

4. The framework for the safety assessment of foods derived from recombinant-DNA plants

Introduction

Recombinant-DNA plants developed for food purposes have undergone safety assessment procedures, as required by various national regulatory systems, since the early 1990s. The frameworks used to structure the safety assessments have been continually developed by international organizations and standard-setting bodies to ensure the safety of products and to promote trade through harmonized regulations. The concept of substantial equivalence was introduced by OECD in 1993 as a feasible way of structuring the safety assessment of recombinant-DNA plants (OECD, 1993). The concept was later adopted by the WHO and FAO as a useful starting point for the safety assessment of recombinant-DNA plants, and now represents an essential component of all regulatory frameworks on a global scale. The rationale behind the concept's utility and adoption is that recombinant-DNA plants developed for food purposes are considered to be essentially equivalent (chemically) to their conventional counterparts, with the exception of the few defined changes that have been introduced.

Extensive general biological characterization and toxicological testing are not therefore thought to be necessary because the comparative approach should reveal relevant biological differences. Safety assessment of recombinant-DNA plants developed for food purposes is nevertheless often based on additional extensive data collected on the immunological and toxicological properties of the new plant variety. The current framework of safety assessment is thus based on both the structured comparative basis enshrined in the concept of substantial equivalence and additional analyses of the toxicological and immunological properties of the intentional and potential unintentional effects of the introduced genetic modifications. The goal of the safety assessment of foods derived from recombinant-DNA plants is to examine the intentional and unintentional consequences of the specific modification on the food components and to establish a comparative safety level by drawing on the history of safe use of the conventional plant counterpart.

The Codex framework of the safety assessment

Based on the Codex “Principles for the Risk Analysis of Foods Derived from Modern Biotechnology” (2003), the Codex “Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants” was introduced in 2003. This training tool provides a detailed introduction to the conduct of food safety assessment based on the Codex framework for the safety assessment of GM foods (CAC/GL45-2003). The stepwise approach to the safety assessment described in the Codex Guideline is presented with reference to Codex guideline paragraphs 18–21.

CODEx GUIDELINE PARAGRAPH 18.

The safety assessment of a food derived from a recombinant-DNA plant follows a stepwise process of addressing relevant factors that include:

- A) Description of the recombinant-DNA plant;
- B) Description of the host plant and its use as food;
- C) Description of the donor organism(s);
- D) Description of the genetic modification(s);
- E) Characterization of the genetic modification(s);
- F) Safety assessment:
 - a) expressed substances (non-nucleic acid substances);
 - b) compositional analyses of key components;
 - c) evaluation of metabolites;
 - d) food processing;
 - e) nutritional modification; and
- G) Other considerations.

CODEX GUIDELINE PARAGRAPH 19. In certain cases, the characteristics of the product may necessitate development of additional data and information to address issues that are unique to the product under review.

CODEX GUIDELINE PARAGRAPH 20. Experiments intended to develop data for safety assessments should be designed and conducted in accordance with sound scientific concepts and principles, as well as, where appropriate, Good Laboratory Practice. Primary data should be made available to regulatory authorities at request. Data should be obtained using sound scientific methods and analysed using appropriate statistical techniques. The sensitivity of all analytical methods should be documented.

CODEX GUIDELINE PARAGRAPH 21. The goal of each safety assessment is to provide assurance, in the light of the best available scientific knowledge, that the food does not cause harm when prepared, used and/or eaten according to its intended use. The expected endpoint of such an assessment will be a conclusion regarding whether the new food is as safe as the conventional counterpart taking into account dietary impact of any changes in nutritional content or value. In essence, therefore, the outcome of the safety assessment process is to define the product under consideration in such a way as to enable risk managers to determine whether any measures are needed and if so to make well-informed and appropriate decisions.

The specific data requirements in the Codex Guideline for describing the features of recombinant-DNA plants are outlined in paragraphs 22–33, and are explained in further detail in the following sections.

Description of the recombinant-DNA plant

A recombinant-DNA plant is produced as a result of successful gene transfer (transformation) followed by stable integration of the recombinant-DNA (transgene) into the nuclear chromosome(s) or organelle genome(s) of the plant. The biotechnologist uses classical plant breeding techniques such as selfing to make this initial plant homozygous at the recombinant locus (loci). The recombinant-DNA can then be stably transferred through generations without segregation. The name of the progeny of such a recombinant-DNA plant is also defined by and refers to the initially produced recombinant-DNA plant. Each plant lineage produced from a successful transfer, plant regeneration and propagation is called an “event” or a “case”.

It is important for the safety assessor to understand the recombinant-DNA plant to be evaluated. For example, a clear understanding of the term “event” is essential to the application of a “case-by-case” safety assessment. Because each “event” represents a unique insertion site (or sites) of the recombinant-DNA (transgene), the resulting phenotypic properties of the regenerated recombinant plants are likely to differ. Thus, whereas the general biological

properties of the recombinant-DNA will be similar across different insertion “events”, potential unintentional effects on the host genome may vary because the insertions may cause different effects depending on their location and insertion number (see Box 4.1). An “event” may represent a plant with a single insert, or with multiple inserts transferred at the same time. For example, a single event may comprise several insertions of recombinant-DNA that encode both insecticide resistance and herbicide resistance, if these traits were transferred at the same time.

Plants containing recombinant-DNA from independent transfer events have “stacked” traits, and are often produced by crossing plant cultivars that each carry unique and well characterized “events”. In this way, more recombinant-DNA insertions (and “events”) that have been selected based

on good performance in their original recipient host can be assembled in a single new plant variety. Plants with stacked recombinant-DNA insertions (transgenes) are also evaluated for potential interactions occurring between the DNA insertions, as a part of the safety assessment.

The first two to three pages of the example dossier extracts provided with this tool contain relevant descriptive information to provide the safety assessor with the key characteristics and intended purpose of the recombinant-DNA plant.

CODEX GUIDELINE PARAGRAPH 22.

A description of the recombinant-DNA plant being presented for safety assessment should be provided. This description should identify the crop, the transformation event(s) to be reviewed and the type and purpose of the modification. This description should be sufficient to aid in understanding the nature of the food being submitted for safety assessment.



Description of the host plant and its use as food

Paragraphs 23–25 request information on the host plant and its known uses for food. A thorough knowledge of the non-modified host plant is necessary to apply the concept of substantial equivalence as a starting point for establishing safety. In the case of food safety assessment, this descriptive knowledge is critical for identifying the natural range and variation of key nutritional components, and of known toxicants (e.g. alkaloids in potatoes and tomatoes, curcubitacin in squash and zucchini), antinutrients and potential allergens. These compounds and their respective concentrations will vary between crops, cultivars and growth conditions in a similar way to those of conventional varieties.

Natural variations in such compounds are known as and described by the “baseline level”. Efforts are underway to establish databases that contain descriptive data on the range of baseline levels for key chemical compounds naturally present in crop plants. Crop plants naturally contain several thousand chemical compounds, of which many will cause undesired effects in toxicological tests if extracted singly and administered in high doses to experimental animals. It is therefore challenging to evaluate the biological effects potentially caused by minor variations or fluctuations in the levels of a particular plant compound. Therefore, knowledge of the natural variation in the baseline level of key compounds in conventional varieties of the plant is of great use in the safety assessment of complex data sets obtained from chemical analysis of recombinant-DNA plants.

Post-harvest processing of plant components may also alter the levels of particular plant compounds that are of nutritional value. Hence knowledge of the use, processing and consumption, as well as the properties, of the final product of the conventional food crop is important in establishing a sound basis for appropriate comparison with the foods derived from recombinant-DNA plants. Such information is provided in the example documents/dossiers.

An information source that provides extensive information on host plant biology is the OECD Consensus Documents. These consensus documents comprise technical information for use during the regulatory assessment of products of biotechnology. They focus on the biology of organisms (such as plants, trees or micro-organisms) or the introduced traits and can be accessed at: http://www.oecd.org/document/51/0,2340,en_2649_34385_1889395_1_1_1_1,00.html

Description of the donor organism(s)

Information about the natural history of the donor organism for the recombinant-DNA sequences is required, particularly if the donor or other members of

CODEx GUIDELINE PARAGRAPH 23. A comprehensive description of the host plant should be provided. The necessary data and information should include, but need not be restricted to:

- A) common or usual name; scientific name; and taxonomic classification;
- B) history of cultivation and development through breeding, in particular identifying traits that may adversely impact on human health;
- C) information on the host plant's genotype and phenotype relevant to its safety, including any known toxicity or allergenicity; and
- D) history of safe use for consumption as food.

CODEx GUIDELINE PARAGRAPH 24. Relevant phenotypic information should be provided not only for the host plant, but also for related species and for plants that have made or may make a significant contribution to the genetic background of the host plant.

CODEx GUIDELINE PARAGRAPH 25. The history of use may include information on how the plant is typically cultivated, transported and stored, whether special processing is required to make the plant safe to eat, and the plant's normal role in the diet (e.g. which part of the plant is used as a food source, whether its consumption is important in particular subgroups of the population, what important macro- or micro-nutrients it contributes to the diet).

CODEx GUIDELINE PARAGRAPH 26. Information should be provided on the donor organism(s) and, when appropriate, on other related species. It is particularly important to determine if the donor organism(s) or other closely related members of the family naturally exhibit characteristics of pathogenicity or toxin production, or have other traits that affect human health (e.g. presence of anti-nutrients). The description of the donor organism(s) should include:

- A) its usual or common name;
- B) scientific name;
- C) taxonomic classification;
- D) information about the natural history as concerns food safety;
- E) information on naturally occurring toxins, anti-nutrients and allergens; for microorganisms, additional information on pathogenicity and the relationship to known pathogens; and
- F) information on the past and present use, if any, in the food supply and exposure route(s) other than intended food use (e.g. possible presence as contaminants).

its genus normally exhibit characteristics of pathogenicity or toxin production, or have other traits that affect human health. If the donor organism contains known allergens particular caution must be exercised (Codex Guideline paragraph 26). When the food derived from recombinant-DNA plants contains genes from such sources, it is assumed that the novel gene product is allergenic unless proven otherwise. The assessment of allergenicity takes this aspect into account. In cases where the recombinant-DNA originates from sources with no history of allergenicity, the current approach to assessing allergenicity or toxicity relies primarily upon amino acid sequence comparisons and the stability of the novel protein to digestion and processing. Notably, this latter comparison is not made with respect to the conventional counterpart, but draws on a broad knowledge base regarding the biological properties of known allergens in food.

Currently, most commercially used DNA sequences inserted into recombinant-DNA plants are collected from commonly occurring soil bacteria and pathogenic plant bacteria and viruses, and hence they often have a known history in agriculture. Establishing prior human exposure to the recombinant-DNA source is useful as a starting point to identify possible toxic and allergenic properties of the gene products. Nevertheless, care should be taken in drawing safety inferences from such information, given the potentially altered expression levels, cellular locations and exposure routes of the recombinant-DNA derived proteins. Information is provided on the donor sources in the example documents/dossiers.

The OECD Consensus Documents also provide information on the biology of gene donors: http://www.oecd.org/document/51/0,2340,en_2649_34385_1889395_1_1_1_1,00.html

Description of the genetic modification(s)

The data requirements related to the genetic modifications serve two purposes: (i) to allow a detailed understanding of the resulting genetic insertions and their locations in the host plant; (ii) to allow unique identifiers to be constructed based on the event-specific insertion sites of the recombinant-DNA in the plant host genome (Codex Guideline paragraph 27). The latter information can be important both for the developer of a recombinant-DNA plant, as a means to ensure commercial distribution and use, and for some countries with mandatory food labelling requirements, to allow event-specific monitoring of recombinant-DNA in the food chain. For the biological safety assessment, it is important to have information on DNA insertion numbers and sites in order to evaluate the effect of the insertions on the host plant genome and to predict potential phenotypic changes. A detailed description of the molecular characteristics of the recombinant-DNA plant is required in order to demonstrate that the developer has critically analysed the plant and its products, including all introduced genes and expressed proteins. It should be noted that the recombinant-DNA plants have undergone extensive selective breeding subsequent to the initial gene transfer event and prior to seeking regulatory approval. Thus, the developer is likely to provide a range of data in the application dossier to demonstrate that the recombinant-DNA plant expresses only the intended phenotypic changes. As seen from the example documents/dossiers, extensive information on the characterization of the genetic modifications is provided.

The method by which the novel traits are introduced into the host plant determines, in part, the information required for the safety assessment of the genetic properties of the plant (Codex Guideline paragraph 28–29). The two principal methods for introducing new genetic

CODEX GUIDELINE PARAGRAPH 27. Sufficient information should be provided on the genetic modification to allow for the identification of all genetic material potentially delivered to the host plant and to provide the necessary information for the analysis of the data supporting the characterization of the DNA inserted in the plant.

CODEX GUIDELINE PARAGRAPH 28. The description of the transformation process should include:

- A) information on the specific method used for the transformation (e.g. *Agrobacterium*-mediated transformation);
- B) information, if applicable, on the DNA used to modify the plant (e.g. helper plasmids), including the source (e.g. plant, microbial, viral, synthetic), identity and expected function in the plant; and
- C) intermediate host organisms including the organisms (e.g. bacteria) used to produce or process DNA for transformation of the host organism.

CODEX GUIDELINE PARAGRAPH 29. Information should be provided on the DNA to be introduced, including:

- A) the characterization of all the genetic components including marker genes, regulatory and other elements affecting the function of the DNA;
- B) the size and identity;
- C) the location and orientation of the sequence in the final vector/construct; and
- D) the function.

material into plant cells are (i) *Agrobacterium*-mediated transformation and (ii) microprojectile bombardment.

(i) ***Agrobacterium*-mediated gene transfer.** *Agrobacterium tumefaciens* is a soil-borne phytopathogen that naturally uses genetic transformation processes to subvert the metabolic machinery of the host plant cell. It does so to divert some of the host's organic carbon and nitrogen supplies to the production of nutrients (opines) that can be specifically catabolized by the invading bacteria. Parasitized cells are also induced to proliferate. Crown gall tumour disease is a direct result of the incorporation of a region of transfer-DNA (T-DNA) from a large (150–250 kb) circular Ti (tumour-inducing) plasmid, carried by *A. tumefaciens*, into the host plant genome. An understanding of this natural transformation process, together with the realization that any foreign DNA placed between the T-DNA border sequences can be transferred to plant cells, led to the construction of the first vector and bacterial strain systems for plant transformation (for a review see Hooykaas and Schilperoort, 1992). Since the first record of a transgenic tobacco plant expressing foreign genes, great progress has been made in understanding *Agrobacterium*-mediated gene transfer at the molecular level. *Agrobacterium tumefaciens* naturally infects only dicotyledonous plants, although methods for *Agrobacterium*-mediated gene transfer into monocotyledonous plants have now been developed for rice (Hiei *et al.*, 1994; Cheng *et al.*, 1998), banana (May *et al.*, 1995), maize (Ishida *et al.*, 1996), wheat (Cheng *et al.*, 1997) and sugarcane (Arencibia *et al.*, 1998; Enríquez-Obregón *et al.*, 1998). A thorough analysis of the strategies for practical application of this method has been published (Birch, 1997). *Agrobacterium*-mediated transformation of plant tissue generally results in a low copy number DNA insertion, small numbers of rearrangements, and higher transformation efficiency than direct DNA delivery techniques such as microprojectile bombardment (Powlowski and Somers, 1996; Gelvin, 1998).

(ii) **Microprojectile bombardment-mediated gene transfer.** Microprojectile bombardment (also known as microparticle bombardment and biolistic transformation) is a technique used to deliver DNA directly to the host genome, and has proven to be useful for the transformation of plant tissues recalcitrant to *Agrobacterium* infection. In short, plasmid or linearized DNA containing the gene(s) of interest is fixed to tungsten or gold particles (microcarriers), which are delivered to host cells at high speed so as to penetrate the plant cells. In the cell, the DNA may separate from the microcarrier and become integrated into the host genome. Microprojectile bombardment can be used to transform tissue explants of most plant species as long as the transformed plant tissue can be regenerated to produce whole plants. As seen from the example documents/dossiers, details on the gene transfer method used and a molecular analysis of the resulting DNA insertion are provided as a standard part of the application for regulatory approval/notification.

Box 4.1. Mechanistic aspects of the transformation process relevant to safety assessment of recombinant-DNA plants

Length and copy numbers of DNA transferred.

It was assumed until 1995 that in *Agrobacterium*-mediated gene transfer the sequences between the left and right borders of the T-DNA were the only transgenic elements transferred to the recipient host. However, Ramanathan and Veluthambi (1995), Wenck *et al.* (1997) and Kononov *et al.* (1997) all demonstrated that plasmid backbone sequences beyond the borders of the T-DNA could be integrated together with the genes of interest. Experiments by Kononov *et al.* (1997) demonstrated that plasmid backbone sequences could be integrated into the host genome coupled with either the right or left border sequences, or as an independent unit unlinked from the T-DNA. The T-DNA can also integrate into the host genome in patterns other than as a single copy at a single site. Multiple copies in direct or inverted repeats and other complex patterns may also occur. The presence of multimeric T-DNA inserts, especially inverted repeat structures, is linked to the phenomenon of transgene silencing (Gelvin, 1998).

In particle bombardment-mediated gene transfer, the transgene integration pattern varies from the full-length introduced transgene to transgene rearrangements that differ in size from the full length insert, occasional concatenation of introduced plasmids carrying the transgene, and variation in copy number among the full-length and partial transgenic elements (Powlowski and Somers, 1996). The copy number of transgene insertions varies from 1 to 20 or more, in addition to the insertion of partial transgene fragments. Multiple copies usually cosegregate as a transgenic locus, indicating that the sequences are either integrated into tightly linked loci or into a single locus, rather than randomly integrated throughout all chromosomes (Powlowski and Somers, 1996). Molecular characterization of transgenic plants produced through microparticle bombardment has provided evidence of

extensive rearrangements of transgenic sequences (Powlowski and Somers, 1996). These rearrangements may be observed in Southern blot analyses as hybridizing fragments of a different size to the full-length DNA insert. Larger fragments are indicative of concatenation (head to head or head to tail)⁸. Larger than full-length fragments of transgenic DNA may also be caused by interspersal of inserted DNA with host DNA. For instance, Powlowski and Somers (1998) reported that each of thirteen transgenic oat lines transformed using microparticle bombardment had intact copies of the transgene, as well as multiple, rearranged, and/or truncated transgene fragments. The number of insertion sites varied from 2 to 12, and all fragments of the transgenic DNA cosegregated. The authors demonstrated that the transgenic DNA was interspersed with host DNA. This phenomenon has also been reported for rice (Cooley *et al.*, 1995).

Variation in gene expression levels based on insertion site.

For both gene transfer methods, plants transformed independently with the same plasmid will commonly have different levels of expression, a phenomenon that is not always correlated with copy number (Gelvin, 1998). Instead, differential expression of transgenes has been attributed by some to positional effects, in which the position of the DNA integration site in the host genome affects the level of transgene expression. However, other research has indicated that factors in addition to, or other than, the position of the site of integration also contribute to the level of transgene expression (Gelvin, 1998). This may be caused by the variable arrangements that transgene sequences may have in the host genome. Variable expression of transgenes, or gene silencing,⁹ is a documented phenomenon in transgenic plants.

Due to commercial business information claims, the exact technical and practical laboratory details of the recombinant-DNA transfer protocols are rarely provided in the application dossier. Some of the general mechanistic aspects of the transformation process that are relevant to safety assessment of the generated recombinant-DNA plants are explained in more detail in Box 4.1.

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⁸ Concatemers of the DNA insert may be detected by extensive Southern blot analysis involving digestion of the genomic DNA with a restriction enzyme that cuts at a single site within the transgenic element; multiple copies of the DNA insert will then be resolved by Southern blot analysis. Concatemers may be formed by homologous recombination of the transformed DNA or by blunt end ligation of cohesive ends produced by limited exonuclease activity. Smaller than full-length fragments are evidence of deletions and truncations.

⁹ Gene silencing can result from interactions between multiple copies of transgenes and related endogenous genes and is associated with homology-based mechanisms that act at either the transcriptional or post-transcriptional level (Matzke and Matzke, 1998). Silencing that results from the impairment of transcription initiation is often associated with cytosine methylation and/or chromatin condensation (Fagard and Vaucheret, 2000) while post-transcriptional silencing (cosuppression) involves enhanced RNA turnover in the cytoplasm (Matzke and Matzke, 1998). A third category of silencing has also been proposed for the consequences of positional effects, in which flanking plant DNA and/or unfavourable chromosomal location exert a silencing effect on the transgene (Matzke and Matzke, 1998). According to Matzke and Matzke (1998), this type of silencing reflects the epigenetic state of host sequences flanking the insertion site or the tolerance of particular chromosome regions to insertion of foreign DNA.



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