Unit, Geneva.

WHO/FAO (2000). Topic 6: Safety testing of food additives and contaminants and the long term evaluation of foods produced by biotechnology, Report of a Joint FAO/WHO expert consultation on foods derived from biotechnology. World Health Organisation, Food Safety Unit, Geneva.