

Project number: 120.72.667.01

Project title: Food and feed safety aspects of cisgenic crop plant varieties

Project leader: E.J. Kok

Report 2010.001

April 2010

Food and feed safety aspects of cisgenic crop plant varieties

T.W. Prins and E.J. Kok

RIKILT - Institute of Food Safety

Wageningen University & Research centre

Akkermaalsbos 2, 6708 WB Wageningen, the Netherlands

P.O. Box 230, 6700 AE Wageningen, the Netherlands

Tel +31 317 480 256

Internet www.rikilt.wur.nl

Copyright 2010, RIKILT – Institute of Food Safety.

The client is allowed to publish or distribute the full report to third parties. Without prior written permission from RIKILT – Institute of Food Safety it is not allowed to:

- a) publish parts of this report;
- b) use this report or title of this report in conducting legal procedures, for advertising, acquisition or other commercial purposes;
- c) use the name of RIKILT – Institute of Food Safety other than as author of this report.

This research was funded by the Dutch Ministry of Housing, Spatial Planning and the Environment.

Steering committee:

- H. Bresser (VROM: Ministry of Housing, Spatial Planning and the Environment)
- D. C. M. Glandorf (RIVM: National Institute for Public Health and the Environment)
- C. van Rossum (Medicines Evaluation Board, Novel Food Unit)
- R. van Ree (AMC, Experimental Immunology)
- J. Kooter (VU, Department of Genetics)
- J. Thio (LNV: Ministry of Agriculture, Nature and Food Quality)
- A. Vioria (VWS: Ministry of Health, Welfare and Sport)
- M. Bovers (COGEM: The Netherlands Commission on Genetic Modification)
- Foreign experts:
 - M. Schauzu (Federal Institute for Risk Assessment, DE)
 - J. Ruprich (National Institute of Public Health, CZ)
 - M. de Loose (ILVO: The Institute for Agricultural and Fisheries Research, BE)
 - A. Depicker (Ghent University, BE)

Distribution list:

All Members of the Steering committee (see above)

<p>This report from RIKILT - Institute of Food Safety has been produced with the utmost care. However, RIKILT does not accept liability for any claims based on the contents of this report.</p>
--

Summary

The present report documents the results of a research project, which has been initiated following a request of the Ministry of Housing, Spatial Planning, and the Environment (VROM), to assess the food and feed safety aspects of cisgenic plants and derived products. The focus will thereby be on the application of cisgenesis in crop plants. The research project consisted of the following parts:

- 1) a desk study that was carried out by experts of RIKILT;
- 2) discussions with national experts as represented in the steering committee of the project and international experts on the basis of draft reports;
- 3) an international web-seminar with other invited experts in the area of i) plant breeding and ii) food and feed safety of genetically modified organisms, with the purpose of further exchanging scientific views on identified food and feed safety aspects of cisgenic plant varieties.

This report presents the results of the discussions that identified food and feed safety aspects of cisgenic plant varieties in comparison to conventional varieties on the one hand and transgenic plant varieties on the other hand. It was concluded that on the basis of the general characteristics of cisgenic plant varieties, there is, from a food and feed safety perspective, no scientific basis for a general reduction of requirements for cisgenic crop plant varieties. At the same time there was large consensus that an optimal food and feed safety assessment for all new plant varieties should be based on the specific characteristics of the individual novel plant variety (product based), rather than on the categorisation of the new plant varieties and their derived products on the basis of (one of) the technologies used to obtain the new plant variety (process based).

Acknowledgements

The authors want to thank the Dutch Ministry of Housing, Spatial Planning, and the Environment (VROM) for financial support. The work was guided throughout the project by the steering committee: Ms H. Bresser, Ms D. C. M. Glandorf, Mr C. van Rossum, Mr R. van Ree, Mr J. Kooter, Ms J. Thio, Ms A. Vilorio and Ms M. Bovers. They are kindly acknowledged for their input. A special thanks is to the foreign experts, Ms M. Schauzu, Mr J. Ruprich, Mr M. de Loose and Ms A. Depicker, and Ms H. Bresser for chairing the project meetings. We are grateful to Mr G. Kleter for his contributions in the formulation of the project proposal and his skills to host the web-seminar.

Many thanks to the active participants of the web-seminar (Adrian Peres [Bayer Bioscience, BE], Andreia Zilio [Univ. of Florianopolis, BR], Anton Haverkort [PRI, NL], Bruce Hammond [Monsanto, US], Dany Morisset, [NIB, SI], Frans Krens [PRI, NL], Hans Bergmans [RIVM, NL], Henk Schouten [PRI, NL], Huw Jones [BBSRC, UK], Jiri Ruprich [National Institute of Public Health, CZ], Kevin Glenn [Monsanto, US], Marc de Loose [Univ. Gent, BE], Matti Sarvas [Nat. Inst. Health and Welfare, FI], Moisés Burachik [Direc. Biotechnology, AR], Patricia Fernandes [PPG Biotechnologia, BR], Piet Schenkelaars [Biotechnology Consultancy, NL], Preben Bach Holm [Aarhus Univ., DK], Wolfram Reichenbecher [Bundesamt für Naturschutz, DE]), and others that sent in their comments afterwards. It should be stressed that the content of this report does not necessarily reflect the opinion of the contributors to the web-seminar.

Contents

- Summary3**
- Acknowledgements4**
- 1 Introduction7**
- 2 Background8**
 - 2.1 Background information, general8
 - 2.2 Background information, safety assessment8
- 3 Approach10**
 - 3.1 Organisation of the study10
- 4 Cisgenesis - definitions11**
 - 4.1 Definition according to the EU Working Group on Novel Techniques11
 - 4.2 ‘Cisgenic-like’ approaches in scientific literature - definitions12
 - 4.3 Considerations on definition aspects (species, crossability, endogenous DNA)13
 - 4.4 Conclusions on aspects of definition14
- 5 Aspects of food and feed safety15**
 - 5.1 Current regulation of GMOs vs conventionally bred new plant varieties15
 - 5.2 Food and feed safety issues of cisgenic vs conventionally bred new plant varieties16
 - 5.3 Food and feed safety issues of cisgenic vs transgenic plant varieties18
 - 5.4 Other safety-related considerations18
 - 5.5 Conclusions safety aspects20
- 6 Discussion and conclusions21**
 - 6.1 Discussion21
 - 6.2 Conclusions23
- Annex I References25**

1 Introduction

The Dutch Ministry of Housing, Spatial Planning, and the Environment (VROM) has requested RIKILT to carry out a research project on the food and feed safety of cisgenic crop varieties. Cisgenesis is a form of genetic modification for which only sequences of the host organism or 'related' organisms are introduced. Cisgenesis is currently also one of the techniques being considered by a working group of the European Commission with regard to its status under the EU legislation on genetically modified organisms (GMOs). The Dutch Ministry of VROM is also actively involved in this European discussion and also liaises on this topic with the Ministry of Agriculture, Nature, and Food Quality, and with the Ministry of Public Health, Welfare, and Sports. The project was started in August 2009.

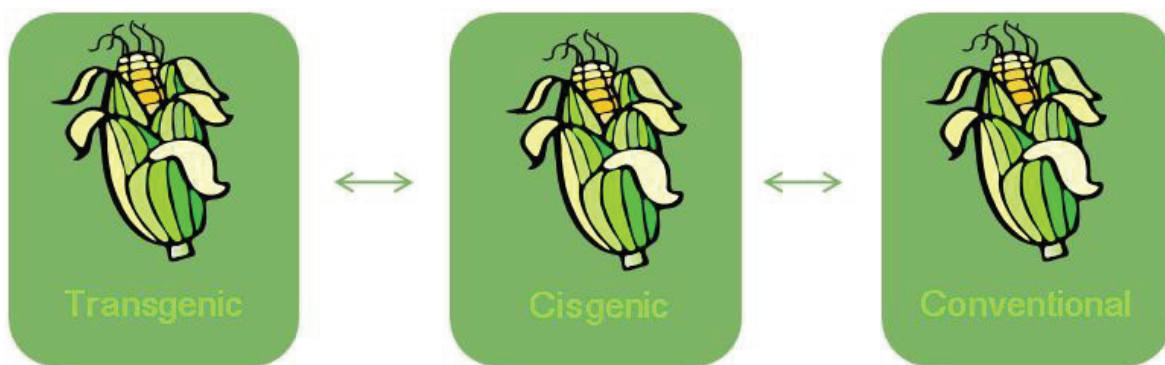


Figure 1. This project aims to compare aspects of food and feed safety of transgenic vs cisgenic vs conventional plant varieties.

The aim of the project was to identify and conclude about food and feed safety aspects of cisgenic genetically modified (GM) plant varieties in comparison to conventional varieties on the one hand and transgenic GM plant varieties on the other hand.

In this report the reader will find the following items:

- a description of background of the project;
- a description of the organisation of the study;
- a discussion part on food and feed safety aspects of the definition of cisgenic plant varieties;
- a concise overview of cisgenesis-like developments in the scientific literature;
- a discussion part on aspects of food and feed safety of cisgenic plant varieties;
- a final discussion on aspects of food and feed safety of cisgenic plant varieties;
- a number of overall conclusions.

2 Background

2.1 Background information, general

Since the first large-scale market introduction of genetically modified crops in 1996, the worldwide area planted with these crops has continuously grown, reaching 134 million hectares in 2009 (James, 2009). The majority of these crops are cultivated outside the European Union (EU), whilst harvested crops and derived products are imported into the EU after their approval. Examples of such genetically modified crops are herbicide-tolerant soybean and insect-resistant maize. These genetically modified crops are transgenic, which means that they have been created with different genetic elements from the same or other species.

Various genetically modified crops that are now in preparation (almost) exclusively contain DNA sequences that naturally occur within the same crop species, the so called 'cisgenic' crops. In the scientific literature no detailed information is yet available on novel cisgenic plant varieties.

Cisgenesis in apple was only briefly described by Gessler et al. (2009) who transformed the *HcrVf2* gene from *Malus floribunda* into commercial cultivars like Gala and Elstar. The authors make use of the selectable marker *nptII*, but remove this marker in a second phase. Some stakeholders assume that this type of modification can alleviate public concerns with regard to genetic modification because, unlike for transgenic GM crops, no boundaries among species are crossed. It is also assumed that this technique does not differ fundamentally from conventional crop breeding, through which, at least in theory, the same results can be obtained. Various scientific and corporate institutions in the Netherlands are working on the development of cisgenic crops, including a Phytophthora-resistant potato. The topic of cisgenesis has captured public attention in a number of ways, including a report by the Netherlands Commission on Genetic Modification (COGEM) on new genetic techniques for crop improvement (COGEM, 2006), societal gatherings, a political discussion within the Dutch Parliament, and a request by the Senate to verify within an EU context if exempting cisgenesis from the current EU GMO legislation (EU Regulation 1829/2003) or reduced requirements for the safety assessment of cisgenic GM crops is possible.

2.2 Background information, safety assessment

Before genetically modified organisms (GMOs) can be released on the market, they will have to be approved, both for their introduction in the environment and for their application as food and feed [Directive 2001/18/EC and/or Regulation (EC) 1829/2003, respectively]. Cisgenic GM crops fall under the definition of a GMO according to EU legislation and therefore would have to be evaluated for their environmental and food/feed safety. The applicant for an authorisation is requested to provide an extensive dossier containing data and information on the donor and host organisms and the genetic modification to demonstrate that the GMO in question does not have adverse effects on human health, animal health, and the environment. The assessment of food and feed safety is based on the internationally harmonized comparative approach, which has been enshrined in Guidance Documents published by the Codex Alimentarius Commission and the European Food Safety Authority (EFSA). The first step is the comparative analysis of molecular, agronomic and morphological characteristics as well as the chemical composition of the GMO and its conventional counterpart with a history of

safe food/feed use. Based on the outcome of this comparison it can be decided which further tests will be needed to assess the food/feed safety. The potential toxicity and allergenicity of newly expressed proteins, consistent unintended effects and horizontal gene transfer, as well as nutritional value of the GMO and/or the introduced elements are subject of further investigations.

Recently, two reports were published by COGEM (COGEM, 2006) and Plant Breeding, Wageningen UR (Jacobsen and Schaart, 2009), respectively, that considered the possible safety issues surrounding T-DNA borders. The underlying thought was that, during cisgenesis by means of T-DNA integration with the aid of *Agrobacterium tumefaciens*, these T-DNA borders would be the sole “foreign” elements that are introduced into the host’s DNA. The potential formation of an epitope (recognized by IgE anti-sera of allergic patients) by the theoretical amino acid sequences corresponding to the T-DNA was thus considered as the potential safety issue. Another issue of integrated T-DNA borders is, although considered to be unlikely, the potential formation of chimerical novel open reading frames. Moreover, the natural occurrence of sequences similar to T-DNA in plant DNA was confirmed, also with the aid of bioinformatics (Jacobsen and Schaart, 2009). Reference was also made to a previous COGEM opinion in which COGEM considered it highly unlikely that integration of T-DNA borders would cause environmental risks. Since no conclusions about the food/feed safety have been drawn in these previous reports, this current report will complete the risk assessment.

RIKILT has provided comments to the above mentioned risk evaluation of T-DNA borders as the principle aspect of the safety assessment of cisgenic GM plant varieties. Another point of interest was, for example, the safety aspects related to the definition of cisgenesis as used by the authors of the report on T-DNA borders, which would also allow for the use of sequences from wild relatives, the latter without having a history of safe use. Also, the safety assessment of cisgenic crops according to the approach applied to GMOs will not be limited to the T-DNA sequences but also considers, amongst others, the possible fusion products on the basis of plant-endogenous sequences and the cisgenic construct. The prior history of exposure to the newly expressed proteins in cisgenic GM plants could, on the other hand, possibly give rise to a less profound set of data requirements for the safety assessment. In summary, RIKILT concluded that no categorical statement can be made with regard to the possibly diminished safety concerns about cisgenesis (Kleter and Kok, 2009).

The present project has mapped, in more detail, the landscape of potential food/feed safety issues surrounding cisgenesis, with a view on possible relaxation of requirements with respect to safety data that need to be supplied by the applicant before market approval as is stipulated in EU Regulation 1829/2003. This has been done by comparing the food and feed safety aspects of cisgenic plant varieties with, on the one hand, conventionally bred plant varieties without the use of recombinant-DNA techniques and, on the other hand, transgenic GM plant varieties as are currently subject to EU Regulation 1829/2003.

3 Approach

3.1 Organisation of the study

This study comprises three different parts:

- 1) A desk study into the available knowledge on cisgenic crops, among others with regard to the types of modifications that have been introduced and the molecular changes that they cause in the DNA of the host organism, as well as the associated change(s) at the level of expression (mRNA, protein, metabolites). These changes will be considered from the perspective of what is already known about the changes that can occur during conventional breeding and transgenic modifications of crops. The desk research has also considered if there is a history of safe use of the elements introduced through cisgenesis (including the T-DNA borders), and also what safety aspects should be considered (similar to what is done during the comparative safety assessment of GMOs).
- 2) Extensive discussions with national experts, members of the steering committee and with international experts in the area of plant breeding and the food and feed safety assessment of complex food products during three subsequent meetings. Draft reports as prepared by RIKILT were the basis for these discussions.
- 3) An international web-seminar that was organised by RIKILT at Wageningen UR with >200 invited experts worldwide in the area of i) plant breeding and ii) food and feed safety of genetically modified organisms. The aim of the web-seminar was to further exchange scientific views on identified food and feed safety aspects of cisgenic plant varieties. Approximately 50 experts signed in for participation, of which 20 provided (interactive) feedback to the web-seminar presentations.

This project report has been compiled on the basis of the combined results of all parts of the project.

4 Cisgenesis - definitions

4.1 Definition according to the EU Working Group on Novel Techniques

It was decided during the first project meeting that this report would use the definitions of cisgenesis and transgenesis, as defined in the draft document by the EU Working Group on Novel Techniques (WGNT), in this report referred to as the WGNT-definitions:

Definition and brief description of cisgenesis and intragenesis based on the current scientific knowledge and current scientific literature

Cisgenesis is a genetic modification of a recipient species with a natural gene from a crossable - sexually compatible - organism (same species or a closely related species). Such a gene includes its introns and is flanked by a native promoter and terminator in the normal sense orientation.

Intragenesis is different from cisgenesis. This is the integration of an intragene. An intragene is commonly a hybrid gene and intragenesis involves the insertion of a reorganized, full or partial coding part of a natural gene frequently combined with another promoter and/or terminator from a gene of the same species or a crossable species.

Cisgenic plants can harbour one or more cisgenes, but they do not contain any transgenes. To produce cisgenic plants, any suitable technique used for production of transgenic organisms may be used. Genes must be isolated, cloned and transformed back into a recipient.

A comparison based on differences and similarities between established WGNT-definitions like transgenesis or traditional breeding can be made to place the concept of cisgenesis into perspective:

Cisgenesis vs. transgenesis:

Both make use of illegitimate recombination to insert a desired DNA sequence into a genome, resulting in the rudimentary deposit of at least one T-DNA border of *Agrobacterium* and possible effects by the recombinational insertion.

Cisgenesis vs. traditional breeding:

In both cases the resulting plants contain DNA sequences that are within the species-range of the recipient genome. Therefore, no new DNA, not belonging to the species' natural gene pool, is being introduced, but insertional mutagenesis may occur and the formation of new fusion proteins can not be excluded. Techniques that belong to 'traditional breeding methods' also comprise mutation breeding and translocation breeding, potentially inducing the same illegitimate recombinations as with cisgenesis (and transgenesis). Interspecific crosses would also not be allowed or at least be questionable within the concept of cisgenics.

An advantage of cisgenesis over traditional breeding is the lack of linkage drag (the unwanted genetic material that is introduced in the progeny besides the desired trait) that would have been introduced into the progeny when a normal crossing would occur. In a normal situation, a crossing would have to be made, followed by recurring back crossings to reduce the linkage drag in the selected germ line while maintaining the desired trait. As this linkage drag may also entail genetic sequences that may have consequences for the food and feed safety of the resulting product, for instance in the case of a conventional cross with a wild relative that has never been used for human consumption, it is clear that, in terms of food and feed safety, in this respect cisgenesis may have advantages over conventional breeding.

4.2 ‘Cisgenic-like’ approaches in scientific literature - definitions

In literature, different terms are used to describe the use of self-genes in transformation events. These are autotransgenesis (Beardmore, 1997; Nam et al., 2008), intragenesis (Rommens, 2004; Rommens, 2007; Russell and Sparrow, 2008), cisgenesis (Schouten et al., 2006; Schouten et al., 2006a) and variations thereof.

Figure 2 is published by Hanley (2009) and depicts three different types of plant genes in different shades of green. Hanley regards cisgenesis as the use of a construct in which the gene is derived from the same species and is flanked with the original flanking regulatory sequences. Intragenics allows shuffling of genes and regulatory sequences. Rommens’ definition of cisgenesis allows that the gene may be introduced by *Agrobacterium*, while Connor limits the definition to the use of a plant-derived intragenic vector system. Last, Hanley presents cisgenics as the generation of a transgenic organism where elements of the organism’s own genome are used instead of elements from other genomes (Hanley, 2008).

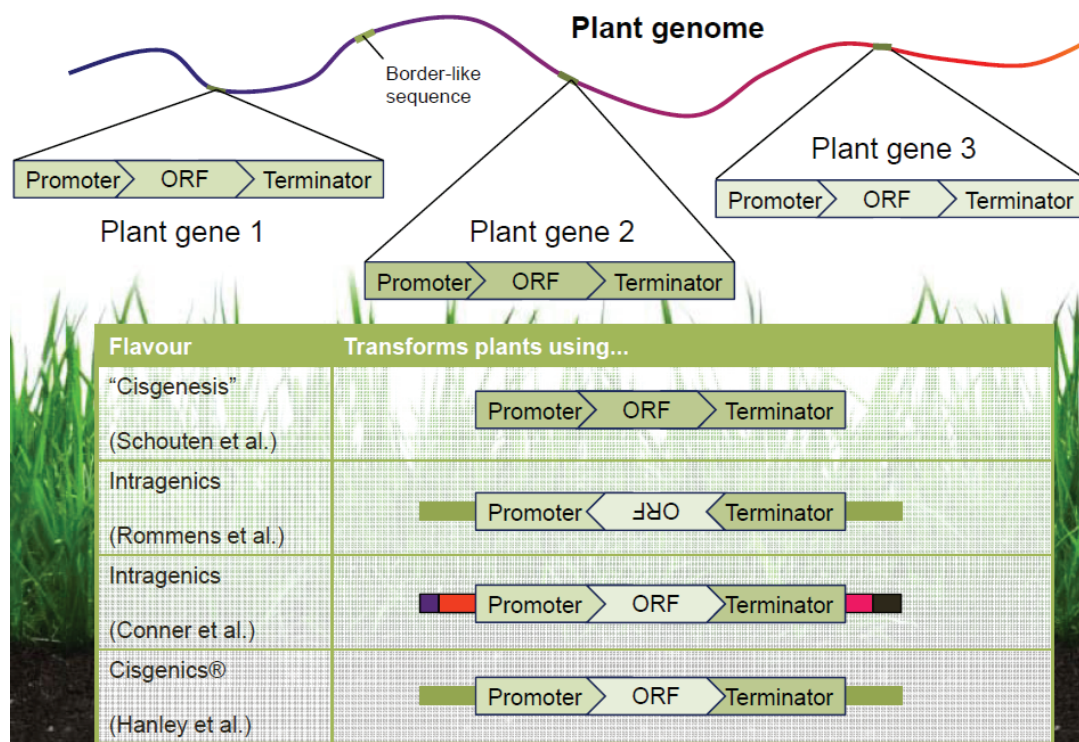


Figure 2: Open reading frame composition of different X-genic principles. Source: (Hanley, 2009)

It shows that different (interpretations of) definitions for cisgenesis and transgenesis are being used in literature. For further reading regarding different aspects of cisgenesis and intragenesis, the reader is referred to a selected set of literature (Jochemsen, 2000; Jacobsen and Schouten, 2007; Rommens, 2007; Rommens et al., 2007; Rommens, 2008; Russell and Sparrow, 2008; Benjamin et al., 2009; Strauss et al., 2009).

4.3 Considerations on definition aspects (species, crossability, endogenous DNA)

As can be seen in the former paragraphs, most cisgenesis-related definitions, including the WGNT-definitions, are broadly formulated and many details remain unspoken, such as:

- The use of endogenous promoters and terminators vs. novel combinations of genes and regulatory elements;
- Tissue-specific expression vs. redirection of the transcribed gene product to a different organelle or plant part;
- Usage of identical codon usage vs. the possibility to codon-optimize the gene;
- One single integration of the gene-of-interest vs. possible rearrangements/partial genes due to the transformation procedure;
- When a cisgene per definition originates from the same Species, Genus or Family, what happens when the classification of a species is shifted?;
- At what point should the definition be applied: does it refer to the intended modification or only to the modification that has actually taken place in the plant. With other words, if a cisgenic construct is transferred to a plant, but rearrangements occur that lead to new genetic combinations in the plant, would this still fall under the same definition?

These unclaritys would be crucial if specific categories of (cisgenic/intragenic) plant varieties would be proposed for diminished data requirements. It is clear that any of these items may, in specific cases, have consequences, in terms of food and feed safety, for the resulting plant products.

Also, the species definition is not a rigid one (<http://en.wikipedia.org/wiki/Species>; Staley (2009)). Some participants in the web-seminar stated that it is possible to establish objective criteria for species and crossability, but in general it was felt that the inclusion of these aspects in the definition in general will lead to 'grey areas' where you do not know the actual degree of crossability of the host and receptor lines of the cisgenic event. During the web-seminar it was also brought forward that, in addition to the exchange of chromosomal DNA, genetic modification may also include the exchange with plastid genes (Bock, 2001; Quesada-Vargas et al., 2005; Noutsos et al., 2007; Craig et al., 2008). It is not clear whether this would fall under the definitions as mentioned above.

A further aspect refers to the (endogenous) character of T-DNA: *Agrobacterium*, the bacterium of choice for delivering gene constructs into plant cells, is a soil bacterium that thrives on symbiosis with (dicotyledonous) plants. It is very likely that redundant T-DNA is present in a large number of plant species, due to a symbiotic relation along evolution. The intragenic vector system, aiming to use only vectors that consist of DNA that originates from the same crop species or related species to which it can be hybridized (Conner et al., 2007), could in theory use these redundant T-DNA sequence motifs that might be present in the plant genome. Currently, in scientific publications the P-DNA approach is applied in which plant-derived transfer DNA (P-DNA) is used (Rommens, 2004; Rommens et al., 2004; Conner et al., 2007). Ergo, once the presence of T-DNA borders has been established in the genome of a plant species that is to be transformed, or any other sequence used in a gene construct, the T-DNA borders may be regarded as 'self-DNA'. The plants derived by this method should still be regarded as intragenic since the (P-DNA) vector is created artificially from reorganised plant-derived sequences.

4.4 Conclusions on aspects of definition

The impossibility to currently define cisgenesis in a ‘watertight’ way will lead to considerable ‘grey areas’ the moment that the group of cisgenic plant varieties would, for instance, be exempted from the EU Directive 2001/18/EC and therefore also from Regulation 1829/2003, or be treated differently in any other way: at that moment it would be crucial to know exactly which novel plant varieties could fall under the definition of cisgenesis and which not. This may also have safety consequences, as current definitions of cisgenesis may include novel plant varieties that, for instance, express proteins that have never been part of the human or animal diet. From a food/feed safety perspective it would not be desirable to approve this type of cisgenic plant varieties for the (European) market without any pre-market safety assessment of at least the newly expressed proteins, or indeed the new plant product as a whole.

5 Aspects of food and feed safety

5.1 Current regulation of GMOs vs conventionally bred new plant varieties

There is a discrepancy between the regulation of GM plant varieties and other novel plant varieties that have been obtained via conventional breeding. As a result of this, there is a discrepancy between the food and feed safety assessment requirements for these two different categories of new plant varieties: All GMOs need to be assessed in detail for their food and feed safety, whereas safety assessment of other categories of new plant varieties is only required in the case of certain concerns on the basis of known alterations in the plant's physiology.

The basic approach for the food and feed safety assessment of GM plant varieties consists of a comparative safety assessment of the novel GM crop with an appropriate comparator (OECD, 1993; FAO/WHO, 1996; OECD, 1996; OECD, 1998; Kok and Kuiper, 2003; König et al., 2004; EFSA, 2006). The comparators are crops that are on the market and already have a so-called history-of-safe-use. In the comparative safety assessment, the GM crop and the comparator(s) are grown under the same conditions. They are compared with regard to their molecular, agronomic and phenotypic characteristics and their chemical composition with the aim to identify differences between the GM crop and the comparable non-GM crop(s) that may be related to the genetic modification. The subsequent safety assessment steps will then focus on the differences that have been identified to see whether these differences may have any (unintended) toxicological, allergenic and/or nutritional consequences. In practice, if differences have been identified, the subsequent steps of the food and feed safety assessment procedure will be decided on a case-by-case basis, depending on the nature of the identified difference(s).

Basic information that will be part of a dossier for food and feed safety analysis in all GMO cases is:

- data on the molecular characteristics of the GM plant with respect to the inserted DNA elements, such as data on all detectable inserts into the plant genome, both complete and partial, and derived phenotype. In the European Union, information also needs to be provided on the place of insertion, i.e. the organization of the inserted genetic material at the insertion site, as well as sequence information on the flanking regions (EFSA, 2006).
- data on gene expression (intended changes). In specific cases, if this is relevant, this may be extended to different developmental stages of the plant (tissue) or to different plant parts. If the molecular characterization has identified the possibility of a fusion protein (unintended effect), it needs to be shown whether this putative fusion protein is actually expressed in the plant parts that will be marketed.
- data on the genetic stability of the inserted sequence in subsequent generations (i.e. a molecular biological characterisation).
- compositional data of, usually, the GM plant and its appropriate comparator(s) in order to detect potential unintended side effects of the genetic modification. The compositional analysis should comprise all key nutrients and anti-nutrients, including natural toxins, of the specific crop under investigation. To this end the OECD (OECD, 2006) has formulated Consensus Documents for many of the major crops describing the crop and the relevant constituents with relation to its food and feed safety. In general, this comparative analysis is done in a two-tiered approach. In the first

step the comparison is made between the GM plant and the direct comparator. If significant differences are detected, the biological relevance is assessed in the second step, by comparing the observed values with data as they are compiled in specific databases, such as the ILSI crop composition database (www.cropcomposition.org), or are documented in scientific literature.

- data on the estimated intake of the GM crop derived foods on the basis of available consumption data of the crop in general and, where relevant, for specific consumer groups.

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

- studies with the newly expressed proteins, usually on the basis of comparisons of the amino acid sequence of the new protein with those of known toxins and both *in vitro* as well as *in vivo* experimental data. If there are new constituents besides proteins, for instance metabolites or higher levels of endogenous compounds as shown by the compositional analysis, these will also have to be assessed.
- in specific cases: toxicological studies with the whole GM plant or derived food/feed products. In general, this will be a sub-chronic toxicity study with rodents.
- studies on potential allergenicity of the newly introduced proteins as well as the entire plant product. This will be done by an initial amino acid sequence comparisons with known allergens. If the plant species contains endogenous allergens (e.g. soybeans) the expression levels in the GM and the respective non-GM plant need to be compared.
- studies on the nutrient composition in the GM plant product(s) versus the comparator(s) in combination with the biological efficacy of the individual components and the dietary intake of the entire product. In specific cases, where relevant questions can not be answered by the nutritional assessment, this may lead to the necessity of a post-marketing monitoring programme to obtain further data for the nutritional evaluation of the GM plant and its products.

Since this concise overview reflects the requirements for GMO safety assessment, it clearly shows the large difference between data requirements for GMOs versus conventionally bred new plant varieties.

5.2 Food and feed safety issues of cisgenic vs conventionally bred new plant varieties

Conventionally bred new plant varieties will in general not have to go through a food and feed safety assessment procedure. In some cases, i.e. in plant species where it is known that high levels of natural toxins may occur, it may be necessary to analyse for these toxic compounds prior to variety registration. This is for instance, the case with glycoalkaloids in new potato varieties, but this is the exception to the rule, in most cases no specific analyses with relation to food and feed safety will have to be performed prior to the marketing of the conventionally bred new plant variety.

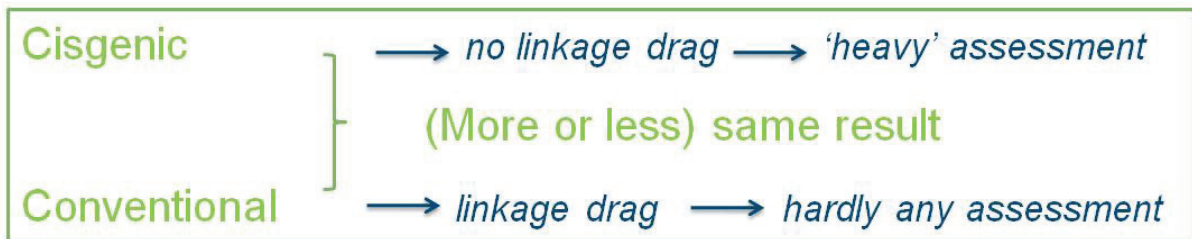


Figure 3: Schematic overview of the phenomenon of 'linkage drag' and the regulatory requirement for food and feed safety assessment in the case of cisgenic versus conventional new plant varieties.

As was observed under 5.1., the discrepancy with current regulatory requirements for the food and feed safety assessment of new (cisgenic) GM plant varieties is very large (Figure 3) and in specific cases this may result in very different safety requirements for conventionally bred or cisgenic GM varieties with basically comparable targeted changes.

In Schouten et al. (2006) it is stated that "The largest advantage of the application of cisgenesis compared to conventional breeding in the case of sexually compatible plants, is that the gene of interest can be transferred in a 'clean' way and the so-called 'linkage drag' of deleterious genes associated with the desired trait in e.g., a wild relative will not hamper or impede the breeding process". As a consequence Schouten et al. claim that cisgenic plants should be exempt from current GMO regulatory requirements (Schouten et al., 2006). In Kok et al. (2008), these claims were discussed from a food and feed safety perspective: "It is not clear, however, how unambiguous the definition of cisgenesis is in terms of food safety, as it may not exclude wild relatives that are not part of the human diet so far that can only be crossed under laboratory conditions. A number of food safety aspects that are the key to novel transgenic varieties, such as the safety assessment of the expressed proteins, may indeed be much less relevant for these cisgenic plant varieties, as the genes involved were already within the available gene pool when using traditional breeding strategies. If the (distant) relative is also being used as a food source, the safety assessment of the newly introduced protein may obviously benefit from the knowledge that it is already part of the human diet. The food safety assessment should take this into account and be conducted accordingly. On the other hand, the wild relative may not form part of the human diet yet, and in that case it would be prudent to assess the safety of the newly introduced nucleotide sequences and protein(s). But indeed this can only be justified if the same approach would be followed when a novel plant variety that would be the result of a traditional cross of the same parent lines, with any (remaining) linkage drag, would also be assessed within the regulatory frameworks of the food safety assessment of new plant varieties." The opinion was endorsed by the present project members.

The inclusion of not only genes from the crop plant itself, but also from 'crossable' plant varieties, indeed opens the possibility to use novel genes from other plant varieties, e.g. wild relatives, that do not yet form part of the food chain. This can be regarded as a safety aspect with relation to the definition of cisgenesis. From a food and feed safety perspective, these expression products may not have a so-called 'history of safe use' and therefore will require an in-depth safety analysis in terms of toxicity/allergenicity prior to marketing. Also, increased expression of 'plant-own' DNA may, in specific cases, affect the food and feed safety of new plant varieties, e.g. in the case of inherent toxic and/or allergenic proteins.

The approach for the safety assessment of conventional breeding – to consider the crop (and the potential change), and assess known natural toxins and/or anti-nutrients, with known hazards – has worked well to date for new plant varieties. However, nowadays also so-called conventional breeding has techniques at hand that can lead to considerable differences in the plant's physiology and thus in crop composition. In fact it can be argued that some conventional breeding methods like induced mutagenesis (Brunner, 1995; Ahloowalia and Maluszynski, 2001) have even a higher probability of unexpected genetic changes (Shirley et al., 1992; Mei et al., 1994) than cisgenesis. On the basis of this it would be prudent to require some form of safety assessment for all novel plant varieties and not just for those now considered to be GM plant varieties.

On the other hand, cisgenic products also have the chance of unintended effects due to insertional mutagenesis at or by the insertion site (see also 5.3), as was confirmed by plant breeding experts, and thus do not differ from GM plants. At the same time it is clear that strategies for homologous recombination and directed mutagenesis become increasingly advanced. With these, in the (near) future it may be feasible to precisely direct the genetic modification, thereby overcoming (part of) the hazards related to effects caused by the random insertion that are linked to genetic modification, both by cisgenesis and transgenesis strategies.

5.3 Food and feed safety issues of cisgenic vs transgenic plant varieties

Cisgenic varieties are currently within the scope of Regulation 1829/2003 (GMO Food and Feed) (EC, 2003a) since the techniques by which they were produced are not excluded from Directive 2001/18/EC. Therefore, the requirements for the safety assessment for cisgenic and transgenic GM plants are the same. These requirements can be divided into two parts: 1) the assessment of the newly introduced gene sequences and their (intended) expression products and derived effects and 2) potential unintended effects caused by the genetic modification (see 5.1). It can be argued that the required studies for the first part of the assessment can be reduced for cisgenic plant varieties where the expression products are known for the specific crop plant and are well-characterised, and expression levels are within 'normal' ranges (to be decided on a case-by-case basis). For the second part, the assessment will not only focus on the introduced T-DNA borders, as has been suggested, but also on the potential fusion proteins derived from the border regions of the introduced sequences and the endogenous DNA. Further, the 5' and 3' genomic sequences flanking the cisgene insert need to be analysed in order to identify whether endogenous genes have been interrupted or its expression level have been changed. Considering the large variety of potential cisgenic crop plant products it is not feasible to formulate generally applicable guidelines and thus, the assessment needs to be done on a case-by-case basis. For this assessment data on the place of insertion as well as on the (biochemical) composition of the plant (parts) that will be consumed need to be supplied, comparable to the assessment of GM plant varieties. If differences are identified, further safety testing may be required for both transgenic and cisgenic plant varieties.

5.4 Other safety-related considerations

A specific aspect that has been discussed in this respect is the safety assessment of T-DNA borders: the introduction of either cisgenic or transgenic DNA sequences by disarmed soil bacterium

Agrobacterium tumefaciens transformation will likely result in the rudimentary deposit of a so-called left border of the T-DNA. The right border is absent in most cases. The size of this left border is 2-24 base pairs. With respect to safety, it can be argued that this sequence might influence the transcription/translation system by either direct translation into a maximum of 8 amino acids, or disturb the reading frame, thereby possibly creating an even larger transcript (Jacobsen and Schaart, 2009). Possible effects can be envisaged due to the presence of this putative peptide (toxicity or selective advantage), or its characteristics in case the gene product appears to be allergenic. The sole presence of the rudimentary T-DNA border itself is not likely to cause unintended effects in terms of toxicity or allergenicity in the cisgenic plant variety due to the limited amount of nucleotides that can encode for a maximum of eight amino acids. In fact, as *A. tumefaciens* is using plants for a symbiotic relationship, it is likely that many plant species already contain identical or similar DNA sequences, as has been determined for *A. rhizogenes* (Intrieri and Buiatti, 2001). The frame shifts, however, that could have been introduced by the T-DNA left border insertion, and that may lead to new (fusion) proteins, can differ from transformant to transformant. The assessment of potentially induced or increased levels of allergenicity/toxicity will be part of the overall food and feed safety assessment on the basis of the molecular characterisation of the entire genetic modification and not just on the T-DNA present, and therefore will have to be performed on a case-by-case basis (See section 5.1).

In cisgenesis (as in conventional breeding and transgenesis) the gene expression level of the introduced gene might deliberately or by accident be out of the natural range of expression variation (i.e. the range found in conventional crop varieties). This might occur even if the regulatory sequences from the same plant are used in the case when multiple copies are generated, or due to position effects (Filipecki and Malepszy, 2006). Due to position effects or the use of other regulatory elements (if from the same species, this would fall under the definition of intragenesis), the level of gene expression might be higher than the naturally occurring level of expression (Kim and Grierson, 2005; Kang et al., 2007; Zheng et al., 2008; Feng et al., 2009). This might have consequences for the safety evaluation. Indeed (intended) changes of protein expression, or derived bioactive compounds, in the same plant species using cisgenesis would be a relevant part of current food and feed safety evaluation. Bioactive compounds today have a positive connotation, but it is known that there are optimal concentration ranges in this respect for health benefit, as was pointed out by web-seminar participants. It was furthermore argued that the experience with the gene product should be taken into account and as a consequence the requirements for the safety assessments could be reduced. If we assume i) no insertional effects and ii) all the secondary effects / cross-talk between metabolic pathways in different varieties of the same species to be comparable, then cisgenesis and traditional crossing could be considered equal. It is known (Filipecki and Malepszy, 2006), however, that insertional effects may occur and that there are differences in e.g. metabolic networks leading to natural toxins in different varieties within species. Furthermore, the copy number and position effects will likely make cisgenic plants different in most cases, as was also pointed out during the web-seminar: a higher copy number or a different position of the newly introduced gene in the plant genome may lead to increased expression, outside of levels as present in conventional varieties.

Finally, both conventional breeding and any GM approach (cisgenesis, transgenesis, etc.) are followed by extensive breeding selection of parental lineage plants based on agronomic and phenotypic characterization (and compositional when appropriate). These recurrent (back) crosses could also eliminate any unwanted side effects of the breeding procedure, either GM or not, since the breeding selection is on the introduced trait. However, unintended side effects of any breeding procedure that

are linked to the new trait, for instance as a result of secondary effects of the expressed product, but will not alter the phenotype of the plant, will not likely be detected in this way.

5.5 Conclusions safety aspects

From the considerations in this chapter it was concluded that the differences in safety aspects between cisgenic and transgenic GM plants may not be restricted solely to the type of insert. Hence, the cisgenic plants cannot be assessed on the basis of reduced data requirements only because of a relatively 'safe' choice of the inserted gene alone. Other factors like method of insertion, place of insertion and possible accompanying (unintended) changes in the genome and physiology of the recipient plant should be taken into account. The exact data requirements will have to be determined on a case-by-case basis, as is the case for transgenic GM crop varieties.

At the same time it was concluded that the current distinction between 'GMOs' and 'other new plant varieties' does not lead to a balanced safety assessment for all novel plant varieties. New plant varieties form a continuum from conventional crosses in the field without any laboratory step via artificial mutagenesis using chemical treatment or radiation up to, for instance, transgenic plant varieties where whole metabolic routes from non-self sources have been introduced (or deleted). Cisgenic varieties are somewhere in the middle of this continuum. An optimal food and feed safety assessment should be based on the specific characteristics of the novel plant variety, rather than to the categorisation of the new plant products on the basis of one of the technologies used to obtain the new plant variety.

6 Discussion and conclusions

6.1 Discussion

On the basis of the project results the following aspects have been identified and discussed:

A With respect to the definition of cisgenesis it was discussed the following aspects are likely to cause a so-called ‘grey area’ when trying to define cisgenesis (or intragenesis):

- The definition of species: it was concluded that there is no watertight definition of a species at this moment, as a result of the current discussions in plant taxonomy as well as a result of the different views on a biological species;
- The (practical) crossability of crop plants: it is clear that a plant can be crossed when this can be achieved in the conventional way by means of sexual reproduction. In some cases this is not feasible and then different laboratory techniques may help to overcome difficulties of crossing in the field. This may result in a viable cross result (F1) in perhaps 50% of the cases and then we consider the lines still crossable, but what if a viable cross result (F1) is only obtained in 1% of the cases or 0.01%, or even less? In the end this will lead to a theoretical discussion on whether it is likely that the cross may ever result in a F1 line, as it will no longer be practical to test this in practice. The result will be a grey area with respect to the definition.
- An extra definition problem may relate to the exchange of plastids: it is not clear whether this would fall under the definition of cis- or intragenesis and thus may create another part of the ‘grey area’.

B Furthermore, there was general agreement that there should be a distinction between ‘true’ cisgenesis, defined as a genetic modification of a recipient species with a natural gene from a crossable-sexually compatible- species, where the gene includes its exon/intron structure and its native promoter and terminator, and intragenesis, where the (order of) sequences are no longer fully ‘native’. It was concluded that cisgenesis and intragenesis can not be regarded as equivalent: small changes in coding sequences and/or regulatory elements may lead to extended changes in the plant’s physiology, and therefore should be assessed separately on a case-by-case basis with respect to potential toxicity and/or allergenicity.

With respect to the safety assessment of cisgenic and intragenic plant varieties, it was determined that in the end only in the case of cisgenesis (assuming that the protein has a history of safe use (see discussions on definitions)) the cisgenic expressed protein may not have to be separately assessed, as it will feature in its ‘conventional’ make-up in the cisgenic plant variety. In the case of intragenic crosses the intragenic expressed protein is likely to be very similar to the conventional protein, but this will have to be assessed on a case-by-case basis as the level of expression and/or the characteristics of the expressed protein may have changed, depending on the composition and structure of the genetic construct. As a result, it was concluded that the introduced gene (and thereby the gene product) in the cisgenic approach, but not necessarily in the intragenic approach, is native and could for that reason be considered as safe. In this respect the assessment of cisgenic plant varieties can benefit from our knowledge of the plant gene and its expression product, and this aspect of the safety assessment can clearly be ‘light’ in the case of cisgenic varieties.

C Both cis- and transgenesis make use of illegitimate recombination and deposit the T-DNA left border in the recipient plant, adjacent to the introduced gene. Therefore, it needs to be assessed in both

cases whether there are any unintended effects because of the insertion (insertional mutagenesis). This aspect may become of less relevance in the near future, when exact positioning of the recombinant-DNA construct may become increasingly feasible. It has been argued that in classical plant breeding many breeding techniques, e.g. chemical or irradiation mutagenesis, are used that probably resort often in more pronounced alterations of the genome than those that are likely to occur in the case of cisgenesis, but this is not a strong argument for cisgenesis being assessed as ‘traditional breeding’. Finally, in the case of intragenic as well as in transgenic GM approaches unintended secondary effects of the expression products of the introduced gene sequences may occur, which may lead to altered levels of proteins and/or secondary metabolites in the plant’s physiology that may require further toxicological and/or nutritional assessment. This is not likely to be the case in cisgenic varieties as the physiological ‘surroundings’ of the gene have not changed. At the same time it was concluded that this leads to the discrepancy that in the case of cisgenic varieties, the resulting plant will have to go through elaborate safety assessments whereas a very similar result of a conventional cross, with potential so-called linkage drag, will in general not be assessed for its food/feed safety.

D The arguments brought forward by participating experts focused on the aspects mentioned under A en B. From the discussions it was clear that in any case it is not yet clear what characteristics the first marketed cisgenic plant varieties actually will have: e.g. whether they indeed will be cisgenic or rather intragenic according to the definitions as applied in this report, whether they will have significant changes in their composition and whether the newly introduced proteins have indeed a so-called ‘history of safe use’. So far we can only make theoretic assessments of novel cisgenic plant varieties, as we lack (safety) data on such varieties. It may therefore be wise to gain expertise with these new developments: assess the derived products on a case-by-case basis, using the knowledge we have on our crop varieties. The safety assessment of cisgenic plant varieties is currently regulated within the European Union, and evaluation of the safety aspects of the different categories of novel cisgenic plant varieties will be performed when we have (more) data to assess for this goal. That will allow a better assessment of the actual differences between cisgenic and transgenic plant varieties, than just merely a theoretic assessment, as is only feasible at this moment in time. On the basis of ‘real’ data it can be assessed whether cisgenic plant varieties show fewer differences compared to conventional varieties than transgenic GM varieties. But even in that case it will be difficult to assess the cisgenic varieties as a different group as for specific aspects of the safety assessment (such as history of use of the expression products and potential insertional mutagenic effects of the genetic construct) the cisgenic varieties will have more in common with novel transgenic plant varieties than with conventional varieties.

E Finally, it was discussed that currently new plant varieties form a continuum from conventional crosses in the field, without any human interference, to artificial mutagenesis resulting in arbitrary mutations, up to transgenic plant varieties which involves extensive DNA technology and, possibly, complex metabolic pathways that have been changed or newly introduced. Cisgenic crop varieties have been created with techniques involving genetic modification, although the resulting cisgenic GM crop might be closer to traditionally bred crops than to GMOs. Depending on the specific modifications and characteristics, cisgenic crop varieties will be somewhere in the middle of this continuum. From a scientific point of view, an optimal food and feed safety assessment for all new plant varieties should be based on the specific new characteristics of the individual novel plant variety, rather than on the categorisation of the new plant varieties and their derived products on the basis of (one of) the technologies used to obtain the new plant variety.

6.2 Conclusions

Existing knowledge on the newly expressed proteins in the cisgenic/intragenic plant varieties will, on a case-by-case basis, already be used within the current regulatory framework for the food and feed safety assessment of GM plant varieties. This thus may already lead to reduced requirements in specific aspects of the food and feed safety assessment. But, taking into account the specific features of cisgenic/intragenic plant varieties in comparison to conventional varieties on the one hand and transgenic GM varieties on the other hand, there is, from a food and feed safety perspective, no scientific basis for a general reduction of requirements for cisgenic crop plant varieties.

This general conclusion is based on the following arguments:

- There is general agreement that it is not possible to determine a watertight definition of cisgenic (or intragenic) plant varieties.
- In the case of cisgenesis, including only cisgene products that are already part of the human or animal diet, the food and feed safety evaluation may be simplified with respect to the specific aspects related to the expression products, as indeed all these gene expression products have a 'history of safe use', but not for other aspects in the safety assessment. This will already be part of the case-by-case approach in the current food and feed safety assessment of new GM plant varieties. For other types of cisgenic/intragenic crop varieties it was concluded that small changes in sequences and/or regulatory elements may lead to extended changes in the plant's physiology, and therefore cisgenic/intragenic plant varieties should be assessed on a case-by-case basis with respect to their potentially toxicological / allergenic / nutritional characteristics.
- Both cis- and transgenesis make use of illegitimate recombination and deposit the T-DNA left border in the donor plant, if the transformation is achieved via the use of the *Agrobacterium tumefaciens* transformation system. Therefore, in both cases risk assessment needs to assess whether the gene insertion has caused any unintended effects (insertional mutagenesis). The current risk assessment of GM plant varieties (including cisgenic varieties) largely focuses on any potential unintended effects of the genetic modification due to insertional effects (of the genetic construct, not just the T-DNA-fragment, and irrespective of the mode of transformation). This would thus apply to both novel cisgenic/intragenic as well as transgenic crop plant varieties.

Finally, it was concluded that new plant varieties form a continuum from conventional crosses in the field, without any laboratory step being part of the breeding procedure (traditional breeding), up to transgenic plant varieties where recombinant-DNA techniques have been used to achieve major changes in the plant's genome. On a biological level, the alterations can range from small changes (adding a single resistance gene) to extensive changes in which complex metabolic routes have been introduced. Depending on their specific modifications and characteristics, cisgenic crop varieties will be somewhere in the middle of this continuum. From a scientific point of view, an optimal food and feed safety assessment in the case of new plant varieties should be based on the specific new characteristics of the individual novel plant variety, rather than on the categorisation of the new plant varieties and their derived products on the basis of (one of) the technologies used to obtain the new plant variety.

Annex I References

- Ahloowalia, B. S. and M. Maluszynski (2001). Induced mutations - A new paradigm in plant breeding. *Euphytica* 118(2): 167-173.
- Beardmore, J. A. (1997). Transgenics: Autotransgenics and allotransgenics. *Transgenic Research* 6(1): 107-108.
- Benjamin, I., D. Kenigsbuch, M. Galperin, J. A. Abrameto and Y. Cohen (2009). Cisgenic melons over expressing glyoxylate-aminotransferase are resistant to downy mildew. *European Journal of Plant Pathology* 125(3): 355-365.
- Bock, R. (2001). Transgenic plastids in basic research and plant biotechnology. *Journal of Molecular Biology* 312(3): 425-438.
- Brunner, H. (1995). Radiation-induced mutations for plant-selection. *Applied Radiation and Isotopes* 46(6-7): 589-594.
- COGEM (2006). Vereenvoudiging van regelgeving bij genetische modificatie met planteigen genen, cisgenese, een reële optie? COGEM signalering CGM/060428-05: 45.
- Conner, A. J., P. J. Barrell, S. J. Baldwin, A. S. Lokerse, P. A. Cooper, A. K. Erasmuson, J. P. Nap and J. M. E. Jacobs (2007). Intragenic vectors for gene transfer without foreign DNA. *Euphytica* 154(3): 341-353.
- Craig, W., P. Lenzi, N. Scotti, M. De Palma, P. Saggese, V. Carbone, N. M. Curran, A. M. Magee, P. Medgyesy, T. A. Kavanagh, P. J. Dix, S. Grillo and T. Cardi (2008). Transplastomic tobacco plants expressing a fatty acid desaturase gene exhibit altered fatty acid profiles and improved cold tolerance. *Transgenic Research* 17(5): 769-782.
- EC. (2003a). EC Regulation No. 1829/2003. from http://eur-lex.europa.eu/pri/en/oj/dat/2003/l_268/l_26820031018en00010023.pdf.
- EFSA (2006). Guidance document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed. *EFSA J.* 99, 1–100 Available from: http://www.efsa.europa.eu/en/science/gmo/gmo_guidance/660.html.
- FAO/WHO (1996). Biotechnology and Food Safety. Report of a Joint FAO/WHO Consultation, Rome, Italy, 30 September-October 1996. FAO Food and Nutrition Paper 61. Rome: Food and Agriculture Organization of the United Nations. <http://www.fao.org/es/esn/gm/biotec-e.htm>.
- Feng, L. L., R. Y. Yang, X. Q. Yang, X. M. Zeng, W. J. Lu and Q. P. Zeng (2009). Synergistic re-channeling of mevalonate pathway for enhanced artemisinin production in transgenic *Artemisia annua*. *Plant Science* 177(1): 57-67.
- Filipecki, M. and S. Malepszy (2006). Unintended consequences of plant transformation: a molecular insight. *Journal of Applied Genetics* 47(4): 277-286.
- Gessler, C., T. Vanblaere, I. Szankowski and G. Brogini (2009). Cisgenic approach to disease resistance in apple. *Phytopathology* 99(6): S42-S42.
- Hanley, Z. (2008). GM approaches without GM outcomes. Retrieved September, 2009, from <http://www.ermanz.govt.nz/tehautu/may08hui/9%20-%20wkshp%20a%20-%20gm%20approaches%20without%20gm%20outcomes%20%28hanley%29.pdf>.
- Hanley, Z. (2009). NZBIO2009 Technology showcase: Forage biotech for NZ - the delivery phase. Retrieved September, 2009, from <http://nzbio2009.co.nz/files/408011190.pdf>.
- Intrieri, M. C. and M. Buiatti (2001). The horizontal transfer of *Agrobacterium rhizogenes* genes and the evolution of the genus *Nicotiana*. *Molecular Phylogenetics and Evolution* 20(1): 100-110.

- Jacobsen, E. and J. Schaart (2009). Risk assessment of T-DNA borders from *Agrobacterium tumefaciens* in cisgenic crops. Letter to minister Cramer (VROM) March 13. 2009, Department of Plant Breeding (WUR).
- Jacobsen, E. and H. J. Schouten (2007). Cisgenesis strongly improves introgression breeding and induced translocation breeding of plants. *Trends in Biotechnology* 25(5): 219-223.
- James, C. (2009). Global Status of Commercialized Biotech/GM Crops: 2009, ISAAA Briefs 41-2009.
- Jochensen, H. (2000). Toetsen en begrenzen. Een ethische en politieke beoordeling van de moderne biotechnologie, Buijten & Schipperheijn (Amsterdam). Lindeboomreeks nr. 12. 263 p.
- Kang, S., K. Kang, K. Lee and K. Back (2007). Characterization of rice tryptophan decarboxylases and their direct involvement in serotonin biosynthesis in transgenic rice. *Planta* 227(1): 263-272.
- Kim, S. H. and D. Grierson (2005). Subcellular localisation and silencing of ripening-associated membrane protein (TRAMP) in tomato *Lycopersicon esculentum* Mill. *Plant Science* 169(6): 1022-1029.
- Kleter, G. A. and E. J. Kok (2009). Antwoord aan VROM over rapport over T-DNA inserties. Briefrapport RIKILT, 6 april 2009.
- Kok, E. J., J. Keijzer, G. A. Meter and H. A. Kuiper (2008). Comparative safety assessment of plant-derived foods. *Regulatory Toxicology and Pharmacology* 50(1): 98-113.
- Kok, E. J. and H. A. Kuiper (2003). Comparative safety assessment for biotech crops. *Trends in Biotechnology* 21: 439-444.
- Konig, A., A. Cockburn, R. W. R. Crevel, E. Debruyne, R. Grafstroem, U. Hammerling, I. Kimber, I. Knudsen, H. A. Kuiper, A. Peijnenburg, A. H. Penninks, M. Poulsen, M. Schauzu and J. M. Wal (2004). Assessment of the safety of foods derived from genetically modified (GM) crops. *Food and Chemical Toxicology* 42(7): 1047-1088.
- Mei, M., H. Deng, Y. Lu, C. Zhuang, Z. Liu, Q. Qiu, Y. Qiu and T. C. Yang (1994). Mutagenic effects of heavy-ion radiation in plants. *Life Sciences and Space Research Xxv(2) - Radiation Biology*. G. Horneck, H. Bucker, A. T. P. Cox, T. C. Yang, B. V. Worbul, M. Donlon, W. Atwell, M. A. Shea, D. F. Smart, R. J. M. Fry, L. W. Townsend, S. B. Curtis and C. E. Swenberg. 14: 363-372.
- Nam, Y. K., N. Maclean, G. Hwang and D. S. Kim (2008). Autotransgenic and allotransgenic manipulation of growth traits in fish for aquaculture: a review. *Journal of Fish Biology* 72(1): 1-26.
- Noutsos, C., T. Kleine, U. Armbruster, G. DalCorso and D. Leister (2007). Nuclear insertions of organellar DNA can create novel patches of functional exon sequences. *Trends in Genetics* 23(12): 597-601.
- OECD (1993). Safety assessment of foods derived through modern biotechnology: concepts and principles. <http://www.oecd.org/dataoecd/37/18/41036698.pdf>.
- OECD (1996). Food Safety Evaluation. Paris: Organisation for Economic Cooperation and Development.
- OECD (1998). Report of the OECD workshop on the toxicological and nutritional testing of novel foods, Aussois, France, 5-8 March 1997. Paris: Organisation for Economic Cooperation and Development. <http://www.oecd.org/ehs/ehsmono/aussoidrEN.pdf>.
- OECD (2006). An introduction to the food/feed safety consensus documents of the Task Force. Series on the Safety of Novel Foods and Feeds, no 14, ENV/JM/MONO(2006)10. Available from: [http://appli1.oecd.org/olis/2006doc.nsf/43bb6130e5e86e5fc12569fa005d004c/f6714efc9beea840c1257156005638c2/\\$FILE/JT03207766.PDF](http://appli1.oecd.org/olis/2006doc.nsf/43bb6130e5e86e5fc12569fa005d004c/f6714efc9beea840c1257156005638c2/$FILE/JT03207766.PDF).

- Quesada-Vargas, T., O. N. Ruiz and H. Daniell (2005). Characterization of heterologous multigene operons in transgenic chloroplasts. Transcription, processing, and translation. *Plant Physiology* 138(3): 1746-1762.
- Rommens, C. M. (2004). All-native DNA transformation: a new approach to plant genetic engineering. *Trends in Plant Science* 9(9): 457-464.
- Rommens, C. M. (2007). Intragenic crop improvement: Combining the benefits of traditional breeding and genetic engineering. *Journal of Agricultural and Food Chemistry* 55(11): 4281-4288.
- Rommens, C. M. (2008). The need for professional guidelines in plant breeding. *Trends in Plant Science* 13(6): 261-263.
- Rommens, C. M., M. A. Haring, K. Swords, H. V. Davies and W. R. Belknap (2007). The intragenic approach as a new extension to traditional plant breeding. *Trends in Plant Science* 12(9): 397-403.
- Rommens, C. M., J. M. Humara, J. S. Ye, H. Yan, C. Richael, L. Zhang, R. Perry and K. Swords (2004). Crop improvement through modification of the plant's own genome. *Plant Physiology* 135(1): 421-431.
- Russell, A. W. and R. Sparrow (2008). The case for regulating intragenic GMOs. *Journal of Agricultural & Environmental Ethics* 21(2): 153-181.
- Schouten, H. J., F. A. Krens and E. Jacobsen (2006). Cisgenic plants are similar to traditionally bred plants - International regulations for genetically modified organisms should be altered to exempt cisgenesis. *EMBO Reports* 7(8): 750-753.
- Schouten, H. J., F. A. Krens and E. Jacobsen (2006a). Do cisgenic plants warrant less stringent oversight? *Nature biotechnology* 24(7): 753.
- Shirley, B. W., S. Hanley and H. M. Goodman (1992). Effects of ionizing-radiation on a plant genome - Analysis of 2 *Arabidopsis* transparent-testa mutations. *Plant Cell* 4(3): 333-347.
- Staley, J. T. (2009). Universal species concept: pipe dream or a step toward unifying biology? *Journal of Industrial Microbiology & Biotechnology* 36(11): 1331-1336.
- Strauss, S. H., V. Busov, C. Ma and K. Van Wormer (2009). Cisgenic and intragenic approaches to genetic modification of growth and form in poplar. *In Vitro Cellular & Developmental Biology-Animal* 45: S24-S25.
- Zheng, P., W. B. Allen, K. Roesler, M. E. Williams, S. Zhang, J. Li, K. Glassman, J. Ranch, D. Nubel, W. Solawetz, D. Bhattaramakki, V. Llaca, S. Deschamps, G. Y. Zhong, M. C. Tarczynski and B. Shen (2008). A phenylalanine in DGAT is a key determinant of oil content and composition in maize. *Nature Genetics* 40(3): 367-372.