Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

20-Sep-2010

English - Or. English

ENV/JM/MONO(2010)41
Unclassified

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

Series on Harmonisation of Regulatory Oversight in Biotechnology No. 51 and Series on the Safety of Novel Foods and Feeds No. 22

CONSENSUS DOCUMENT ON MOLECULAR CHARACTERISATION OF PLANTS DERIVED FROM MODERN BIOTECHNOLOGY

This Consensus Document was jointly developed by both the OECD Working Group on the Harmonisation of Regulatory Oversight in Biotechnology and the OECD Task Force for the Safety of Novel Foods and Feeds.

JT03288491

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Series on Harmonisation of Regulatory Oversight in Biotechnology
No. 51

Series on the Safety of Novel Foods and Feeds
No. 22

Consensus Document on Molecular Characterisation of Plants Derived from Modern Biotechnology

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 2010

ABOUT THE OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 33 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in ten different series: **Testing and Assessment**; **Good Laboratory Practice and Compliance Monitoring**; **Pesticides and Biocides**; **Risk Management**; **Harmonisation of Regulatory Oversight in Biotechnology**; **Safety of Novel Foods and Feeds**; **Chemical Accidents**; **Pollutant Release and Transfer Registers**; **Emission Scenario Documents;** and the **Safety of Manufactured Nanomaterials**. More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (http://www.oecd.org/ehs/).

This publication is available electronically, at no charge.

For this and many other Biosafety and Novel Food & Feed Safety publications, consult the OECD's World Wide Web site (www.oecd.org/biotrack/)

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FOREWORD

The Working Group on the Harmonisation of Regulatory Oversight in Biotechnology and the Task Force for the Safety of Novel Foods and Feeds are implementing closely-related programmes of work at the OECD. Both of them develop science-based ***consensus documents***, which are mutually acceptable among member countries. These consensus documents contain information for use during the regulatory assessment of products derived from modern biotechnology.

In the area of plant biosafety (dealt with by the Working Group), consensus documents are being published on information on the biology of certain plant and animal species, selected traits that may be introduced into plant species, and environmental safety issues arising from certain general types of modifications made to crops, trees or micro-organisms.

In the area of food and feed safety (dealt with by the Task Force), consensus documents are focused on the nutrients, anti-nutrients or toxicants, the use as a food/feed and other relevant information on particular products. Reference is made to the concept of substantial equivalence, as it is considered that a comparative approach focusing on the determination of similarities and differences between the genetically engineered food and its conventional counterpart aids in the identification of potential safety and nutritional assessment.

This consensus document is the first one that was jointly developed by both the Working Group and the Task Force. It addresses the issues linked to molecular characterisation in a risk/safety assessment. It describes the background and purpose of molecular characterisation, transformation methods, inserted DNA, insertion site and expressed material, inheritance and genetic stability. A summary is provided under section V of the document.

Canada served as the lead country in the preparation of the document, in collaboration with a Steering Group composed of experts from eight national delegations and one observer organisation, and the draft has been revised on a number of occasions based on the input from other member countries and stakeholders.

The Working Group and the Task Force endorsed this document during a joint session held at the OECD Headquarters on 9 June 2010. The document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

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PREAMBLE

As plants and other organisms derived from modern biotechnology are being increasingly released worldwide, and derived food and feed products are being commercialised and marketed, the OECD member countries identified the need for detailed technical work aimed at establishing appropriate approaches to the safety assessment of these products.

The environmental risk/safety assessment of transgenic organisms is normally based on the information on the characteristics of the host organism, the introduced traits, the environment into which the organism is introduced, the interaction between these, and the intended application. The OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on identifying parts of this information, which could be commonly used in countries for environmental safety/risk assessment to encourage information sharing and prevent duplication of effort among countries. Biosafety Consensus Documents are one of the major outputs of its work. They are intended to be a "snapshot" of current information on a specific host organism or trait, for use during regulatory assessments. They are not intended to be a comprehensive source of information on everything that is known about a specific host or trait; but they do address the key or core set of issues that member countries believe are relevant to risk/safety assessment. This information is said to be mutually acceptable among member countries. To date, 40 Biosafety Consensus Documents have been published. They include documents which address the biology of crops, trees and micro-organisms as well as those which address specific traits which are used in transgenic crops.

Regarding the risk/safety assessment of food and feed derived from transgenic organisms, at a Workshop held in Aussois, France (OECD, 1997), it was recognised that a consistent approach to the establishment of substantial equivalence might be improved through consensus on the appropriate components (*e.g.* key nutrients, key toxicants and anti-nutritional compounds) on a crop-by-crop basis, which should be considered in the comparison. It was also acknowledged that the components may differ from crop to crop. The OECD Task Force for the Safety of Novel Foods and Feeds therefore decided to develop consensus documents on phenotypic characteristics and compositional data. These data are used to identify similarities and differences following a comparative approach as part of a food and feed safety assessment. They should be useful to the development of guidelines, both national and international and to encourage information sharing among OECD member countries. These documents, 19 published to date, are a compilation of current information that is important in food and feed risk/safety assessment. They provide a technical tool for regulatory officials as a general guide and reference source, and also for industry and other interested parties and complement those of the Working Group on Harmonisation of Regulatory Oversight in Biotechnology. They are mutually acceptable to, but not legally binding on, member countries. They are not intended to be a comprehensive description of all issues considered to be necessary for a safety assessment, but a base set for an individual product that supports the comparative approach. In assessing an individual product, additional components may be required depending on the specific case in question.

The present *Consensus Document on Molecular Characterisation of Plants Derived from Modern Biotechnology* constitutes the first result from a joint collaborative project implemented from 2003 to 2010 by the Working Group on Harmonisation of Regulatory Oversight in Biotechnology and the Task Force for

the Safety of Novel Foods and Feeds. Paragraph 10 of the document explains the scope of the text as follows:

> "*The purpose of molecular characterisation is to inform the risk/safety assessment of plants derived from modern biotechnology. Such characterisation provides knowledge at the molecular level of the inserted DNA within the plant genome*[1]*, the insertion site and the expressed material (ribonucleic acid [RNA] and proteins), and may provide information on intended and possible unintended effects of the transformation. Molecular characterisation of the genotype*[2] *contributes to a rigorous assessment of the potential impacts of transformation on the food, feed and environmental risk/safety of a recombinant-DNA plant. It assists in the prediction of the phenotype and the phenotype will ultimately determine whether the recombinant-DNA plant poses any risk/safety concerns."*

The Consensus Documents are of value to applicants for commercial uses of transgenic organisms, regulators in national authorities as well as the wider scientific community. As each of the documents may be updated in the future as new knowledge becomes available, users of Consensus Documents are encouraged to provide the OECD with new scientific and technical information, or opinions regarding the contents, and to make proposals for additional areas to be considered. A short pre-addressed questionnaire is attached at the end of this document that can be used to provide such comments to the OECD.

The published Consensus Documents are also available individually from OECD's website (http://www.oecd.org/biotrack) at no cost.

[1] Genome includes genetic material from both the nucleus and organelles.

[2] Genotype is defined as the genetic constitution of an organism.

SECTION I – BACKGROUND

A. Molecular Characterisation and Risk/Safety Assessment

1. Molecular characterisation is one component of the science-based multi-disciplinary approach used in food, feed and environmental risk/safety assessment of plants derived from modern biotechnology. The molecular characterisation of these plants is used to gain an understanding of the genetic material introduced and expressed in them. The purpose of this document is to explain the scientific basis underlying the application of molecular characterisation to the food, feed and environmental risk/safety assessment of these plants.

2. This document is meant to inform a risk/safety assessor on the use of molecular characterisation data and information, which is one component of an overall risk/safety assessment. The document does not discuss which data and information should be considered by the competent authority conducting the risk/safety assessment because the use of the data and information considered may depend on the type of risk/safety assessment being performed as well as characteristics of the product. The document does not provide an exhaustive list of analytical techniques that may be used for molecular characterisation. Where examples of analytical techniques are given, these serve only to provide a better context for an aspect of molecular characterisation discussed and do not imply that specific techniques are recommended or necessary.

3. Modern biotechnology has been defined as "the application of a) *In vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or b) Fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection," in the Cartagena Protocol on Biosafety (SCBD, 2000) and by the Codex Alimentarius Commission (Codex, 2003a).

4. Notwithstanding the fact that plant varieties produced through all techniques, including conventional breeding methods, can pose risks, the scope of this document will be limited to plants produced using recombinant-DNA (rDNA) techniques and direct injection of nucleic acid into cells or organelles, referred to herein as recombinant-DNA plants[3]. More specifically, this document will examine the transformation process and vectors used during transformation; the genetic material delivered to the recipient plant; and the identification, inheritance and expression of the genetic material in the recombinant-DNA plant.

5. This document focuses on the subset of recombinant-DNA plants intended for commercialisation, unconfined or full release that is subject to risk/safety assessments.

6. For context, this subset of recombinant-DNA plants, subject to regulatory evaluation, has typically passed through a post-transformation screening and selection process. The development of new recombinant-DNA plants begins with the production of a large number of transformants (Padgette

[3] Other terms such as genetically modified plants, genetically engineered plants, transgenic plants and transformed plants are often used interchangeably with the term recombinant-DNA plant. For the purposes of this document, the term recombinant-DNA plant will be used specifically as defined in paragraph 4.

et al., 1995; Zhou *et al*., 2003; Heck *et al.*, 2005). Plants derived from the initial transformants are cultivated over several propagation cycles in order to identify those plants that stably express and inherit the intended phenotype[4] while maintaining desirable agronomic characteristics such as growth characteristics, fertility and yield. This screening and selection process helps developers identify plants exhibiting pleiotropic effects resulting from the transformation process. With each successive propagation cycle, crop developers discontinue development of plants that have unexpected or undesired traits. This process results in the selection of recombinant-DNA plants intended for commercialisation, unconfined or full release; the risk/safety assessment is performed on these recombinant-DNA plants.

B. National and International Experience

7. Many national authorities with a history of regulating products of biotechnology have put in place standards and procedures for the pre-market assessment of recombinant-DNA plants and the products derived from them. The expertise and experience developed at the national level have been shared in a number of intergovernmental forums such as the Organisation for Economic Co-operation and Development (OECD), the World Health Organization (WHO) and the Food and Agriculture Organization (FAO). The scientific principles and approach to risk/safety assessment, developed through consultation at the international level, are currently applied by regulatory agencies around the world. This document complements existing guidance developed by national authorities and international organisations in this area.

8. In the context of environmental risk/safety, several guidance documents have been developed that focus on an approach to evaluating environmental risk/safety, such as the *Safety Considerations for Biotechnology: Scale-up of Crop Plants* published by the OECD (1993). In addition, many other OECD documents, developed through consensus of the member countries, have provided the basis for environmental risk/safety assessment of recombinant-DNA plants.

9. In the context of food risk/safety, the Codex Alimentarius Commission, under the Joint FAO/WHO Food Standards Programme, has adopted several documents developed by the Codex *Ad Hoc* Intergovernmental Task Force on Foods Derived from Biotechnology, including the *Principles for the Risk Analysis of Foods Derived from Modern Biotechnology* (Codex, 2003a) and the *Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants* (Codex, 2003b). In the context of feed risk/safety, the OECD has published *Considerations for the Safety Assessment of Animal Feedstuffs Derived from Genetically Modified Plants* (OECD, 2003). In addition, many other OECD documents, developed through consensus of the member countries, have provided the basis for food and feed risk/safety assessment of recombinant-DNA plants.

C. The Purpose of Molecular Characterisation

10. The purpose of molecular characterisation is to inform the risk/safety assessment of plants derived from modern biotechnology. Such characterisation provides knowledge at the molecular level of the inserted DNA within the plant genome[5], the insertion site and the expressed material (ribonucleic acid

[4] Phenotype is defined as an observable characteristic or trait of an organism that is determined by interactions between its genotype and the environment, and may include but is not limited to physical, morphological, physiological and biochemical properties.

[5] Genome includes genetic material from both the nucleus and organelles.

[RNA] and proteins), and may provide information on intended and possible unintended effects of the transformation. Molecular characterisation of the genotype[6] contributes to a rigorous assessment of the potential impacts of transformation on the food, feed and environmental risk/safety of a recombinant-DNA plant. It assists in the prediction of the phenotype and the phenotype will ultimately determine whether the recombinant-DNA plant poses any risk/safety concerns.

11. As it is generally considered by regulatory authorities, and in international consensus-building exercises, molecular characterisation encompasses a number of discrete considerations, including:

- The transformation method

  A description of the transformation method, together with a detailed description of any DNA sequences that could be potentially inserted into the plant genome;

- The inserted DNA, the insertion site and expressed material

  A description of the inserted DNA, including any genetic rearrangements, deletions or truncations that may have occurred as a consequence of the transformation, and the RNA and/or proteins expressed from the inserted DNA in various plant tissues and/or at different times during plant development; and

- Inheritance and genetic stability

  This addresses not only inheritance of the inserted DNA but also stability (*e.g.* translation or transcription) over multiple propagation cycles.

12. Molecular characterisation of the inserted DNA may be relevant in predicting possible unintended effects relevant to risk/safety, but it is not typically the primary means to detect such unintended effects. Other components of the risk/safety assessment including allergenicity and toxicological assessment of new substances (*e.g.* proteins, metabolites), changes in the levels of nutrients and anti-nutrients and of endogenous toxicants and allergens, or changes in plant fitness are integral for detecting unintended effects relevant to risk/safety.

13. Molecular characterisation for food, feed and environmental risk/safety assessment of recombinant-DNA plants is based on methods that target specific sequences and expressed products. New profiling technologies can provide information on many components at a particular level of biochemical/molecular organisation (*e.g.* transcriptomics – RNA; proteomics – proteins). While many of these new profiling technologies are under development, they are not as yet applied by national authorities in risk/safety assessment of recombinant-DNA plants. However, such technologies may serve as supplementary tools in risk/safety assessment in the future, provided they are sufficiently developed and validated. The potential applications of profiling technologies in the risk/safety assessment as well as the challenges associated with such applications have been discussed in several reviews (*e.g*. Kuiper *et al.*, 2003; Chassy *et al*., 2004) and are not addressed further in this document.

14. For context, unintended effects could arise from any form of plant breeding. For recombinant-DNA plants, these unintended effects may be due to the disruption of genomic sequences by the insertions, the action of transformation-induced genomic deletions and rearrangements, including within the inserted DNA, or pleiotropic effects caused by the new trait. Unintended effects may result in off-types that would be eliminated during the post-transformation screening and selection process. While both recombinant-

[6] Genotype is defined as the genetic constitution of an organism.

DNA plants and conventionally bred plants, including those generated using techniques of mutagenesis, may be evaluated and selected for agronomic and morphological traits, typically most conventionally bred plants do not undergo a risk/safety assessment comparable to that performed for recombinant-DNA plants.

15. In conclusion, molecular characterisation is considered an important part of risk/safety assessment; however it is only one component in the overall approach to risk/safety assessment. Molecular characterisation complements other components of the risk/safety assessment, such as environmental, chemical, nutritional, allergenicity and toxicological data to compare the recombinant-DNA plant with its appropriate comparator. Of interest for the risk/safety assessment is whether plant transformation could inadvertently increase the potential toxicity or allergenicity of the recipient plant, alter its nutritional quality, have negative environmental impacts or confer other undesirable traits. The totality of the available information relevant to risk/safety enables regulatory authorities to determine if a recombinant-DNA plant meets appropriate risk/safety standards.

SECTION II - TRANSFORMATION METHODS

A. Introduction

16. Transformation is the process of inserting DNA sequences of interest into a plant genome. Different transformation methods are available and each method has associated characteristics that could influence the inserted DNA sequences that are integrated into the plant genome. For instance, the integration process could lead to rearrangements, deletions or multi-copy insertions as well as the insertion of 'other' sequences originating from either plasmid (vector) or chromosomal DNA. The presence of these 'other' DNA sequences is relevant to risk/safety assessment in so far as such sequences may result in the presence of new substances in the recombinant-DNA plant and may also lead to altered levels of RNAs and proteins. In this section, focus is put on DNA integration that might occur as a result of the particular transformation method employed.

17. Various methods are available for introducing DNA into the plant genome (reviewed by Hansen and Wright, 1999). The most commonly used bacterial-mediated plant transformation methods employ disarmed *Agrobacterium* spp. Other plant-associated bacteria outside the *Agrobacterium* genus might become important in plant transformation (Broothaerts *et al.*, 2005). Direct transformation methods include particle bombardment (also termed biolistics) and electroporation. Alternative methods (*e.g.* microinjection, electrophoresis) have been specifically designed for recalcitrant plant species or specific target tissues (Hansen and Chilton, 1996; reviewed by Rakoczy-Trojanowska, 2002). This section will focus on the most widely practiced transformation methods.

B. *Agrobacterium*-mediated Transformation

18. During *Agrobacterium*-mediated transformation, a DNA region, termed T-DNA, flanked by short specific DNA stretches (*i.e.* T-DNA borders), is transferred and integrated in the plant genome (for review see Gelvin, 2003). Besides the T-DNA border sequences, virulence (*vir*) genes play a key role in the processing, export and integration of the T-DNA from the bacterium to the plant. In addition to their naturally *cis*-acting function, Vir proteins have been shown to be able to act in *trans*. Based on the latter finding, the so-called binary vector system, comprising i) a plasmid containing the DNA construct[7] flanked by T-DNA border sequences, and ii) a disarmed helper plasmid delivering the *vir* gene functions, has been developed. In order to disarm helper plasmids, T-DNA regions are removed. The binary vector system is nowadays most frequently applied in *Agrobacterium*-mediated transformation (Hellens *et al.*, 2000).

19. The *Agrobacterium* strain and helper plasmid used can be identified, and if previously uncharacterised a description can be provided. Information can also be provided on how the helper plasmid used was disarmed. In addition, the plasmid containing the DNA construct can be described. This information will reveal DNA sequences potentially transferred.

[7] For the purposes of this document the term 'DNA construct' refers to the DNA intended for insertion into the plant genome.

20. *Agrobacterium*-mediated transformation of plant tissue usually results in a low copy number of the DNA construct at a single insertion site. In some recombinant-DNA plant varieties reaching commercialisation T-DNAs have been found to be inserted as tandem repeats (direct or inverted in structure) at a single locus (reviewed by Smith *et al.*, 2001). Integration of incomplete T-DNA sequences is also occasionally seen. Integration may be accompanied by several types of rearrangements of the DNA construct (duplications, inversions and interspersion with plant DNA) and of plant genomic DNA at the insertion site (duplications, inversions and translocations). The insertion of plasmid backbone sequences from outside the T-DNA borders is also sometimes observed (reviewed by Smith *et al.*, 2001), either with the right or the left T-DNA border sequences or as an independent unit unlinked from the T-DNA (Kononov *et al.*, 1997). Further consideration of the risk/safety assessment of these phenomena is given in Section III.

C. Direct Transformation

21. Direct transformation of plant cells involves introducing the DNA sequences of interest directly to plant cells with the use of various techniques (*e.g.* particle bombardment, electroporation) that allow transport of the exogenous material across the cell wall and cell membrane. There is a possibility of introducing other DNA sequences not intended for transfer such as bacterial chromosomal DNA, depending on the purity of the DNA used for transformation. A description of the vector DNA, its preparation and its purity can be provided to reveal DNA sequences potentially transferred.

22. Direct transformation can be used with plant species not amenable to *Agrobacterium*-mediated transformation to successfully introduce new traits (see Taylor and Fauquet, 2002). Single integrants may be obtained if minimal expression cassettes (promoter, open reading frame and terminator) are used (Fu *et al.*, 2000). Particle bombardment may lead to insertion of multiple copies of the DNA construct (in direct or inverted repeat structure) at a single or multiple loci (Jackson *et al.*, 2001; reviewed by Smith *et al.*, 2001). Multiple copies of the DNA construct at a single insertion site may have short stretches of plant genomic DNA interspersed between them. In some cases, the introduced DNA may have undergone deletions or rearrangements, such as concatamerisation (reviewed by Smith *et al.*, 2001). Vector backbone DNA might also be present in recombinant-DNA plants produced using whole plasmids or in cases where purified expression cassettes were used for transformation and the expression cassettes were not sufficiently purified.

D. Conclusions

23. A description of the transformation method employed provides information about the DNA sequences potentially transferred to the plant genome and can be valuable for identifying changes to the plant in order to focus subsequent aspects of the risk/safety assessment.

SECTION III - INSERTED DNA, THE INSERTION SITE AND EXPRESSED MATERIAL

A. Inserted DNA and Insertion Site

24. In a risk/safety assessment, the analysis of the inserted DNA can be used to characterise the genotype arising from the transformation. Data defining whether deletions and/or rearrangements have occurred in the DNA construct or at the insertion site can be used to identify whether there may be potential effects other than the intent of the original transformation. In this section, information on the inserted DNA and the changes at the insertion site resulting from the transformation are discussed.

25. It should be noted that in this section the analysis of the inserted DNA is considered to be part of an assessment where the inserted DNA is stably inherited in recombinant-DNA plants intended for commercialisation, unconfined or full release, as discussed in paragraph 6.

Integration and copy number

26. Insertion of a DNA construct can either occur in the nuclear plant genome or in the genome of organelles, such as chloroplasts. Information on whether an insertion is located in the nucleus or an organelle can inform the environmental risk/safety assessment with regard to the potential dispersal of the gene of interest in relation to the reproductive biology of the recombinant-DNA plant. If the inserted DNA is located in the chloroplasts, it will most likely only be inherited maternally [most higher plants transmit their chloroplast DNA (predominantly) maternally rather than through pollen dispersal (Bock, 2007)]. Inserted DNA will be inherited both maternally and paternally when located in the nucleus. Molecular analysis and inheritance studies can provide information on the location of the inserted DNA (see also Section IV).

27. Depending on the transformation method used, the number of insertion sites might vary. In addition, there may be multiple copies of the DNA construct at each insertion site (see also Section II). Although plants with a single copy of the DNA construct are typically selected, in some cases plants with multiple copies of the DNA construct may be more efficacious as they result in higher expression levels. Copy number may influence gene silencing; however, copy number may not be as relevant as the homology of the introduced DNA to endogenous genes (Flavell, 1994).

28. Using appropriate controls, experimental data (*e.g.* Southern blot analysis) may reveal information such as the number of insertion sites, the copy number at each site and the genetic elements (*e.g.* promoters, enhancers) that have been inserted.

Presence of plasmid backbone sequences

29. Integration of DNA vector backbone sequence into the plant genome can occur with both *Agrobacterium*-mediated and direct transformation methods (see also Section II). Incorporation of DNA vector backbone sequences may be important if it results in the expression of additional proteins (for discussion see paragraph 33) or alters endogenous gene expression. Therefore, Southern blots of genomic DNA may be probed with DNA sequences from vector backbone(s) to determine if these elements have been inserted.

Organisation of transforming DNA and sites of insertion

30. The DNA used for transformation may be rearranged during the process of integration into the plant genome. Sequence analysis, polymerase chain reaction (PCR) analysis of the inserted DNA and Southern blotting are techniques that can be used to identify such rearrangements. If experimental results indicate a complex insert, such as one with rearrangements or deletions, further analysis may be useful to characterise the inserted DNA for the purposes of determining whether new substances may be present in the plant that could be relevant to the phenotype of the plant. These rearrangements may not necessarily be significant with regard to food, feed and/or environmental risk/safety.

31. T-DNA integration into an endogenous gene's coding or regulatory sequence and deletions or rearrangements of plant genomic DNA at the insertion site may cause loss of endogenous gene function or alteration of endogenous gene expression. This may result in changes in the plant which may or may not be significant with respect to risk/safety. Analysis of the regions flanking the inserted DNA may be used to determine if the DNA construct has been inserted in an endogenous gene's coding or regulatory sequence, and for the identification of any potential effects on plant gene function. The ability to analyse changes at the insertion site regarding the loss of plant gene function is, however, often compromised by lack of knowledge of most gene functions. Characterisation of insertion sites could inform the subsequent analyses for unintended effects that are part of the agronomic, phenotypic and compositional assessment of the plant (as discussed in paragraph 12).

32. New open reading frames (ORFs) might be formed as a result of transformation, potentially leading to the production of new proteins. DNA sequence analysis of the regions spanning the inserted DNA-genomic DNA junctions may reveal the presence of new ORFs as well as the presence of regulatory sequences upstream or downstream of the new ORF.

B. Expressed Material

33. Expression of the inserted DNA is taken into account in order to evaluate the risk/safety of the new gene products on food, feed and the environment. Expression of vector backbone sequences and new ORFs may also be considered. Data obtained through molecular analysis should reveal whether the inserted vector DNA can be transcribed and translated. If potential new ORFs are identified, bioinformatics tools can assist to determine the likelihood of RNA formation, the possibility for transcription and translation to occur and the amino acid sequence of the putative new protein. If it is found that new proteins are likely produced, their potential impact on risk/safety should be fully characterised. The risk/safety assessment of any new protein is outside the scope of this document.

34. In some cases the intended goal of the insertion of the DNA construct is to suppress or down regulate the transcription of an endogenous target gene. In these cases, protein expression of the endogenous target gene will be reduced or inhibited. In some cases gene silencing constructs may also influence, as an unintended effect, the transcription or translation of other endogenous genes sharing significant sequence similarity.

Transcription and translation

35. Successful transfer of a DNA construct into a new plant variety does not necessarily mean the construct will be expressed (Gelvin, 2003). Several factors can influence the level and stability of expression of the inserted DNA. The copy number of the insert, the structure of the inserted DNA (*e.g.* presence of inverted repeats) and the insertion site have been shown to affect transcription (Flavell, 1994**;** Gelvin, 1998; Matzke and Matzke, 1998**)**. Moreover, where and when the inserted DNA is actively

transcribed depends, in part, on the promoters used (*e.g.* tissue-specific promoters may limit expression to desired tissues), the developmental stages (*e.g.* flowering, seed setting) of the plant and the environment in which the recombinant-DNA plant is grown (Bregitzer and Tonks, 2003; Zhu *et al*., 2004).

36. Expression of the inserted DNA can be determined by use of either nucleic acid techniques such as northern blotting to detect recombinant RNA or by antibody-based methods such as western blotting to detect protein encoded by the inserted DNA. When performing analyses to characterise the expression of the inserted DNA, care should be taken to ensure that the conditions used for analysis (such as the tissues examined and the growth conditions used) are relevant to the risk/safety assessment. Once identified, the expression products from the inserted DNA can be characterised and assessed for risk/safety.

37. Expression of the inserted DNA in relevant tissues and under relevant environmental conditions is taken into consideration when assessing exposure and is considered as part of the subsequent risk/safety assessment. The stable integration in the plant genome does not imply that inserted DNA expression would, nor should, be expected to occur at steady state levels through the life cycle of the recombinant-DNA plant. Analysis of plant tissues at key developmental stages for proteins encoded by the inserted gene would reveal the amount of proteins produced at those developmental stages relevant to the risk/safety assessment, such as whether the protein is present in food and feed, or at which developmental phases environmental exposure will be most significant (*e.g.* expression of the protein in pollen).

Post-translational modification

38. Following translation, a protein can undergo further modifications. Identifying and characterising the proteins encoded by the inserted gene(s) can provide information useful in confirming that the substances expressed are those that the developer intended to express. Characterising these proteins can create a link to the history of safe use, where relevant, by showing that the proteins expressed *in planta* are not meaningfully different from the proteins when expressed in their native hosts. This is necessary in order to ensure that the data and information about the proteins in their native hosts that may be referenced in the risk/safety assessment of the recombinant-DNA plant are relevant. Algorithms to identify potential post-translation modification such as N- and O-glycosylation sites, Ser/Thr/Tyr phosphorylation sites and (iso)prenylation have been developed (Blom *et al*., 2004; Maurer-Stroh and Eisenhaber, 2005). Protein analysis studies applying specific staining methods, radioactive labelling studies or matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) may demonstrate the presence of the predicted post-translational modifications (Jensen, 2000) that are deemed relevant to the risk/safety assessment. While some of these post-translational modifications might impact on the risk/safety of the protein, these considerations fall beyond the scope of molecular characterisation but should be considered as part of the overall risk/safety assessment.

C. Conclusions

39. The analysis of the inserted DNA can be useful in the characterisation of the genotype arising from the transformation. Deletions and/or rearrangements that may have occurred in the DNA construct or at the insertion site may result in effects other than the intent of the original transformation. Analysis of expressed products is important for the assessment of the phenotype; however, it must be considered in the context of a complete risk/safety assessment.

SECTION IV - INHERITANCE AND GENETIC STABILITY

A. Introduction

40. Information regarding the inheritance and genetic stability of the inserted DNA is used to extend the conclusions of a risk/safety assessment conducted for a particular propagation cycle of the recombinant-DNA plant to subsequent genetic descendants. Therefore, information regarding the inheritance and genetic stability of the inserted DNA is important and necessary in the assessment of food, feed and environmental risk/safety.

41. Inheritance is defined as the pattern of transmission of genotype and phenotype into genetic descendants. The stability of a genetic modification is defined as maintenance of the integrity of the original structure and function of the modification over time and over propagation cycles. Genetic stability can be confirmed by conducting genotypic analysis at the insertion site and/or by phenotypic analysis for expression of the desired trait in the course of plant production and propagation.

B. Inheritance and Genetic Stability in Risk/Safety Assessment

42. Genetic stability and inheritance of introduced traits within and across propagation cycles are considered as part of the risk/safety assessment. Analysis of inheritance includes consideration of whether the inserted DNA is located on a nuclear plant chromosome or in plant organelles and whether it is transferred into offspring maternally or paternally. Demonstrating that the inserted DNA has been stably integrated into the genome provides some assurance that a risk/safety assessment performed on an early propagation cycle of the plant is applicable to future propagation cycles of the plant. For context, when selecting plants for commercialisation, unconfined or full release, developers typically look for plants in which the inserted DNA has been stably integrated into the genome.

Patterns of inheritance

43. In the case of insertion of the DNA construct into the nucleus, predictable patterns of inheritance are typically reflected in Mendelian segregation ratios for phenotype and genotype. Deviations from Mendelian inheritance are potential indicators of genetic instability, especially for chromosomal genetic modifications of the nuclear genome in diploid, sexual plants that form the majority of new plants typically encountered by regulators. However, the patterns of inheritance applicable to a particular plant species depend on the mechanisms of inheritance that exist for the subject plant species such as the reproductive strategy, the ploidy and whether nuclear or organelle genomes are involved.

44. Mendelian inheritance would not be expected for all asexual, vegetatively propagated plants, some polyploids and all genetic modifications of plastid or mitochondrial genomes. Such expected instances of non-Mendelian inheritance should not be interpreted as genetic instability.

Factors of genetic stability

45. As in all plants, genotypic change may occur over the course of mitotic or meiotic cell division and the transmission of genes into resulting progeny. Spontaneous mutations could occur due to errors in

base pair incorporation during DNA replication and chromosome doubling prior to mitotic cell division. The pairing of homologous chromosomes during meiosis can lead to crossing over, a recombination that may result in a new grouping of genes. The stability of the inserted DNA may also depend on the sequence and structure of the introduced or modified genes and on characteristics of the insertion site.

Methods to determine the stability of a genetic modification

46. The stability of a genetic modification may be analysed at the phenotypic and/or the genotypic level. The stability of phenotypic expression may be determined by trait characterisation or by analysis of sufficient samples, where appropriate, of RNA or protein expression. Some phenotypic traits (*e.g.* resistances) may be quantified under testing conditions with the intact plant. As with other plant genes, expression of inserted DNA will be influenced by the environment. This should be taken into account during a phenotypic consideration of stability. Changes in patterns of expression or expression levels can be quantified in a biochemical reaction mediated by an expressed enzyme or by detection of the expressed protein with specific antibodies (*e.g.* enzyme-linked immunosorbent assay [ELISA], western blot analysis).

47. The stability of a genetic modification at the genotypic level may be documented through comparative analyses of the structure of the genetic modification using techniques such as Southern blot, PCR or other types of genetic analysis of multiple plants within and across propagation cycles. Genotypic changes across propagation cycles in the recombinant-DNA plant should be considered in the context of the normal variation that occurs with plant breeding.

C. Conclusions

48. Inheritance and genetic stability can inform the food, feed and environmental risk/safety assessment. This information is important in extending the conclusions of a risk/safety assessment conducted for particular propagation cycles of the recombinant-DNA plant to subsequent genetic descendants.

SECTION V - SUMMARY

49. Molecular characterisation encompasses consideration of the transformation method employed, the inserted DNA and expressed material, and the inheritance and genetic stability of the inserted DNA. Molecular characterisation in and of itself is not a sufficient means of predicting the risk/safety of recombinant-DNA plants. However, molecular characterisation may be useful in focusing other components of the risk/safety assessment that assess the phenotype of the plant, such as characterisation of the levels of nutrients, anti-nutrients, endogenous toxicants or allergens, or changes in plant fitness. To date, the most appropriate available scientific procedures and technology have been used in the molecular characterisation of recombinant-DNA plants. Experience from the use of these procedures and technology form the basis of this document. Based on the current pace of technological advancement, it is expected that new methodologies may be applied to the molecular characterisation of recombinant-DNA plants should such technologies prove to have added value as a mechanism of hazard identification in food, feed and environmental risk/safety assessments.

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