The views expressed in this report are those of an expert working group and do not necessarily represent those of the European Commission or the Competent Authorities. Only the European Court of Justice can give a binding opinion on EU law.

New Techniques Working Group

FINAL REPORT

1.0 Introduction

Directive 90/220/EEC (repealed and replaced by Directive 2001/18/EC) on the deliberate release of genetically modified organisms (GMOs) into the environment and Directive 90/219/EEC (amended by Directive 98/81/EC and repealed and replaced by Directive 2009/41/EC[1]) on the contained use of genetically modified micro-organisms (GMMs) establish the basis for GMO/GMM regulation in Europe. Both pieces of legislation were adopted in parallel in 1990.

Biotechnology has found considerable application in plant breeding. For the most part, the purpose of using biotechnology in plant breeding is to introduce new traits and to achieve this in a more precise and cost-effective manner. This is the objective irrespective of whether the technique used is considered to result in genetic modification.

New breeding and genetic modification techniques have evolved at a rapid pace since the introduction of the legislation in 1990 with the result that in some instances it is unclear whether it falls under the scope of the GMO legislation. There is growing interest in using biotechnology in such a way that the resulting organism does not contain any genetic material from an organism that it could not hybridise/ breed with naturally or indeed, contain any new genetic material at all. Furthermore, in some cases the resulting changes are similar to those achievable with conventional breeding techniques.

The techniques addressed in this report were selected because it is unclear whether they would result in a GMO and whether the resulting products fall under the scope of the existing GMO/GMM legislation.

2.0 Legal context

In accordance with the deliberate release and contained use legislation[2], a GMO/GMM is defined as *"an organism/ micro-organism ....in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination*[3]*"*. Techniques, which are or are not considered to result in genetic

[1] This Directive regulates activities involving GMMs (such as genetically modified viruses or bacteria and cell cultures of higher organisms) under conditions of containment, i.e. in a closed environment in which contact with the population and the environment is limited.
[2] Directive 2001/18/EC on the deliberate release into the environment of GMOs, and Directive 2009/41/EC on the contained use of GMMs.
[3] Article 2 of Directive 2001/18/EC

modification, or are excluded from the scope of the GMO legislation, are listed in the respective annexes of Directives 2001/18/EC and 2009/41/EC.

An organism (*any biological entity*) and a micro-organism (*any microbiological entity, cellular or non-cellular*) *must be capable of replication or of transferring genetic material*.

Some techniques may include a step in the process where Directive 2009/41/EC applies. For example:

- If plant cells (in a cell culture) containing a recombinant vector are used, the cell culture may be regarded as a GMM according to the definition in Article 2 Directive 2009/41/EC. This usually takes place in a laboratory or other contained area.
- If a GMM (i.e. recombinant *Agrobacterium* or plant virus) is used in or on plants, its use is covered by Directive 2009/41/EC as "GMM in plants". This usually takes place in a greenhouse or growth chamber.

Neither of the described steps necessarily affects whether the grown plant is classified as a GMO or not, if it is released into the environment; its status as a GMO will need to be considered for deliberate release under Directive 2001/18/EC.

Both Directives list techniques that:

- give rise to genetic modification (Annex I, Part A of Directive 2009/41/EC and Annex IA Part 1 of Directive 2001/18/EC);
- are not considered to result in genetic modification (Annex I, Part B of Directive 2009/41/EC and Annex IA Part 2 of Directive 2001/18/EC);
- yield organisms that are excluded from the Directive (Annex II Part A of Directive 2009/41/EC and Annex IB of Directive 2001/18/EC)

Annex I Part A of Directive 2009/41/EC and Annex IA Part 1 of Directive 2001/18/EC (techniques giving rise to genetic modification) use the wording '*inter alia*' and therefore provide indicative lists of techniques leading to genetic modification. The remaining Annexes provide exhaustive lists. Therefore, the techniques leading to genetic modification are not limited to those listed under Annex I Part A of Directive 2009/41/EC and Annex IA Part 1 of the Directive 2001/18/EC.

The Annexes of both Directives are broadly similar with one exception. Directive 2009/41/EC excludes self-cloning from the scope of the legislation. Self-cloning is where a nucleic acid is removed from the cell of an organism and all or part of the nucleic acid is reinserted into cells of the same or a phylogenetically closely related species (see Appendix 4).

3.0 Establishment of the New Techniques Working Group (NTWG)

At the request of the Competent Authorities (CA) under Directive 2001/18/EC, a working group (WG) was established (October 2007) to analyse a non-exhaustive list of techniques for which it is unclear whether they would result in a GMO. CAs were invited to nominate two experts per Member State. A first list of techniques was

1 compiled by the CAs (section 3.2). In addition, the CAs contributed to and approved a
2 mandate for the WG (Appendix 1).
3
4 The findings of the WG are applicable to the relevant techniques in general but are
5 focussed primarily on the implications for plant breeding. The report also refers to
6 micro-organisms produced using new techniques in biotechnology. However, apart
7 from the section on synthetic genomics, the focus is on plant breeding. This reflects
8 the techniques that have been identified for consideration.
9
10 The mandate does not take account of safety issues. Nevertheless, it was agreed at the
11 initial meeting that comments on safety aspects would be taken into consideration
12 where such comments were raised.
13
14 The WG met nine times (15 December 2008, 9 February 2009, 24 April 2009, 18/19
15 June 2009, 21/22 September 2009, 21/22 January 2010, 26/27 January 2011, 23/24
16 February 2011 and 27/28 April 2011[4]). It is the function of the WG to make its
17 findings available to CAs in the form of technical advice. Therefore, in accordance
18 with the mandate the outcome of the WG will be presented to a meeting of the
19 Competent Authorities under each Directive or to a joint meeting of the Competent
20 Authorities under the two Directives. The MS experts who participated in the
21 deliberations of the WG are provided in Appendix 2.
22
23 It is envisaged that the results of the WG will be viewed in parallel with the results of
24 the evaluation exercise of the GMO legislation on food/feed and cultivation, which
25 was finalized and published on 28 October 2011. It will also be viewed in conjunction
26 with a study led by the European Commission's Joint Research Centre (JRC) Institute
27 for Prospective Technological Studies (IPTS) in cooperation with the JRC Institute for
28 Health and Consumer Protection (IHCP)[5]. This study, which was published in May
29 2011, investigates the degree of development and adoption of new plant breeding
30 techniques by the breeding sector and discusses drivers and constraints for further
31 commercial adoption. It also highlights studies on food, feed and environmental safety
32 and evaluates difficulties for detecting and identifying crops produced by the new
33 plant breeding techniques.
34
35 The Commission has asked EFSA to address the following considerations in separate
36 opinions per technique or per groups of techniques, as appropriate:
37 - Determine whether there is a need for new guidance or whether the existing
38 guidance on risk assessment should to be updated or further elaborated, in
39 anticipation of the placing of products on the market through the application of
40 the listed techniques.
41 - What are the risks in terms of impact on humans, animals and the environment
42 that the eight techniques listed could pose, irrespective of whether or not they
43 fall under the GMO legislation? This latter request should consider the most
44 recent scientific literature and knowledge of plant breeding experts and
45 compare plants obtained by these new techniques with plants obtained by

[4] No meeting of the working group took place between January 2010 and January 2011 due to the change of competences within the Commission and the need for delivering on other priorities.
[5] http://ftp.jrc.es/EURdoc/JRC63971.pdf.

conventional plant breeding techniques and secondly with plants obtained with currently used genetic modification techniques.

3.1 Approach taken by the experts

The WG considered the most recently available scientific data to define and analyse the techniques under consideration. As established in the mandate, each 'new technique' was evaluated in the context of the GMO/GMM definition and the annexes[6] of each Directive. The WG considered each technique to determine whether the definitions in the GMO/GMM legislation applied and whether the resulting organism has been altered at all. The WG also took into account whether and if so, to what extent, the techniques were captured by the examples listed in Annex 1A of Directive 2001/18/EC and Annex 1 Parts A and B of Directive 2009/41/EC and the characteristics of the resulting organism with respect to terms used in these two annexes. For instance, by considering whether new combinations of genetic material have been generated, whether new nucleic acid molecules have been inserted into the organism or whether they are present transiently and not capable of continued propagation i.e. heritable/inherited. In addition, experts considered whether the alteration in the resulting organism could be produced by traditional techniques or in nature and the extent to which the resulting organisms could be distinguished from organisms resulting from the use of traditional techniques.

The experts recognised that the annexes in the Directives were drafted before these techniques were developed and as such are not necessarily fit for purpose. Since some of the techniques analysed are not properly captured by the examples of GM techniques nor by the list of techniques that are not considered to result in genetic modification, the experts have also provided additional remarks that could be used by regulators in the follow-up to the report. More specifically, given that the existing Directives exempt organisms obtained through an exhaustive list of techniques of genetic modification, the WG indicated whether and why some techniques could qualify for inclusion into such list as a result of potential future revisions of Annex IB of Directive 2001/18/EC and Annex II Part A of Directive 2009/41/EC or for possible guidelines relating to the Annex I of Directive 2001/18/EC and Annex II of Directive 2009/41/EC (among other possible follow up actions to this report).

Experts noted that some of the techniques under consideration are processes that involve multiple steps. Whether the whole process or the different technical steps within it are defined as 'techniques of genetic modification' may be significant in terms of determining the status of the organisms produced at these different stages. Therefore, where multiple steps are associated with a 'new technique', experts considered it valuable and transparent to assess these individual steps separately to determine whether or not they yield GMOs as defined in the Directive(s).

[6] Annex IA and IB of Directive 2001/18/EC and Annex I and Annex II Part A of Directive 2009/41/EC – see section 2.0 of this document for further interpretation.

3.2 'New techniques' considered by the WG

The following techniques were proposed by the CAs for consideration by the "new techniques working group":

a. Oligonucleotide Directed Mutagenesis (ODM)
b. Zinc Finger Nuclease Technology (ZFN) (comprising ZFN-1, ZFN-2 and ZFN-3 as defined in this report)
c. Cisgenesis (comprising Cisgenesis and Intragenesis)
d. Grafting
e. Agro-infiltration
f. RNA-dependent DNA methylation (RdDM)
g. Reverse Breeding
h. Synthetic Genomics

4.0 Terms and issues discussed

There are certain terms in use in the legislation that influence considerations on whether a specific technique and or a resulting organism falls within the scope of the legislation, depending on the interpretation of the term. These terms were discussed separately in order to gain clarity and common agreement within the group and in order to make the conclusions of the group on the specific techniques more logical and robust.

In addition, issues such as 'transient presence/effect' and 'GMO offspring' were deemed equally relevant to the discussions and were similarly discussed.

When analysing organisms produced by a particular technique in the light of the criteria set out in Annex IB of Directive 2001/18/EC and Annex II Part A of Directive 2009/41/EC, a key consideration was whether these techniques involve the use of recombinant nucleic acid molecules or GMOs/GMMs other than those produced by mutagenesis and cell fusion (including protoplast fusion) and in the case of GMMs, self-cloning.

4.1 "..altered in a way…."

The term "*altered in a way*" is used in Article 2(2) of Directive 2001/18/EC and Article 2(b) of Directive 2009/41/EC which define GMO and GMM respectively as an "*organism*" or "*micro-organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination*". The techniques ("process") leading to the generation of GMOs are listed under Annex IA Part 1 of Directive 2001/18/EC and Annex I, Part A of Directive 2009/41/EC, and under Annex IB of Directive 2001/18/EC and Annex II Part A of Directive 2009/41/EC (Section 2.0 of this document provides an overview of the annexes and their respective techniques).

The experts recognized that the term "altered in a way" could be interpreted in one of two ways:

1. The emphasis is on the technique; the resulting organism is a GMO, even if the same modification or an identical organism could be obtained by the techniques listed in Annex I A Part 2 or Annex I B of Directive 2001/18/EC and Annex I Part B or Annex II Part A of Directive 2009/41/EC.
2. The emphasis is on the resulting organism; if the resulting organism is indistinguishable from an organism obtained from natural processes, conventional breeding or by application of the techniques listed in Annex IA Part 2 or Annex IB of Directive. 2001/18/EC and Annex I Part B or Annex II Part A of Directive. 2009/41/EC, then it cannot be considered as a GMO and would therefore be considered out of the scope of the Directives. (Article 2)

Experts also discussed the degree of change that should be considered an alteration according to the Directives, given the plasticity of genomes and the genetic variation that occurs naturally and through traditional breeding techniques. A minority of experts considered a single base pair change was sufficient to be captured by the legislation.

4.2 Nucleic acids and recombinant nucleic acid

The term "nucleic acid" is used in Annex IA Part 1(1) of Directive 2001/18/EC and Annex I Part A(1) of Directive 2009/41/EC.

Nucleic acid molecules e.g. DNA and RNA can be produced naturally or by synthetic means. In some of the techniques proposed for consideration, synthetic analogues of nucleic acids or nucleic acids containing synthetic nucleotide analogues are used, such as locked nucleic acids (LNA) and peptide nucleic acids (PNA).

LNA and PNA have different chemical properties from DNA and RNA and can behave differently *in vivo* compared to nucleic acid molecules e.g. in replication, transcription and translation. It was noted that LNA or PNA are used in the same way as nucleic acids, i.e. as tools to alter the genetic material in cells for example for the purpose of site-directed mutagenesis.

The term "recombinant nucleic acid molecules" is used in Annex IB of Directive 2001/18/EC and Annex II Part A of Directive 2009/41/EC. In line with the aforementioned Directives, a recombinant nucleic acid molecule is created outside the cells through the formation of a new combination of genetic material/nucleic acid molecules. There was a discussion on how many nucleotides could constitute a new combination of genetic material/nucleic acid molecules in this context. A majority of experts concluded that in order to form a new combination, a nucleotide sequence of at least 20 bp is required. A minority of experts were of the opinion that under the current definition, the replacement of only one nucleotide in a nucleic acid molecule could be interpreted as producing a recombinant nucleic acid.

The definition of a recombinant nucleic acid molecule is important because Annex IB of Directive 2001/18/EC and Annex II Part A of Directive 2009/41/EC both stipulate that techniques/methods of genetic modification yielding organisms to be excluded from the Directive, may only be excluded on the condition that they do not involve the use of recombinant nucleic acid molecules.

4.3 Heritable material

The term heritable material is used in Annex IA, Part 1(2) and Part 1(3) of Directive 2001/18/EC and Annex I, Part A(2) and Part A(3) of Directive 2009/41/EC. In both instances the term is used in the context of genetic material, which, when introduced directly (i.e. without the involvement of a vector system) into an organism (micro-organism) by procedures including micro-injection, micro-encapsulation or cell fusion, will result in a "technique of genetic modification" in the sense of the Directives. Although heritable material is not clearly defined in the Directive, there are two possible interpretations:

i. "heritable material" must be inherited in the case in question. The argument being that the first indent in the list of Annex IA Part 1[7] involves the use of vectors and refers to the transfer of genetic material into a host organism and continued propagation. In order for this to be consistent with the second indent in the list of Annex IA Part 1[8] heritable material should be interpreted as being propagated through the host organism and not just being transiently present (see section 4.4); and,
ii. "heritable material" has simply to be capable of being inherited.

GMOs that have been authorised to date, and into which 'heritable material prepared outside of the organism' has been introduced directly, have in all cases been capable of passing this material onto their offspring. Whereas, nucleic acid introduced into cells using some of these new techniques will not be inherited e.g. in the case of ODM, ZFN-1 ZFN-2 and RNA-dependent DNA methylation. However, the changes they impart will be inherited (although this is limited in the case of RNA-dependent DNA methylation).

The consequences for taking these different approaches when considering whether ZFN and ODM are captured by Annex 1A Part 1 are addressed in the specific sections (5.1 and 5.2, respectively).

4.4 GMO offspring

Offspring is a product of reproduction of organisms. Reproduction can be sexually or asexually

- In the case of sexual propagation in plants, the offspring are the product of two parents.
- In the case of vegetative propagation in plants, the offspring is the product of a single parent.
- In the case of animals it is essentially the same, but asexual propagation is rare, e.g. parthenogenesis, or does not happen naturally, e.g. "cloning".

[7] "recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside the organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation"

[8] "techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation";

- In the case of prokaryotic micro-organisms and eukaryotic cell cultures, reproduction occurs by cell division (asexual).
- In the case of eukaryotic micro-organisms, such as fungi, reproduction can occur sexually as well as asexually.
- In the case of viruses and other non-cellular entities such as viroids, reproduction occurs by biosynthetic copying, e.g. of the viral nucleic acids in a host cell, such as plant, animal or microbial cells.

Some of the techniques discussed in this report involve intermediate organisms or cells that contain new DNA. Further on in the process, however, some of these intermediate organisms or cells give rise to offspring that no longer contain the new DNA. In the NTWG different views were expressed on whether and when the offspring of a GMO can be classified as a non-GMO. This aspect is not considered in the Directives.

For the techniques described in this report all experts agree that unintended changes in the DNA sequence due to the (former) presence/insertion of the new DNA may have taken place, and would probably appear at very low frequency[9]. If such unintended changes appeared, they would be comparable to natural or induced changes not involving techniques that lead to GMO. All experts agreed that once it is established that the 'foreign' genetic material is no longer present in the resulting organism it is no longer considered a GMO, however, some expert raised concerns reading the Directives. Clear criteria would be needed to establish whether the 'foreign' genetic material is no longer present in the resulting organism in order for the offspring to be designated non-GMO.

For other techniques the offspring of a GMO may still be considered a GMO even when the new DNA is no longer present. The main underlying argument being that also unintended changes in the DNA sequence due to the (former) presence/insertion of the new DNA may have taken place.

4.5 Transient Presence (with respect to exogenous nucleic acid molecules in the cell/transient presence of recombinant DNA)

Despite reference in Annex IA Part 1(1) of Directive 2001/18/EC (and Annex I Part A(1) of Directive 2009/41/EC) to the "*insertion of nucleic acid molecules…into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation*", the legislation makes no reference to the term transient presence or effect. However, it does refer to ‘capable of continued propagation' and this is linked to the discussion on heritability. Since this term can have a bearing on whether the final organism is considered a GMO, it is considered important to discuss it.

Sometimes techniques that introduce genetic material into a cell do not result in a stable genetic transformation. Instead, the introduction can result in an effect for a

[9] EFSA Journal 2011; 9(5): 2149 "Guidance on selection of comparators for the risk assessment of genetically modified plants and derived food and feed"

period of time (varying from hours to generations). This can occur because the genetic material that is introduced into the cell is lost.

The transient presence of nucleic acid molecules in cells can be utilised to (over) express a foreign gene (e.g. synthesis of foreign proteins) temporarily or to change the expression level of a gene in the recipient cell, by modifying its regulation (e.g. agroinfiltration). In such cases, the trait is not inherited and does not by itself alter the sequence of the recipient cell's genetic material. However, the transient presence of nucleic acids in cells can also be used to induce mutations (ZFN) or changes in the methylation of DNA (RdDM). These changes in the recipient cell's genome are either permanent genetic alterations (mutations) or can remain in the progeny for some generations (epigenetic alterations like methylation of DNA).

A majority of experts consider that if the 'foreign' genetic material is present but is not able to replicate the organism should not be considered a GMO. A minority of experts agreed that as long as this 'foreign' genetic material is present even if it's not able to replicate the organism should be considered a GMO. All experts agreed that once it is established that the 'foreign' genetic material is no longer present in the resulting organism it is no longer considered a GMO.

See heritable material (section 4.3)

4.6 Organism

In accordance with Article 2(1) of Directive 2001/18/EC, *an organism means any biological entity capable of replication or of transferring genetic material*.

In accordance with Article 2 (a) of Directive 2009/41/EC, *a micro-organism means any microbiological entity, cellular or non-cellular, capable of replication or of transferring genetic material, including viruses, viroids, and animal and plant cells in culture.*

All experts agreed that plasmids as such are not 'organisms' as defined in the Directives. Instead they are a component of an organism i.e. of the bacterium from which they have been extracted. As long as the plasmid cannot replicate by itself outside of bacterial cells and it is not capable of transferring genetic material by itself, it is not an organism. This contrasts with viruses, which infect cells.

With regard to the use of vectors in cell culture, as long as the vectors remain present in the cells, the technique used will fall under the scope of Directive 2009/41/EC.

For the purpose of this report, experts have used the term "resulting organism" to denote an organism that results after having gone through all the steps of the particular technique. This could e.g. be a plant or seed intended for deliberate release or placing on the market or a microorganism intended for contained use.

Experts have used the term "intermediate organism" to denote any organism that is generated in the steps leading to the resulting organism. It should be recognized that intermediate steps could involve the introduction of heritable material into cells, as well as the loss of such material. In several cases it was essential for the assessment of

a technique to conclude whether or not an organism in which a genetic modification is no longer present, is a GMO as defined in the Directive(s). Therefore, experts concluded that it was important to discuss the intermediate organisms for the techniques dealt with in this report.

Synthetic biology and more particularly synthetic genomics, which is based on the chemical synthesis of an entire genome or of large portions of the genome followed by its introduction into a cell or cell-free environment where its replication is enabled, has appeared since the writing of the Directives. Whether or not a synthetic entity will be considered as an organism should be analysed in the context of the above mentioned definitions. The definition of an "organism" states that only entities capable of replication or of transferring genetic material fit under this term. Experts considered that if a synthetic entity fulfils the condition of capability of replication and/or transferring genetic material, then it should be defined as an organism in the context of the Directives.

4.7 Similarity

The Directives list techniques that give rise to genetic modification, that are not considered to result in genetic modification or that yield organisms to be excluded from the Directives. In order to classify the status of the 'new techniques', experts considered whether they were similar to the listed techniques.

Experts also discussed whether the changes (intended and unintended) induced by these 'new techniques' can also be produced by traditional breeding techniques, natural processes or techniques listed in Annex 1B of Directive 2001/18/EC and Annex II Part A of Directive 2009/41/EC. These discussions took into account whether novel combinations of genetic material could be generated and in one case, whether inserted DNA should be considered heterogenous rather than cisgenic.

5.0 Evaluation of the Techniques

5.1 Oligonucleotide-directed mutagenesis (ODM)

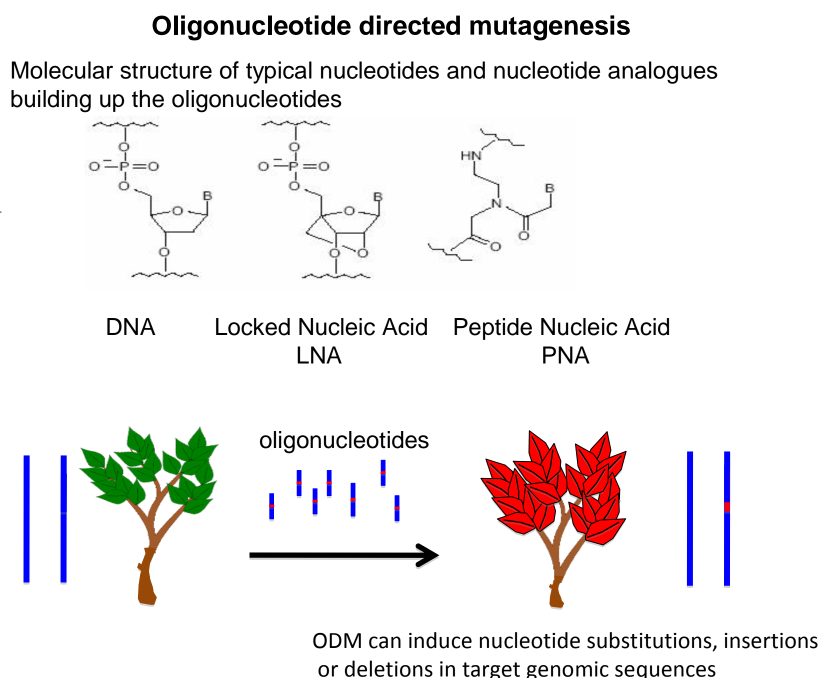

Fig. 1 Oligonucleotide-directed mutagenesis

The top row shows some examples of typical molecules used for oligonucleotide-directed mutagenesis. The bottom row shows the oligonucleotides above the black arrow; short sequences identical to plant genomic sequences are blue; red indicates a small sequence change. The resulting plant on the left (red) stably inherits the change to subsequent generations.

5.1.1 Definition and brief description of the technique based on the current scientific knowledge and current scientific literature

Oligonucleotide directed mutagenesis (ODM) employs oligonucleotides for targeted (site-specific) induction of point mutations[10].

Oligonucleotides of approximately 20 to 100 nucleotides are delivered to the cells by methods suitable for the different cell types (including electroporation, polyethylene-glycol-mediated transfection, natural uptake). The technique exploits the sequence specific interaction of the oligonucleotide with the resident DNA of the cells, resulting in gene targeting. This directs the attempted genetic modification to a specific region in the DNA or even to a specific base pair. The genetic modification can be the induction of a point mutation or reversion of an existing mutation which may lead to changes in the expression of a gene.

Four different types of oligonucleotides have been used so far:

[10] replacement of one or a few base pairs or introduction of short deletions

- single-stranded homologous DNA with a mismatch to the target sequence;
- chimeric oligonucleotides consisting of RNA stretches within single-stranded DNA;
- triple helix-forming oligonucleotides (TFOs) which form relatively stable associations with duplex DNA via Hoogsteen hydrogenbonds (parallel and antiparallel)[11];
- RNA oligonucleotides were recently investigated to induce RNA-mediated, targeted DNA nucleotide sequence changes and RNA-templated DNA repair resulting in point mutations;
- LNA or PNA or any other nucleic acid analogues could also be used.

5.1.2 Application of the technique

The induction of point mutations has been successfully performed in agriculturally important plants including rapeseed, maize, tobacco, rice, and wheat (e.g. herbicide tolerance) and it is being explored in animals including sheep and cattle (e.g. genetic improvement of livestock animals). The technique has been used successfully in bacteria and yeast mainly as a tool to perform basic research on gene expression, regulation and genetic recombination, and in human cells for gene therapy.

5.1.3 Other issues

Potential impacts of the technique

If the oligonucleotide and the experimental protocol are adequately designed, the mutation induced by ODM should be highly specific.

5.1.4 Relevant issues for classification

- **Similarity to mutagenesis:**
  - Oligonucleotide-directed mutagenesis (ODM) is a form of mutagenesis induced by oligonucleotides.
  - Only oligonucleotides with sequence similarity or analogy to the recipient's genome are used (they may be modified chemically to improve stability).
  - During the application of ODM, modifications are made to the organism's genetic material by the hosts own repair mechanisms.
  - The induced point mutations are site-specific. Similar mutations can occur spontaneously in nature or may be induced by conventional mutagenesis (chemical or radiation).
- **Nucleic acids:** A majority of experts were of the view that oligonucleotides in this technique cannot be considered as recombinant nucleic acids in the sense of Annex I Part B of the Directive 2001/18/EC or Directive 2009/41/EC. For a minority of experts it is not possible to arrive at this conclusion.

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[11] Chemical modification of the sugar backbone of TFOs (giving so called locked nucleic acids, LNA) increased the stability of the oligonucleotide and of the triplex. PNAs were also employed as TFOs. The introduction of a genetic change by TFOs close to or at the site of binding is thought to involve the cellular DNA repair complex and in some instances the simultaneous presence of an oligonucleotide with the desired point mutation sequence. A special application of TFOs with a reactive group attached to them is also being explored. The reactive group can be a radioactive isotope, e.g. I(125), causing a localized DNA strand break upon decay, or a chemical agent forming DNA adducts or crosslinks after activation, e.g. psoralen derivatives plus photoactivation. Thus, the oligonucleotide can trigger a site-directed DNA damage leading to induced mutagenesis or recombination.

5.1.5 Coverage by GMO legislation

A Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the technique may be attributed

There are two possible interpretations about the coverage of ODM by the GMO legislation (see in particular sections 4.2, 4.3 and 4.4):

1. ODM is not captured by Annex IA Part 1 on the grounds that oligonucleotides introduced into the cell are not recombinant nucleic acid molecules capable of continued propagation (No 1[12]) and they are not heritable material (No 2[13]). Furthermore, the resulting organisms from ODM are captured by Annex IB because the technique entails mutagenesis. Mutagenesis is listed as one of the techniques yielding organisms to be excluded from the application of Directive 2001/18/EC and Directive 2009/41/EC. This represents the view of a majority of experts.
2. ODM is similar to techniques listed in Annex IA, Part 1, because ODM is a recombinant nucleic acid technique that (i) leads to a new combination of genetic material resulting in a heritable change in the DNA sequence (point 1) and (ii) it involves the direct introduction of heritable material prepared outside of the organism (point 2). On this basis, ODM falls under the scope of Directive 2001/18/EC and Directive 2009/41/EC. This represents the view of a minority of experts.

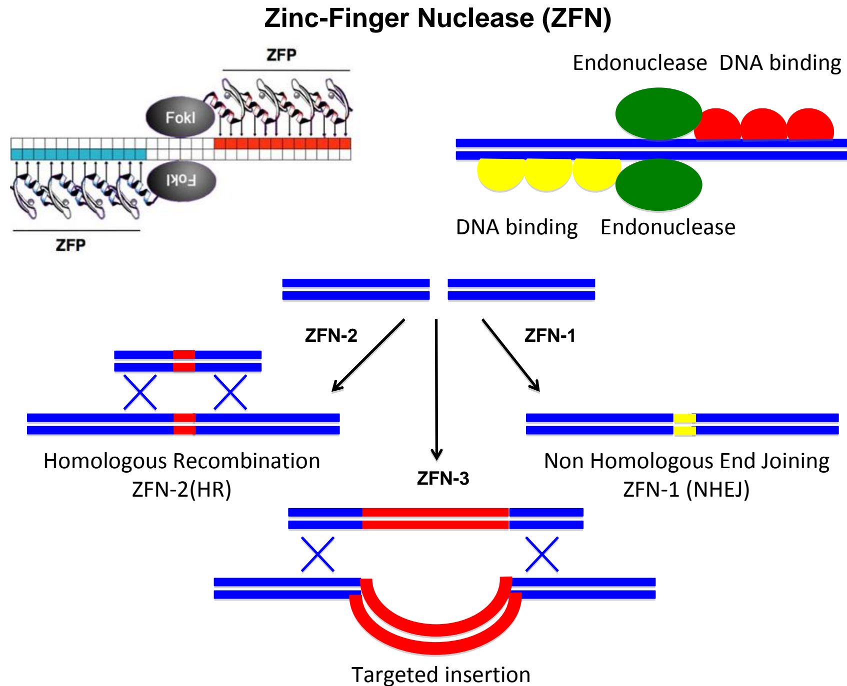
B Concluding remarks for regulation on the status of the technique

- **Directive 2001/18/EC/ Directive 2009/41/EC**
  All experts agree that ODM results in changes in organism that can be obtained with other forms of mutagenesis. They also noted that ODM is expected to generate fewer unintentional changes or effects than those introduced into organisms by irradiation or chemical mutagenesis, which is listed under indent 1 of Annex IB/Annex II Part A "as a technique of genetic modification yielding organisms to be excluded from the Directives". Therefore, ODM is captured by Annex IB/ Annex II Part A. This was a majority opinion.
- **Detection**
  Organisms developed through ODM cannot be distinguished at the molecular level from those developed through “conventional” mutation techniques (using chemicals or ionizing radiations) or through selection in natural populations.

[12] Indent 1 of Annex I A Part 1: "recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not occur nut in which they are capable of continued replication".

[13] Indent 2 of Annex I A Part 1: "techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation".

1 2
5.2 Zinc Finger Nuclease and related techniques



3 4 5
Fig. 2 Zinc Finger Nuclease technique

6 7 8 9
Schematic representation of the three different ways the ZFN technique may be used (see text for details). Blue bars represent the host double-strand DNA; red bars represent heterologous double-strand DNA; FokI = restriction endonuclease, the cleavage domains in ZFNs; ZFP = Zinc-finger protein

10 11 12 13
5.2.0 Introduction

14 15 16 17 18 19 20 21 22 23 24
Zinc Finger Nucleases (ZFN) are protein chimeras comprised of a zinc finger based DNA binding domain linked to a DNA cleavage domain introducing a single-strand cut. Zinc Finger domain(s) can be custom-designed to bind to a specific site within a given locus, thereby providing a highly specific targeting tool. Two ZFN proteins with neighbouring targets on opposite DNA strands (see Figure 2) are necessary to produce a DNA double-strand break (DSB). The genes for the ZFN proteins are delivered for instance by electroporation with plasmids or by infection with viral vectors into the cells; *Agrobacterium*-mediated transfer can also be used in plants. ZFNs are typically expressed transiently from a non-replicating vector (plasmid, virus) however, they may be delivered directly as proteins or as mRNA.

25 26 27 28 29 30 31 32
In the cell, the ZFN proteins recognise the target DNA site as a heterodimer and generate a DSB. Depending on the number of zinc fingers in the ZFN, the target DNA site may be (currently) up to 24 nucleotides long. Such a length potentially makes it feasible to target any gene within a eukaryotic genome specifically. The DSBs are capable of triggering the cell's natural DNA-repair processes: homologous recombination and non-homologous end-joining, thus facilitating site-specific mutagenesis.

As of now, this technique may be used in three different ways, which for practical reasons are designated ZFN-1, ZFN-2 and ZFN-3 in this report. ZFN-1 generates site-specific random mutations by non-homologous end-joining conferring changes of a single or a few base pairs, short deletions and insertions. In ZFN-2, a short homologous repair template is used together with the ZFN-complex, to introduce specific nucleotide sequence changes by homologous recombination. In ZFN-3 a large stretch of DNA (up to several kilobases) with ends homologous to the DNA sequences flanking the DSB site is introduced together with the ZFN-complex. This allows insertions of entire genes at specific locations and ZFN-3 might therefore be used for transgenesis as well as cisgenesis and intragenesis (see section 5.3).

With regard to plants, ZFN techniques have been shown to function in tobacco, Arabidopsis and maize.

ZFN techniques have many applications for humans and animals including gene inactivation, reversion of mutations ("gene repair") and insertion of new genes. In addition, ZFN techniques have been shown to function also in fish, amphibians and invertebrates. The efficiency varies depending on the species.

ZFN techniques are not relevant in prokaryotes for which homologous recombination works and where more cost-effective methods have been established for site-specific mutagenesis. The method works well in yeast, and has potential applications for other eukaryotic microbes although it has not yet been exploited to its full potential.

Instead of ZFN, other nucleases targeting specific sequences of 16 to 22 nucleotides or more are presently being developed and their application explored. These nucleases include an increasing number of engineered meganucleases (homing nucleases from genetic elements; Grizot et al., 2010; Nucleic Acids Res.) and of transcription activator like (TAL) nucleases engineered from transcription-activator-like proteins and a nuclease (Christian et al., 2010; Genetics). What is discussed in this section relating to ZFN-1, ZFN-2, and ZFN-3 similarly applies to the use of these other sequence-targeting nucleases.

5.2.1 Zinc Finger Nuclease-1 (ZFN-1) / Zinc Finger Nuclease-2 (ZFN-2)

ZFN-1 and ZFN-2 are considered together owing to the similarities between the techniques.

5.2.1.1 Definition and brief description of the technique based on the current scientific knowledge and current scientific literature

A Zinc Finger Nuclease-1 technique (ZFN-1) generates site-specific random mutations (changes of single base pairs, short deletions and insertions) by non-homologous end-joining.

During ZFN-1, no repair template is provided to the cells together with the ZFN. The DSB is repaired by non-homologous end-joining which is a natural DNA-break repair mechanism in the cell. This often (though not always)

results in a single or a few base substitutions or small localized deletions or insertions. In the case of insertions, the inserted material is derived from the organism's own genome i.e. it is not exogenous. The DNA end (from the strand break) may also become joined to a completely unrelated site, which results in chromosomal translocation.

B Zinc Finger Nuclease-2 (ZFN-2) generates site-specific the desired point mutation by DNA repair processes through homologous recombination, (specific nucleotide substitutions of a single or a few nucleotides or small insertions or deletions). During ZFN-2, a continuous stretch of DNA is delivered to the cells simultaneously with the ZFN. This template DNA is homologous to the targeted area, spanning a few kilo base pairs (kbp), and overlaps the region of the DSB. The template DNA contains the specific base pair alteration(s) to be introduced into the target DNA or chromosome. The exogenous repair DNA competes with the sister chromatids as a repair template and - with a low frequency - leads to replacement of the original nucleotide sequence. In most studies, the aim has been to replace one or a few bp. There are indications that efficiency of repair decreases where the number of mismatches increases in the template DNA with increasing distance from the DSB. The result is thus comparable with some other site-specific mutagenesis methods.

5.2.1.2 Application of the technique

ZFN-1 and ZFN-2 can be used for targeted random (ZFN-1) or nucleotide-specific (ZFN-2) mutagenesis. In principle, ZFN-1/2 can be applied to any cell line or organism provided there is the possibility to introduce the ZFN proteins or their coding nucleic acids.

5.2.1.3 Other issues

A Potential impacts of the technique

Same as ODM (see 5.1.1.3).

5.2.1.4 Relevant issues for classification

- **Transient presence/effect**:
  Until recently, the approach has been to insert recombinant nucleic acids encoding ZFN into a vector. Provided the construct does not replicate or integrate, its presence in the cell is transient (see section 4.4). In the case of ZFN-2, the repair template is always provided as a specific fragment of DNA. Both the construct and the repair template are transiently present in the recipient cell. The ZFN can also be introduced into the cell as mRNA. It should be noted that recent technical developments allow the ZFN protein to be delivered directly into the cells, thus avoiding the need for DNA recombinant vectors.
- During the application of ZFN-1/ZFN-2, the ZFN encoding nucleic acids give rise to a permanent heritable change owing to a change in the DNA nucleotide sequences. Such modifications to the organism's genetic material are made by the host's own repair mechanisms.

- **Heritable material**:
  With regard to ZFN-2, there was a discussion as to whether oligonucleotides (in this case, the DNA template) constitute heritable material in the sense of the Directives, point on which experts did not agree. (see section 4.3)
- **Resulting organism/Offspring**:
  There was a discussion whether the technique leads to an organism falling within the scope of Directive 2001/18/EC, in particular, if this only depends on whether the GMO offspring does not carry the transgenic construct (see section 4.6).
- **Nucleic Acids:**
  Currently the technique tends to make use of recombinant nucleic acid molecules (vector with genes encoding the two ZFN proteins) or the corresponding mRNAs. Therefore, in accordance with the chapeau of Annex IB of Directive 2001/18/EC and Annex II Part A of Directive 2009/41/EC, the technique can not yield organisms to be excluded from the Directive even if it results in a genetic modification that may happen spontaneously or through other forms of mutagenesis. This conclusion is not valid when the ZFN proteins are delivered directly into the cells (see first bullet under "Transient presence/effect).
- **Similarity to mutagenesis**:
  The same mutations may occur spontaneously or through other forms of mutagenesis (chemical or physical).

5.2.1.5 Coverage by GMO legislation

A. Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the technique may be attributed

- All experts agreed that when the ZFN proteins (concerns ZFN-1) are introduced directly into the cell as a protein, the technique is fully captured by Annex IB (Directive 2001/18/EC) or Annex II Part A (Directive 2009/41/EC) (under which organisms resulting by the listed techniques are excluded from scope of the Directives under certain conditions[14].
- A majority considers the intermediate organisms containing non-replicative constructs or mRNA are not GMOs. A minority of experts agrees that these organisms, containing nucleic acids encoding ZFN proteins (concerns both ZFN-1 and -2), irrespective of its heritability and its capability of continued propagation, is covered by Annex IA, Part 1 of Directive 2001/18/EC and Annex 1 Part A of Directive 2009/41/EC.
- A majority of experts agreed that in the case of ZFN technique, when ZFN proteins are introduced together with repair template directly into cells, the technique is also fully captured by the Annex IB (Directive 2001/18/EC) or Annex II Part A (Directive 2009/41/EC) and therefore the resulting organisms are out of the scope of the Directives through

[14] Chapeau of Annex IB of Directive 2001/18/EC/of Annex II of part A of Directive 2009/41/EC: "… on the condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms [or GMM, resp.] other than those produced by one or more of the techniques/methods listed below…"

the exclusions that these Annexes provide. A majority of experts were of the view that oligonucleotide templates in this technique cannot be considered as recombinant nucleic acids in the sense of Directive 2001/18/EC or Directive 2009/41/EC. For a minority of experts it is not possible to arrive at this conclusion.

- All experts agree that ZFN-1/ZFN-2 results in changes in organism that can be obtained with other forms of mutagenesis. For a majority of experts organisms produced by ZFN-1/ZFN-2 are "to be excluded" in line with Annex IB of Directive 2001/18/EC and Annex II Part A of Directive 2009/41/EC. When recombinant nucleic acid molecules are used (the prevalent technique), for a minority of experts, however, the Directive is clear that organisms produced by ZFN-1/ZFN-2 cannot be excluded on this basis since they do not meet the conditions of the chapeau of Annex IA part 2 of Directive 2001/18/EC and Annex II Part A of Directive 2009/41/EC.

B Concluding remarks for regulation on the status of the technique

- All experts agreed that the organisms resulting from ZFN-1/ZFN-2 are similar to organisms resulting from mutagenesis already identified for exclusion under Annex IB of Directive 2001/18/EC or Annex II Part A of Directive 2009/41/EC. They also noted that these techniques are expected to generate fewer unintentional changes or effects than those introduced into organisms by irradiation or chemical mutagenesis.
- There is a general agreement that the resulting organism from the use of ZNF-1/ZFN-2 is a GMO, but it should be excluded from the Directive. Some experts considered that ZNF-1/ZFN-2 are already captured by Annex IB and thus excluded from the Directive (see above). Other experts feel that exclusion could be achieved by specific inclusion of ZNF-1/ZFN-2 in Annex IB of Directive while further experts are of the opinion that this could be done by clarifying that "mutagenesis" actually includes ZNF-1/ZFN-2.
- **Detection**
  Organisms developed through ZFN-1/ZFN-2 cannot be distinguished at the molecular level from those developed through “conventional” mutation techniques (using chemicals or ionizing radiations) or selection from natural diversity.

1 **5.2.2 Zinc Finger Nuclease-3**
2
3 **5.2.2.1 Definition and brief description of the technique based on the current scientific**
4 **knowledge and current scientific literature**
5
6 Zinc Finger Nuclease-3 technique (ZFN-3) targets delivery of transgenes (insertions)
7 by homologous recombination.
8
9 DNA fragments or gene cassettes up to several kbp in length can be inserted precisely
10 to a desired site in the genome or a gene. In practice, a recombinant DNA molecule is
11 constructed in which the DNA fragment or the gene cassette of interest (donor DNA)
12 is sandwiched between stretches of DNA that are homologous with the DNA
13 sequences flanking the DSB site. Donor DNA can come from any species and it is
14 delivered to the cell, along with the ZFN, and it is targeted to the desired site of the
15 genome and inserted into the DSB site.
16
17 **5.2.2.2 Application of the technique**
18
19 There has been an emphasis to develop and use the technology on human cells
20 although it has also been seen as a powerful tool for genetic engineering of animals.
21 Furthermore, potential applications of the technique include targeted gene addition,
22 replacement and trait stacking in plants. As demonstrated recently (reference), it is a
23 breakthrough to insert DNA into a specific site in the plant (e.g. maize[15]) genome.
24 Previously, this has not been possible.
25
26 **5.2.2.3 Relevant issues for classification**
27
28 - <u>**Nucleic acids and recombinant nucleic acids**</u>:
29 New combinations of genetic material are introduced into the cell when the donor
30 DNA fragment is integrated into the recipient genome and is continuously
31 propagated.
32 - <u>**Similarity to self-cloning**</u>**:**
33 The technique may meet the criteria of self-cloning when:
34 - the donor DNA (or template of the synthetic DNA) originates from an
35 organism of the same species as the recipient or from a phylogenetically
36 closely related species;
37 - the recombinant vector has an extended history of safe use in the
38 particular micro-organism;
39 - the resulting micro-organism is unlikely to cause disease to humans,
40 animals or plants, (see also relevant comments on cisgenesis).
41
42 **5.2.2.4 Coverage by GMO legislation**
43
44 **Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the technique**
45 **may be attributed**
46

[15] *Shukla et al., 2009, Nature 459. 437-441*

- All experts agreed that ZFN-3 is covered by Annex IA, Part 1 of Directive 2001/18/EC since it is a recombinant nucleic acid technique involving the formation of new combinations of genetic material. Organisms developed through this technique therefore fall within the scope of the Directive.
- The technique may in some cases meet the criteria of self-cloning (see 5.2.2.3 above) as described in Annex II, Part A(4)[16] of Directive 2009/41/EC and when that is the case it may be considered to fall outside the scope of Directive 2009/41/EC.

5.2.2.5 Other issues

Potential impacts of the technique

Same as cisgenesis, intragenesis and currently used techniques of genetic modification, depending on the transferred gene

B. Concluding remarks for regulation on the status of the technique

- While the ZFN-3 technique generally is within the scope of Directives 2001/18/EC and 2009/41/EC, in some cases it could meet the criteria of self-cloning as described in Annex II, Part A of Directive 2009/41/EC and when that is the case it may be considered as falling outside the scope of Directive 2009/41/EC.
- **Detection**
  Detection and identification of organisms modified by ZFN-3 technology are possible through the amplification based methods (PCR) currently used for GMO detection, with the prerequisite that prior adequate DNA sequence information on the introduced modification is available.

[16] Self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism which may or may not be followed by reinsertion of all or part of that nucleic acid (or a synthetic equivalent), with or without prior enzymic or mechanical steps, into cells of the same species or into cells of phylogenetically closely related species which can exchange genetic material by natural physiological processes where the resulting micro-organism is unlikely to cause disease to humans, animals or plants. Self-cloning may include the use of recombinant vectors with an extended history of safe use in the particular micro-organisms.

5.3 Cisgenesis and Intragenesis

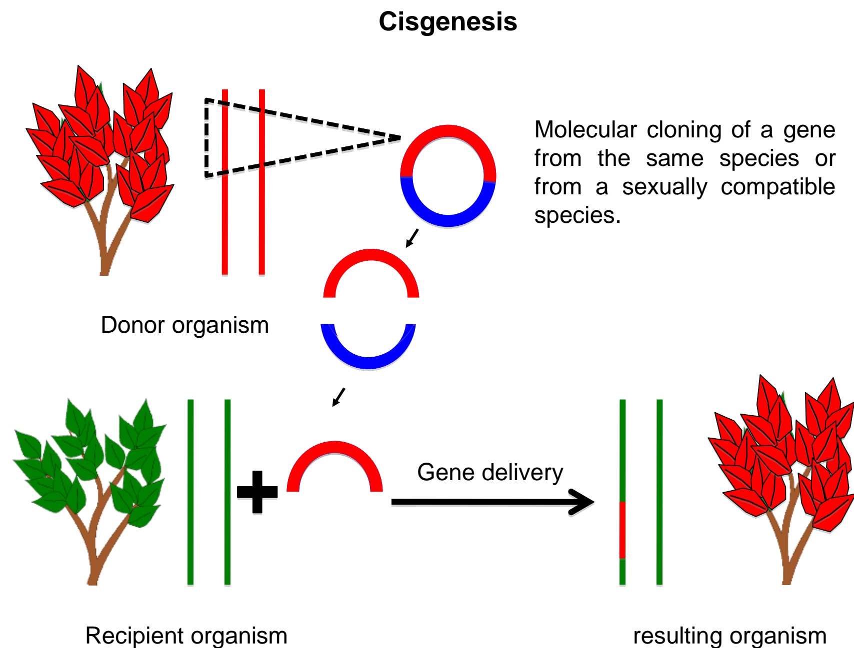


Fig. 3 Cisgenesis

Schematic representation of cisgenesis. Blue: nucleic acid from the vector; red: DNA sequences from the donor plant; green: DNA sequences from the recipient plant. Donor and recipient organism belong to species which are sexually compatible.

5.3.1 Definition and brief description of the technique based on the current scientific knowledge and current scientific literature

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

Cisgenic plants can harbour one or more cisgenes, but they do not contain any parts of transgenes or inserted foreign sequences. To construct cisgenic plants the same molecular biology techniques used for construction of transgenic organisms may be used. Genes must be isolated, cloned or synthesized and transferred back into a recipient where stably integrated and expressed.

Sometimes the term cisgenesis is also used to describe an *Agrobacterium*-mediated transfer of a gene from a crossable - sexually compatible – plant. If T-DNA borders remain in the resulting organism after transformation, the technique is referred further in the text as cisgenesis with T-DNA borders.

Intragenesis is a genetic modification of a recipient organism that involves the insertion of a reorganised, full or partial coding region of a gene frequently combined with a promoter and/or terminator from another gene (intragene) of the same species or a crossable species. These may be arranged in a sense or antisense orientation compared to their orientation in the donor organism.

5.3.2 Application of the technique

Often, the genome of an undomesticated line of a plant species may be a source of a valuable gene, e.g. in determining disease resistance. In order to transfer such a gene of interest to a domesticated plant line of that species, e.g. of a high yielding variety, it is necessary to perform a consecutive line of several crosses and backcrosses. Thus it may take 10 - 15 years (for annual plants) or more to develop a cultivar possessing the desired gene from the non-adapted plant by traditional breeding. The application of cisgenesis would condense this otherwise lengthy process. In addition, cisgenesis and intragenesis prevents so-called linkage drag of unwanted or undesirable genes, which is often observed in traditional breeding. Cisgenesis and intragenesis are currently mainly applied in plants, however the technique may be similarly used in animal breeding. According to the experts current commercial applications relate more frequently to intragenesis than to cisgenesis.

5.3.3 Other issues

A. Potential impacts of technique[17]

Cisgenesis may lead to the disruption of existing ORFs or creation of new one(s), due to random insertion of the gene in any part of the genome, or over expression of a native (cis-)gene. This could lead to altered biochemical properties, allergenicity, and/or toxicity. However, similar effects can sometimes take place in traditional breeding or via normal biological processes.

5.3.4 Relevant issues for classification

- **Similarity to currently used techniques of genetic modification:** The random insertion of a cisgene or intragene could induce alterations in the plant genome in a similar manner to transgenes, e.g. the creation of new open reading frames (ORFs) and the disruption of existing genes. There is also the possibility of multiple insertions and over-expression of the inserted gene(s).
- **Similarity to traditional breeding:** Cisgenesis yields organisms similar to those obtained by traditional breeding techniques or via normal biological processes of reproduction. This is also the case with cisgenesis with T-DNA borders, where the T-DNA border sequence is identical to or has high similarity (≥85% identity) to DNA sequences already present in the genome of the plant species.

[17] Transgenic Atlantic salmon that grows faster than ordinary farmed salmon has already been developed, and it is likely that cisgenic salmon with similar characteristics can be produced.

1 **5.3.5 Coverage by GMO legislation**
2
3 **A Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the**
4 **technique may be attributed**

5 • All experts concluded that cisgenesis and intragenesis are covered by
6 Annex IA, Part 1 of Directive 2001/18/EC. Organisms developed through
7 these techniques therefore fall within the scope of Directive 2001/18/EC.
8 • All experts indicated that cisgenesis is similar to self-cloning. As regards
9 cisgenesis with T-DNA border sequences, all experts agreed that if
10 sequences identical to T-DNA border sequences or with high similarity
11 (≥85% identity) are already present in the genome of the same or sexually
12 compatible species, the resulting organism may be considered as equivalent
13 to organisms resulting from self-cloning on the basis that these
14 modifications could also be obtained by traditional plant breeding
15 techniques, or take place via normal biological processes of reproduction.
16 If the T-DNA borders are not present in the same or sexually compatible
17 species, all expert agreed that the organism cannot be obtained by
18 traditional breeding and the technique cannot be considered as self-cloning.
19 • All experts agreed that intragenesis cannot be considered as yielding GM
20 organisms equivalent to organisms resulting from self-cloning, and such
21 organisms cannot be obtained by traditional breeding. Micro-organisms
22 developed through self-cloning are covered by Annex II, Part A(4) of
23 Directive 2009/41/EC and are thereby excluded from the scope of
24 Directive 2009/41/EC. This exclusion only applies where "… the resulting
25 micro-organism is unlikely to cause disease to humans animals or plants".
26 It similarly applies to animal and plant cells in culture.[18]
27
28 **B Concluding remarks for regulation on the status of the technique**

29 • The technique may in some cases meet the criteria of self-cloning as
30 described in Annex II, Part A of Directive 2009/41/EC and when that is the
31 case it may be considered as falling outside the scope of Directive
32 2009/41/EC. More specifically, all experts considered that cisgenesis and
33 cisgenesis with T-DNA borders (ONLY with border sequences identical or
34 highly similar (≥85% identity with sequences) already present in the same
35 or sexually compatible species) may be considered as yielding organisms
36 equivalent to those resulting from self-cloning and therefore that could be
37 considered as out of the scope of Directive 2009/41/EC.
38 • The co-legislators treated self-cloning differently in Directive 2001/18/EC
39 and in Directive 2009/41/EC. Assuming that the logic for having excluded
40 self-cloning from Directive 2009/41/EC was that it yields micro-organisms
41 with modifications that could also be obtained by traditional breeding, all
42 experts wondered about the justification for not excluding them from
43 Directive 2001/18/EC.
44 • **Detection**

[18] Article 2(a) of Directive 2009/41/EC defines micro-organism as "…any microbiological entity, cellular or non-cellular, capable of replication or of transferring genetic material, including viruses, viroids, and animal and plant cells in culture"

1 The introduction of cisgenes and intragenes into plants can be specifically
2 detected if a sufficient part of the sequence of the insert as well of the
3 adjacent sequence is known.
4
5

5.4 Grafting

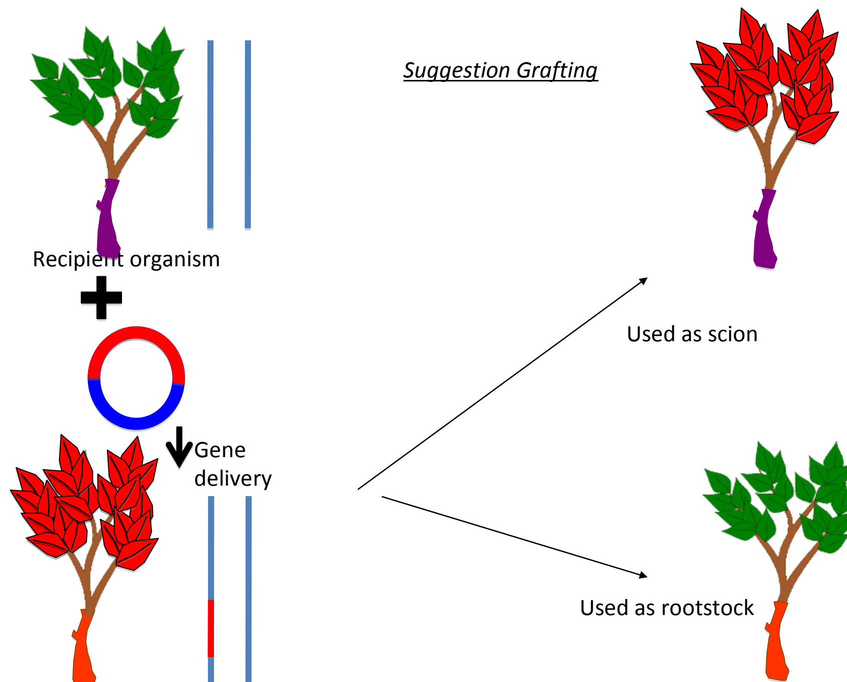


Fig. 4 Grafting
Schematic representation of grafting using both conventionally bred and GM plants. Top left: a conventionally bred plant line; bottom left a transgenic plant line derived thereof; right two different chimera, where either the transgenic plant line or the conventionally bred plant line (bottom) are used as root stock or scion, respectively.

5.4.1 Definition and brief description of the technique based on the current scientific knowledge and current scientific literature

Grafting is an ancient technique used to combine desired traits of two different plants. It is a method whereby a vegetative top part (*the graft or scion*) of one plant is attached to a rooted lower part (*the rootstock*) of another plant. A callus develops in the junction area and the callus cells become differentiated into the same type of cells as those in the vicinity. In particular, the vascular systems of both parts are connected, which allows the transfer of sap between the rootstock and the graft. Although different parts of the plant consist of genetically distinct cells, the whole plant forms a single organism ('chimera[19]').

Two possibilities were considered:

- Grafting a non-GM scion onto a GM rootstock;
- Grafting a GM scion onto a non-GM rootstock.

Proteins and RNAs can conceivably be transported from the rootstock through the graft junction and into the scion and vice versa. This can affect the gene expression and phenotype of the upper (or lower) part of the plant and is independent of whether the rootstock (or the scion) is genetically modified or not. Although transport of macromolecules may occur from the lower part of the GM plant (rootstock) to the

[19] A chimera is an organism consisting of at least two genetically different kinds of tissue.

non-GM scion, these are not transmitted to the next generation via the seeds as they do not lead to genetic modification.

5.4.2 Application of the technique

Grafting has been extensively applied to fruit trees, but grafting is also used on for example vegetables, especially within the genera Cucurbita and Solanum.

Cultivation of grafted vegetables has been practiced in many countries for several decades and is increasing in Europe. Watermelon and tomato are the two major vegetables where grafting is important worldwide. Grafting is also routinely used with other vegetables such as cucumber, melon, oriental melon, greenhouse squash, eggplant, grapevine and pepper. One of the major advantages of using grafted plants is e.g. to utilize the resistance of rootstocks to certain soil-borne diseases like *Fusarium* wilt in cucurbits and tomato, *Phytophthora* disease in pepper, and virus in tomato. Watermelons, for example, are grafted onto rootstocks of squash and gourd varieties that are resistant to *Fusarium* and other soil-borne diseases that would otherwise devastate the watermelons.

5.4.3 Other issues

A Potential impacts of technique

- Risk assessment considerations: Where a non-GM scion is grafted onto a GM rootstock, the scion (including any tissue or organisms derived from it) is considered non-GM since no change in coding sequence is present in the scion. However, there may be a change in gene expression or in protein/metabolite composition in the scion due to the transport of molecules across the graft. For environmental and food/feed safety purposes, the risk assessment should consider the entire plant and take into account potential effects of the GM rootstock on the scion. The assessment should take into account the consideration that the same GM rootstock may be used in combination with different non-GM scions, or that the same non-GM scion may be used in combination with different GM rootstocks. If effects on the scion are identified conditions may be posed on the use of the GM rootstock or the whole plant. The above-mentioned considerations would also apply to the case of a non-GM rootstock combined with a GM scion.
- Following risk assessment of a GM rootstock, specific conditions can be taken into consideration on the use of the non-GM scion on a case-by-case basis, depending on the nature of the modification of the GM rootstock and its impact on the non-GM scion.

5.4.4 Relevant issues for classification

- The chimeric plant consists of a GM part (e.g. GM-rootstock), fused to a non-GM part (e.g. non-GM scion).
- Where a non-GM scion is grafted onto a GM rootstock, the whole plant is a chimera in which the flowers, seed, fruits, and parts of the scion like the leaves are non-GM. However, where the rootstock entails e.g. a woody crop, suckers

may grow from the rootstock and produce flowers and seeds (fruits). These seeds or fruits are by definition GM.

- Where a GM scion is grafted onto a non-GM rootstock, the whole plant is a chimera in which the flowers seeds, fruits, and parts of the scion like the leaves are GM.
- In some cases, grafting could be used as a tool to obtain an effect through RNA interference. In this case, the conclusions of the WG for RNA-dependent DNA methylation should also be taken into account (see point 5.4).

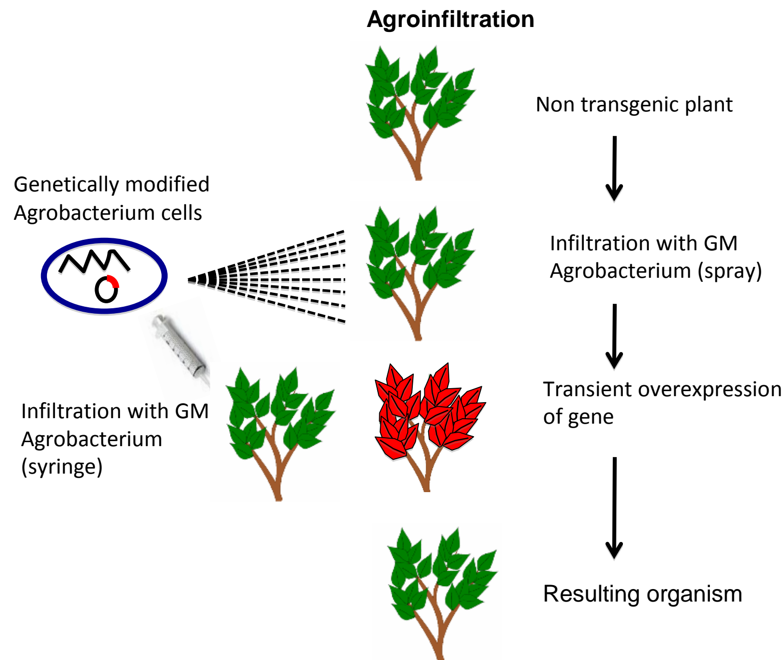
5.4.5 Coverage by GMO legislation

- There is a general agreement that the whole plant, which is a chimera, falls within the scope of Directive 2001/18/EC.
- Where the fruit/seed/offspring are being considered and where a non-GM scion is grafted onto a GM rootstock, the resulting fruit/seeds/offspring derived from the scion do not fall under the scope of Directive 2001/18/EC.
- Where a GM scion is grafted onto a non-GM rootstock, the resulting fruit/seeds/offspring fall under the scope of Directive 2001/18/EC.
- **Detection**

  The transgenic part of the chimera can be specifically detected if a sufficient part of the sequence of the insert as well of the adjacent sequence is known. As the DNA sequence of the non-GM scion is not modified, detection and identification of the GM rootstock on the basis of the harvested product (part of the non-GM scion) is currently not possible and is very unlikely to be developed in the near future.

Final

1 **5.5. Agro-Infiltration**
2
3



4
5 Fig. 5 **Agro-infiltration:**
6 Schematic representation of agro-infiltration. The syringe and the spray indicate two alternative ways of
7 application. The black arrows on the right indicate the steps in the procedure. The transient nature of agro-
8 infiltration is reflected by the green plant at the bottom of the figure, where the red leaf colour has disappeared.
9 Note that only very few, if any, plant cells at the site of infiltration are transformed, thus the red plant here does
10 not indicate that the plant is transgenic, but the transient expression of the infiltrated gene.
11
12
13

14 **5.5.1 Definition and brief description of the technology based on the current scientific**
15 **knowledge and current scientific literature**
16
17 Bacteria in the genus *Agrobacterium*, and especially *A. tumefaciens*, have the
18 capability to transfer a part of their DNA (the so called T-DNA) into the nucleus of
19 plant cells. Agro-infiltration aims to use *Agrobacterium* to inject large numbers of
20 foreign DNA molecules into the plant cells, so that they can be used as templates for
21 the cell's transcription/translation machinery with no need for replication or
22 integration.
23
24 To perform agro-infiltration, plant tissues are infiltrated (*in vivo* or *ex vivo*) with a
25 liquid suspension of *Agrobacterium* sp. containing a genetic construct in order to
26 promote localised expression of a given genetic material. The benefits of agro-
27 infiltration over stable transformation are speed, convenience, and the high level of
28 expression usually reached.
29
30 In some *in vivo* applications the T-DNA may contain replicative material (in the form
31 of either fully functional virus genome, or as “replicons" not able to spread within the

plant) in order to obtain expression in parts of the plant other than the infiltrated area or to further increase the expression level.

5.5.2 Application of the technique

Depending on whether or not the plant tissues contain germline cells/tissues, two types of agro-infiltration can be distinguished:

1. **Agro-infiltration "*sensu stricto*":**
   Non-germline tissues (typically, leaf tissues) are agro-infiltrated in order to obtain localised expression, for instance:
   i. to obtain large amounts of a given protein expressed in plant tissues;
   ii. to test the phenotypic effect of a given gene product in plants (for instance, when an avirulence[20] gene or similar factor is expressed, the possibility to rapidly screen plant populations for the presence of the cognate resistance gene has potential applications in the breeding industry).
2. **"Floral dip":**
   Flowers or inflorescences containing germline cells are agro-infiltrated in order to obtain stable transformation of some embryos that can then be selected at the germination step. Nowadays this is the technique of choice to transform the model plant *Arabidopsis thaliana*, and it can also be used for other species in the same family (including rapeseed, cabbage, mustard etc).

Agro-infiltration takes place under conditions of containment. Progeny, if produced, is grown in a greenhouse and may be grown outdoors in some cases, for instance when agro-infiltration is used as a tool in the process of selecting commercial varieties with a given phenotype.

5.5.3 Relevant Issues for classification of the resulting plant

- The agro-infiltrated plant tissues contain *Agrobacterium* cells in their intercellular space. The *Agrobacterium* cells do not enter the plant cells. Generally they remain localised in the infiltrated area, where they survive and can even multiply since this situation is similar to their ecological niche. Some movement of the bacterial cells to non-infiltrated areas of the plant (including the reproduction organs) is conceivable but not expected.
- These *Agrobacterium* cells contain a plasmid with a T-DNA region in which, typically, foreign DNA has been inserted by biotechnological techniques. The bacteria actively deliver the T-DNA into the plant cells.
- The T-DNA accumulates but does not replicate in the plant cell. It undergoes nuclear transcription and the derived mRNA undergoes translation. Integration into the genome is a rare event that can occur in a small number of cells in each infiltrated area: the selection of these rare events to regenerate stable transformants

[20] Plants have an innate immunity system based on Resistance (R) genes that, in the presence of cognate Avirulence (Avr) genes of their pathogens, trigger a localised response involving cell death and systemic acquired resistance; ectopic expression of the Avr gene in cells bearing the R gene in their genome leads to cell death. Agro-infiltration may be used to screen plant populations for the presence of the R gene on the basis of induction of a localised necrosis in the infiltrated area.

is not the aim here except in the case of floral dip. In the unlikely event that T-DNA is delivered to germline cells, in non-infiltrated areas of the plant integration into the genome of a germinal line could occur. Again, while this is conceivable, it is highly unlikely (it has never been observed to date).

- The nature of the foreign DNA inserted within the T-DNA region may have a bearing on its ability to replicate and /or whether it integrates into the plant genome, and if it occasionally does, with what frequencies. For instance, it is the aim of applications where the T-DNA contains replicative material (such as viruses) to achieve expression in the entire plant.
- In most applications, the plant (or plant tissues) subjected to agro-infiltration *sensu stricto,* is not allowed to set seed, but is destroyed at the end of the procedure (purification of the expressed protein).
- Sometimes progeny could be produced when agro-infiltration is used in the process of screening plant populations for a desired phenotype (i. e. *sensu stricto*).
- In the case of floral dip a progeny is generally produced and selected for stable integration of the T-DNA in its genome.

5.5.4 Coverage by GMO legislation

A Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the technique may be attributed

- The recombinant *Agrobacterium.* It is a GMM and falls within the scope of Directive 2009/41/EC (Annex I, Part A) when it is present.
- The plants that were subjected to agro-infiltration. Integration of the foreign DNA into the genome of a few plant cells of the infiltrated area is a rare event that needs to be examined on a case-by-case basis. Furthermore, cells in which T-DNA integration into the genome has occurred will not be selected for the regeneration of entire plants (even in the case of floral dip, this selection process occurs in the following generation). On this basis, there was a divergence of opinion among the experts as to whether plants subjected to agro-infiltration should formally be considered to fall under the scope of the Directives. However all experts agreed that these plants fall under the scope of Directive 2009/41/EC since they contain GMMs (see above).
- The progeny of the plants subjected to **agro-infiltration "*sensu stricto*".** Plants derived from lines where the absence of a stably integrated event into the genome is supported by relevant data, should be considered as falling outside the scope of Directive 2001/18/EC.
- The progeny of the plants subjected to **"floral dip".** Since floral dip is actually designed to produce plant lines with stable integration events, the progeny lines that are shown to harbour a stable integration event fall clearly within Annex IA, Part 1 of Directive 2001/18/EC.

B Concluding remarks for regulation on the status of the technique

- It was underlined by some experts that agro-infiltration is just one amongst several techniques used to introduce DNA into a few cells of a pluricellular organism in order to obtain localized expression of a foreign DNA. Elements of the opinion above could also be helpful to assess these techniques.
- The recombinant bacterial vector and the progeny of the plants subjected to floral dip clearly fall within the scope of Directive 2009/41/EC (Annex I, Part A) and Directive 2001/18/EC (Annex IA, Part 1) respectively. The experts had divergent opinions regarding the status of the agro-infiltrated plants with regard to Directive 2001/18/EC (exclusion or inclusion on the basis of a few isolated cells usually bound for destruction), but agreed that the large bacterial population hosted in its intercellular space fall within the scope of Directive 2009/41/EC (Annex I, Part A). In situations where the agro-infiltrated plants produce a progeny, this progeny should be considered to fall outside the scope of Directive 2001/18/EC once the absence of a stable integration event is shown.
- **Detection**
  If the constructs introduced into plants by agro-infiltration are not replicated and/or integrated, their presence is transient and can be detected only in the agro-infiltrated plant itself. These DNA fragments will not be transferred to the next generation so they cannot be detected or identified in the progeny plant and the products derived thereof. Detection and identification of such products from agro-infiltration or from agroinoculation is therefore not possible. Detection and identification of agroinfiltrated plants and progeny plants that contain stably inserted fragments is possible with the same methodologies that are currently developed and used for GMO detection, which also implies that adequate information needs to be available.

  In the case of **floral dip**, the aim is to select for stable integration into the germline, leading to a genetically modified plant, which means that detection and identification are possible with the methods currently available for GMO detection (PCR), and also implies that adequate information needs to be available.

1 **5.6 RNA-dependent DNA methylation (RdDM)**
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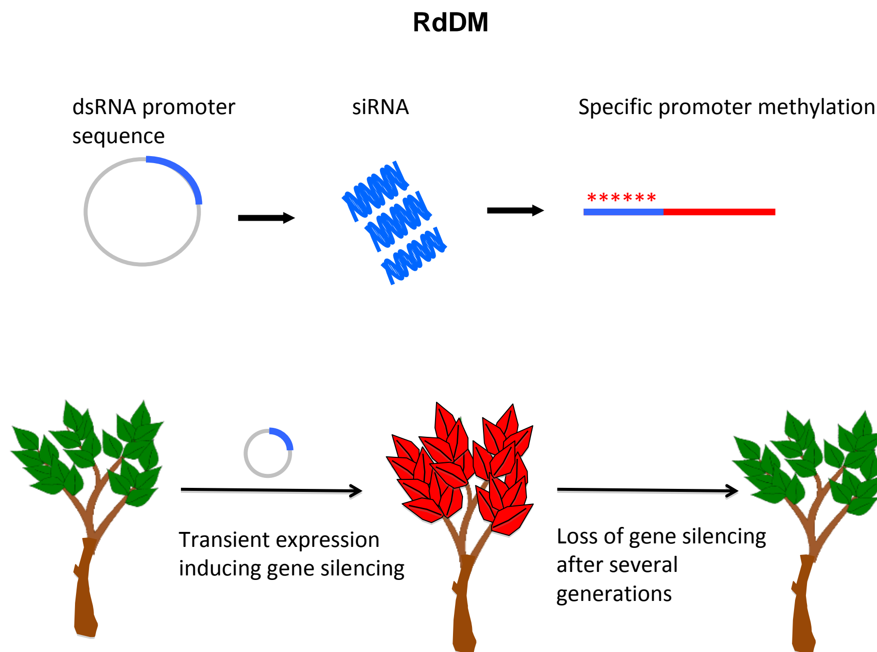

3
4 **Fig. 6 RNA-dependent DNA methylation**
5 Schematic representation of RNA-dependent DNA methylation. SiRNA can be introduced via a vector (top left),
6 or introduced directly. Specific methylation of plant genes is indicated by asterisks above the DNA sequence.
7 The red plant in the bottom indicates the (transient) silencing of plant. This does not necessarily mean that the
8 plant is transgenic.
9
10
11
12 **5.6.1 Definition and brief description of the technique based on the current scientific**
13 **knowledge and current scientific literature**
14
15 RdDM is a technique that uses the effect of small RNA sequences e.g. micro RNA
16 (miRNA) or small/short interfering RNA (siRNA) to alter gene expression through
17 methylation of specific DNA sequences without changing the nucleotide sequence
18 itself (epigenetic change). The purpose could be to shut down expression of specific
19 genes. This gene silencing obtained by the methylation can be inherited through some
20 generations, but will eventually disappear.
21
22 The mechanisms by which the DNA methylation and eventually the gene silencing
23 takes place are part of a complex and naturally occurring mechanisms that organism
24 use in their cell regulation.
25
26 Gene silencing through DNA methylation can be accomplished in a cell or in an
27 organism by introducing a gene which, once transcribed, gives rise to the formation of
28 double stranded RNAs (dsRNA) and further into small interfering RNA. If these
29 interfering RNA molecules share homology with a promoter region, they can
30 specifically induce methylation resulting in the silencing of the gene regulated by the
31 promoter. This technique allows for highly targeted gene silencing. DNA methylation
32 patterns can be maintained, even after clearance of the initially new inserted gene e.g.

due to segregation. However the effect will decrease through subsequent generations and will eventually fade out restoring the original expression of the gene(s) involved.

5.6.2 Application of the technique

In mammalian cells, nematodes and flies, RdDM can be induced by direct introduction of double stranded RNAs (e.g. by liposomes or naked siRNA covalently conjugated with protein).

In contrast, RdDM in plants is usually established by transformation with a DNA construct that encodes hairpin RNAs. For the purpose of the further discussion below certain scenarios can be envisaged:

- Scenario 1): the RdDM is accomplished by insertion of DNA that contains new combinations and that are inherited to the next generation. The resulting organism contains this new DNA and also the new methylations and expression pattern.
- Scenario 2): the RdDM is accomplished by insertion of DNA that is capable of continued propagation but only present in the intermediate organisms. The resulting organism, however, is free of this new DNA by segregation but still contain the new methylations which eventually fades off.
- Scenario 3): the RdDM is accomplished by the insertion of DNA or RNA that are not capable of continued propagation. The resulting organism is free of this new DNA/RNA but contain the new methylations.

A possible limitation of the technique in some applications may be that the epigenetic changes fade out and in that sense the duration of the effect is unpredictable over time. Changes over generations are expected to last longer in plants than in animals.

5.6.3 Relevant Issues for classification

- **Similarities to natural processes**: DNA methylation leading to changes in gene expression and phenotype can also occur naturally.
- **Intermediate organism and resulting organism**: both the insertion of the DNA or RNA resulting in RdMD can be accomplished in different ways (e.g. direct/indirect, with/without vectors involved) and the resulting organisms can be selected in different ways (e.g. with/without inserted DNA). All these factors can influence on the interpretation on the GMO status of the intermediate as well as the resulting organisms and therefore lead to many different views from the experts (se also 4.6 offspring section).

5.6.4 Coverage by GMO legislation

A Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the technique may be attributed

All experts agreed that the technique used in scenario 1 is covered by Annex IA, Part 1 of Directive 2001/18/EC and Annex I Part A or Directive 2009/41/EC and the resulting organisms are GMOs.

All experts also agree that the new methylation itself is not regulated by the Directives since methylation of nucleotides is not considered as an alteration of the genetic material in the sense of the Directives. Therefore, for most of the experts the resulting organisms in scenario 2 and 3 is not covered by Annex 1A, Part 1 of Directive 2001/18 or Annex I Part A or Directive 2009/41/EC because neither of these organisms (with the new methylation) contains any heritable changes in nucleotide sequences. This conclusion is reached even if the intermediate organisms are considered GMOs. The arguments for this conclusion are related to the discussion about transient presence and offspring in chapter 4.4 and 4.5.

A majority of experts considered that intermediate organisms (scenarios 2 and 3) do not fall under the definition of GMO of either Directives if the RNA is directly delivered into the cell without being able to replicate. This was based on the grounds that the selected offspring organism does not contain the inserted gene coding for siRNA and no heritable changes in nucleotide sequences are created. The resulting organisms are thus not GMOs.

A few experts considered that if the intermediate organism is considered a GMO the resulting organism is also a GMO in the sense of the Directive. This was based on the ground discussed in the section on GMO offspring.

All experts agreed that the resulting organism (e.g from scenarios 2 and 3), not containing the inserted gene coding for RNAi and having no heritable changes in nucleotide sequences, should logically be outside the scope of the Directives due to the fact that the resulting organisms are comparable to organisms obtained with natural processes.

B Additional remarks for regulation on the status of the technique

The current and future status of the technique depends on the interpretation of the legislation as regards the use of intermediate organisms considered GMOs.

5.6.5 Other issues

A Potential impacts of the technique

Since RdDM-associated transcriptional silencing is apparently influenced by the local surrounding genomic condition of the target promoter locus, reduction of promoter activity may significantly vary among the endogenous promoters of interest, ranging from no reduction to an almost complete knockdown level by the effects of the surrounding genomic sequences.

A possible limitation of the technique may be envisioned in the fact that the epigenetic changes are unstable and in that sense unpredictable over time.

B Concluding remarks

- The current and future status of RdDM depends on the interpretation of the legislation as regards the use of intermediate organisms considered GMOs.

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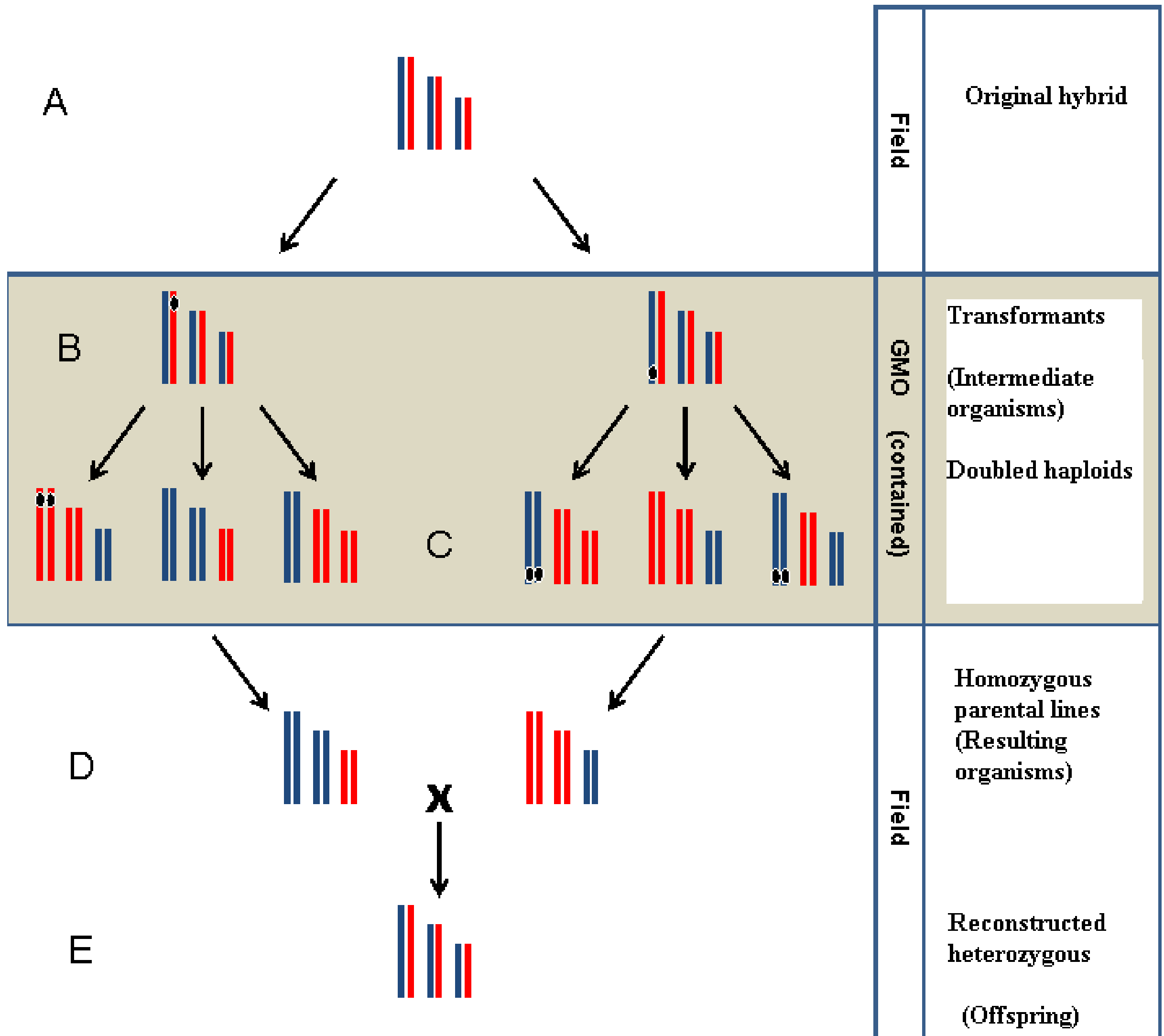
- **Detection**
  The characteristics (phenotype and methylation) can be used for identification only as long as the effect from earlier generations continues.

Final

1 **5.7 Reverse breeding**
2



3
4 **Fig. 7 Reverse Breeding**
5 Schematic explanation of the steps involved in reverse breeding. For details see text.
6
7
8

9 **5.7.1 Definition and brief description of the technology based on the current scientific**
10 **knowledge and current scientific literature**
11

12 Reverse breeding allows to produce specific F1 hybrids in a much shorter timeframe
13 and ambient numbers in comparison with conventional plant breeding techniques. In
14 reverse breeding (Fig. 7), an individual heterozygous plant is chosen for its elite
15 quality (Fig. 7, A), and, subsequently, homozygous parental lines are derived from this
16 plant (Fig. 7, D), which upon crossing, can reconstitute the original genetic
17 composition of the selected heterozygous plant (Fig. 7, E) from which the lines were
18 derived.
19

20 During reverse breeding, a genetic modification step is employed to suppress
21 recombination during meiosis. However, the final heterozygous plants (and their
22 homozygous parental lines) are non-transgenic (devoid of any new DNA).

To obtain the homozygous parental lines the following steps are taken:

- First, in cells derived from the chosen heterozygous plant, meiotic recombination is suppressed through RNAi (RNA interference)-mediated down-regulation of genes involved in the meiotic recombination process. The RNAi construct is integrated into one chromosome of the cells, (Fig. 7, B).
- The next step is regeneration of these cells into plants (Fig. 7, C). During flowering of this plant with suppressed recombination, haploid microspores (immature pollen grains) are formed.
- The genomes of these haploid spores will subsequently be doubled upon specific treatments. The diploid microspores can eventually develop into embryos and subsequently into homozygous plants (so-called doubled haploids), the homozygous parental lines.

Crossing appropriate pairs of those homozygous plants (Fig. 7, D) will create the desired heterozygous genotype (Fig. 7, E). Using homozygous parental lines for the cross that do not contain the transgenic RNAi-constructs ensures that the resulting final heterozygous plants are non-transgenic (Fig. 7, E). This plant breeding approach offers advantages over existing methods in that in principle any heterozygous plant can now be commercially exploited through reconstitution of suitable parental lines.

5.7.2 Application of the technique

Reverse breeding allows breeders to produce specific F1 hybrids in a much shorter timeframe, in comparison with conventional plant breeding techniques. Furthermore, it provides more flexibility in combining desired traits in a heterozygous setting.

5.7.3 Relevant Issues for classification

- **Resulting organism and GMO offspring:** following suppression of meiotic recombination, GM (containing the gene for RNAi production) and non-GM doubled haploid plants are generated. Screening is performed to use only non-GM doubled haploids for crosses (which can result in the final hybrids which are the same as the original plant) and on this basis what should be considered non-GM.

5.7.4 Coverage by GMO legislation

A Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the technique may be attributed

- All experts agreed that the specific step where RNAi-mediated suppression of recombination is performed, gives rise to an intermediate organism falling within the scope of Directive 2001/18/EC according to Article 2 and Annex IA, Part 1 of this Directive, and also Directive 2009/41/EC provided it is a microorganism.
- All experts agree that no alteration has been made in the resulting organism (the homozygous parental lines, Fig. 7, D, selected not to carry the genetic modification) and their offspring (Fig. 7, E): are non-GM plants and therefore

may be considered as outside the scope of Directives 2001/18/EC and 2009/41/EC.

B Concluding remarks for regulation on the status of the technique

The resulting plants and their offspring are not GM and therefore may be considered as not within the scope of Directives 2001/18/EC and 2009/41/EC on the following grounds:

- The genetic material of the resulting organisms and their offspring have never contained any inserted foreign DNA;
- the genetic composition of the offspring is the same as the original organism or plant material; and
- the resulting organisms and their offspring can be obtained by traditional breeding techniques.

This interpretation is only valid on the condition that only non-GM homozygous plants (doubled haploids) are chosen after the genetic modification step, in order to perform crosses to obtain the desired hybrids.

Detection

Detection is not possible as the end-products of reverse breeding are free of genetic modification-related sequences.

5.8 Synthetic Genomics

5.8.1 Definition and brief description of the technology based on the current scientific knowledge and current scientific literature

Synthetic genomics is a field within synthetic biology that may include techniques of genetic modification. It involves the synthesis of stretches of DNA molecules and their combination into functional larger synthetic DNA molecules which are then transferred into a recipient structure. The synthesis of building blocks enables the easy introduction of changes into the genetic material, including mutations (exchanges, deletions and insertions of specific nucleotides), gene fragments or complete genes including those without any natural template.

Synthetic genomics also paves the way for the introduction of redesigned or newly designed combinations of biological parts that do not necessarily exist in nature and that, for instance, enable the reconstruction of new biological pathways. Alternative genetic codes that are composed of chemically modified nucleic acids ("xeno nucleic acids"[21]) and not currently found in life forms could also be used (field called Xenobiology), emphasizing that synthetic genomics merges with other fields of synthetic biology.

Given the fact that synthetic genomics (and more generally synthetic biology) is a fast-evolving field with a potential for very new developments as compared to what can be achieved with gene modification techniques currently listed in the Directives, the WG felt that the subject was too broad to discuss in this context. The analysis and opinion below therefore focuses on very specific and already available applications of synthetic genomics involving the combination of DNA fragments.

5.8.2 Application of the technique

The technique is used for basic research as well as for biotechnological applications.

With regard to basic research, the use of synthetic genomics may contribute to an improved understanding of existing biological systems or the interaction between pathways. For instance, the smallest known bacterial genome, that of *Mycoplasma genitalium*, has been synthesised recently from synthesised pieces of about 6 kb long as a proof-of-concept experiment.

As regards biotechnological applications, the construction of a minimal genome and its use as a basic framework to introduce biological parts could lead to the development of products such as biofuels, pharmaceutical products, cosmetics or products for bioremediation.

Apart from synthetic genomes transplanted into a chassis derived from natural bacteria, no examples of a synthetic genome being able to sustain life and to reproduce itself has been reported so far in the literature, although this is expected to be achieved in the next few years. Currently, large synthetic genomes are expected to be

[21] "Xeno nucleic acids" (XNA) are nucleic acids encompassing diversifications of the nucleic bases, the leaving-group and/or the backbone motif.

introduced into host cells by so-called genome transplantation. As it has been shown, complete bacterial genomes can be introduced into a living cell where through selection, the parent genome can be replaced by the introduced one.

5.8.3 Relevant Issues for classification

The creation of synthetic genomes and their transplantation into recipient hosts appears to meet the definition of genetic modification as laid out in Directives 2001/18/EC and 2009/41/EC. However, the following elements should be taken into consideration:

- Synthetic genomes can be introduced into a recipient such as cell extracts or protocells. Since these recipients as such, are not capable of replication (in the meaning of the Directives) or of transferring genetic material they do not meet the definition of an organism or microorganism.
- Once synthetic genomes are introduced into a host then the resulting entity meets the definition of an organism or micro-organism, if it is capable of replication or of transferring genetic material in the meaning of the Directives.
- Point 2 of Annex I Part A of Directive 2009/41/EC (and of Annex IA Part 1 of Directive 2001/18/EC), which refers to techniques involving the direct introduction of heritable material prepared outside the organism, could be interpreted as including introduction of full genomes into a recipient structure.

5.8.4 Coverage by GMO legislation

A Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the technique may be attributed

To date most of the work on synthetic genomics has been undertaken under contained use. However, it could be envisaged that medicinal products (e.g. vaccines, gene therapy) or environmental sensors could be developed that would be considered for deliberate release.

There are two possible interpretations as regards how the technique should be covered by the GMO legislation, depending on whether:

(a) the emphasis is on the recipient (cell extracts or protocells), which is not considered as a (micro-)organism (see above). In this case, the technique falls outside the scope of the Directives;

(b) the emphasis is on the resulting entity, which is considered to be a (micro-)organism. In this case, the technique falls under the scope of the Directives in the meaning of Point 2 of Annex I Part A of Directive 2009/41/EC (and of Annex IA Part 1 of Directive 2001/18/EC), which refers to techniques involving the direct introduction of heritable material prepared outside the organism. When recombinant nucleic acid molecules are used, Point 1 of the above-mentioned annexes may also apply.

B Concluding remarks for regulation on the status of the technique

The future status of the technique and its resulting organisms will therefore depend on the interpretation (either a or b above) that is considered to be more appropriate.

- Given the fact that synthetic genomics (and more generally synthetic biology) is a fast-evolving field with a potential for very new developments as compared to what can be achieved with gene modification techniques currently listed in the Directives, the WG raised the question whether the GMO legislation would be the most appropriate place to deal with this technique, and with synthetic biology in general.
- The WG did not discuss detection issues for synthetic genomics.

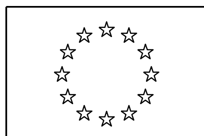
5.8.5 Other issues

Potential impact of the technique

The application of this technique and its potential for the development of novel synthetic (micro-)organisms may lead to some challenges as regards certain steps in the risk assessment, such as the difficulty of identifying suitable or appropriate comparator or of distinguishing the resulting (micro-)organism from those naturally present in the environment. These aspects should be taken into consideration when addressing the legal status of the technique.

Appendix 1	**Terms of Reference**
Appendix 2	**Experts nominated by the Competent Authorities for participation in the New Techniques Working Group**
Appendix 3	**Annex IA, Part 1 and Part 2 and Annex IB of Directive 2001/18/EC and Annex I, Part A and Part B and Annex II Part A of Directive 2009/41/EC**
Appendix 4	**Abbreviations**
Appendix 5	**References**

Appendix 1
Terms of Reference



EUROPEAN COMMISSION
DIRECTORATE-GENERAL
ENVIRONMENT

Directorate B - Protecting the Natural Environment

Brussels,
ENV B3/AA/ D(2008)

WORKING GROUP ON THE ESTABLISHMENT OF A LIST OF TECHNIQUES FALLING UNDER THE SCOPE OF DIRECTIVE 2001/18/EC ON THE DELIBERATE RELEASE OF GENETICALLY MODIFIED ORGANISMS INTO THE ENVIRONMENT AND DIRECTIVE 90/219/EEC ON THE CONTAINED USE OF GENETICALLY MODIFIED MICRO-ORGANISMS

TERMS OF REFERENCE

Introduction

At the meeting of the Competent Authorities under Directive 2001/18/EC[22] which was held on 16 April 2007, the Dutch delegation proposed that a Working Group (WG) be set up to address new techniques which are applied in plant breeding and modification of organisms in general. The background for this request is that Member States increasingly deal with questions from stakeholders whether newly applied techniques result in a genetically modified organism (GMO). These questions have led to different interpretations among Member States. In order to harmonize the approach of Member States in this issue, the Dutch delegation proposed that the WG address whether these (new) techniques lead to GMOs as defined under Directives 2001/18/EC and 90/219/EEC[23]. The proposal was welcomed by BE, DK, DE, SE, FI, FR, AT, MT, NO and SL.

Terms of reference

Directive 2001/18/EC and Directive 90/219/EEC provide for a general definition of a Genetically Modified Organism (GMO) and a Genetically Modified Micro-organism (GMM) respectively. These Directives include annexes which provide additional information regarding the techniques that result in genetic modification, that are not considered to result in

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[22] Directive 2001/18/EC[22] of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 17.4.2001, p. 1

[23] Directive 90/219/EEC, Council Directive of 23 April 1990 on the contained use of genetically modified micro-organisms

genetic modification, or that result in genetic modification but yield organisms that are excluded from the scope of the Directives. The following parts of Directive 2001/18/EC and Directive 90/219/EEC as amended by Directive 98/81/EC[24] have to be taken into account:

- Definition of a GMO : *"genetically modified organism (GMO)"* means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination" (Directive 2001/18/EC Article 2)
- Definition of a GMM: ""*genetically modified micro-organism (GMM)"* shall mean a micro-organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination" (Directive 90/219/EEC, Article 2)
- List of the techniques leading to genetic modification (Annex I Part A of Directive 90/219/EEC and Annex IA, Part 1 of Directive 2001/18/EC)
- List of the techniques which are not considered to result in genetic modification (Annex I, Part B of Directive 90/219/EEC and Annex IA Part 2 of Directive 2001/18/EC)
- List of the techniques of genetic modification yielding organisms to be excluded from the Directives (Annex II Part A of Directive 90/219/EEC and Annex IB of Directive 2001/18/EC)
- The objective of the legislation is to protect human health and the environment (Directive 2001/18/EC, Article 1; Directive 90/219/EEC Article 1)

The techniques listed in the Annexes (Annex IA Part 1, Annex IA Part 2 and Annex IB of Directive 2001/18/EC and Annex I Part A, Annex I Part B and Annex II Part A of Directive 90/219/EEC) could be complemented with relevant new techniques, if that is considered necessary, and according to the appropriate procedures foreseen for a possible review of Directive 90/219/EEC and Directive 2001/18/EC.

As stipulated by Article 31(1) of Directive 2001/18/EC, Member States and the Commission shall meet regularly and exchange information on the experience acquired with regard to the prevention of risks related to the release and placing on the market of GMOs. The establishment of a specialised WG focusing on techniques leading to genetic modification can serve the purpose of risk prevention in the sense of the above provision.

Objective(s)

The WG shall evaluate a list of new techniques for which it is unclear whether they result in genetic modification. These techniques will be discussed in the light of:

- the definition of a GMO/GMM;
- the techniques listed in the Annexes of the Directives; and,
- the most recent available scientific data.

[24] Council Directive 98/81/EC of 26 October 1998 amending Directive 90/219/EEC on the contained use of genetically modified micro-organisms.

1 The WG shall discuss the possible consequences on the list of the techniques presented in the
2 aforementioned Annexes.

3 The WG should compare the definitions of GMM/GMO and the annexes in the two Directives
4 and analyse potential discrepancies.

5 This work may represent the basis for future guidelines or new categorisation of techniques of
6 genetic modification. The most appropriate means to present the findings of the WG will be
7 discussed in the course of the meetings of the Competent Authorities under Directives
8 2001/18/EC and 90/219/EEC or in a joint meeting under both Directives.

9 The WG is an ongoing working group, which may be consulted in the light of new scientific
10 information and methodological progress.

11 **Approach**

12 ▪ The WG should include experts under Directive 90/219/EEC and Directive
13 2001/18/EC.

14 ▪ A list of (new) techniques will be proposed and categorized as an initial working tool
15 of the WG. In this context a priority list of techniques shall be established by the
16 Member States. Priority will be given to techniques that are already in use or will
17 become important in the near future.

18 ▪ Techniques will be discussed by technical experts of each Member State in the light of
19 the definitions (and Annexes) of the Directives in order to determine whether or not
20 they would result in genetic modification in the context of the Directives and to what
21 extent they can be compared to techniques already listed in the Annexes of the
22 Directives

23 ▪ The findings of the WG may be referred to EFSA for their opinion

24 ▪ The terms "recombinant nucleic acid techniques" will be interpreted in the light of the
25 aforementioned list of new techniques.

26 ▪ The structure and substance of the work, as well as the prioritisation of the tasks
27 assigned to the WG should be decided by the Commission in consultation with the
28 Competent Authorities under Directive 2001/18/EC and Directive 90/219/EEC.

29 ▪ The outcome of the WG will be presented to the meetings of the Competent
30 Authorities of both Directives or to a joint meeting of the Competent Authorities
31 under the two Directives. It shall be reviewed and updated in the light of new
32 scientific information and methodological progress.

33 **Setting up of the working Group**

34 The Competent Authorities were required to nominate national experts as members of the
35 WG by 30 September 2008 at the latest.
36 The work of the WG will be carried out by WG meetings preceded and followed by work co-
37 ordinated via e-mail.
38 Further information as regards the structure and the substance of the work progress will be
39 provided on a regular basis to Member States.

1 **Proposed Work Plan and Agenda**

2 During the first meeting of the New Techniques WG, the experts will compile and
3 complement the list of techniques to be analysed. A starting point will be the list of
4 techniques already provided by the Member States in 2007. A prioritisation of the tasks shall
5 be defined. It is planned to work in sub-groups dealing with different categories of techniques,
6 if suitable. Each sub-group will nominate a rapporteur. The outcome of the first meeting shall
7 be submitted to and agreed by the Competent Authorities under Directive 2001/18/EC and
8 Directive 90/219/EEC.
9 The sub-groups will work via e-mail co-ordination. The results will be presented at the
10 subsequent meeting of the WG. It is planned to commence the activity of the group in
11 December 2008. A document outlining the state of play of group's work will be presented to
12 the Competent Authorities on a regular basis.
13

1 **Appendix 2**
2
3 **Experts nominated by the Competent Authorities for participation in the New**
4 **Techniques Working Group**
5
6

Mr Mag. Dr. Alois Haslinger	Bundesministerium f. Wissenschaft und Forschung	Austria
Dr. Dietmar Vybiral	Bundesministerium f. Gesundheit, Familie und Jugend	Austria
Dr. Didier Breyer	Scientific Institute of Public Health - Division of Biosafety and Biotechnology (SBB)	Belgium
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Appendix 3
GMO Definitions in Directives 2001/18/EC and 2009/41/EC

A. Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC

Article 2 (Definitions)

For the purpose of this Directive:

(1) "organism" means any biological entity capable of replication or of transferring genetic material;
(2) 'genetically modified organism (GMO)' means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination;

Within the terms of this definition:
(a) genetic modification occurs at least through the use of the techniques listed in Annex I A, part 1;
(b) the techniques listed in Annex I A, part 2, are not considered to result in genetic modification.
(3) ... (8)

Annex I A

Techniques referred to in Article 2 (2)

Part 1

Techniques of genetic modification referred to in Article 2 (2) (a) are *inter alia*:

(1) recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation;
(2) techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation;
(3) cell fusion (including protoplast fusion) or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally.

Part 2

Techniques referred to in Article 2 (2) (b) which are not considered to result in genetic modification, on condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms made by techniques/methods other than those excluded by Annex I B:

(1) in vitro fertilisation,
(2) natural processes such as: conjugation, transduction, transformation,
(3) polyploidy induction.

Article 3 (Exemptions)

1. This Directive shall not apply to organisms obtained through the techniques of genetic modification listed in Annex I B.

2. [carriage of genetically modified organisms by rail, road, inland waterway, sea or air].

Annex I B

Techniques/methods of genetic modification yielding organisms to be excluded from the Directive, on the condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms other than those produced by one or more of the techniques/methods listed below are:

(1) mutagenesis,
(2) cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods.

B. Directive 2009/41/EC of the European Parliament and the Council of 6 May 2009 on the contained use of genetically modified micro-organisms (Recast)

Article 2
For the purposes of this Directive the following definitions shall apply:
(a) ….
(b) 'genetically modified micro-organism' (GMM) means a micro-organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination; within the terms of this definition:
  (i) genetic modification occurs at least through the use of the techniques listed in Annex I, Part A;
  (ii) the techniques listed in Annex I, Part B, are not considered to result in genetic modification;

Article 3
1. Without prejudice to Article 4(1), this Directive shall not apply:
(a) where genetic modification is obtained through the use of the techniques/methods listed in Annex II, Part A;
(b) ….

Annex I

Part A

Techniques of genetic modification referred to in point (b)(i) of Article 2 are, inter alia:

1. Recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation.
2. Techniques involving the direct introduction into a micro-organism of heritable material prepared outside the micro-organism, including micro-injection, macro-injection and micro-encapsulation.
3. Cell fusion or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally.

Part B

Techniques referred to in point (b)(ii) of Article 2 which are not considered to result in genetic modification, on condition that they do not involve the use of recombinant-nucleic acid molecules or GMMs made by techniques/methods other than the techniques/methods excluded by Part A of Annex II:

1. *in vitro* fertilisation;
2. natural processes such as: conjugation, transduction, transformation;
3. polyploidy induction.

Annex II

Part A

Techniques or methods of genetic modification yielding micro-organisms to be excluded from this Directive on condition that they do not involve the use of recombinant-nucleic acid molecules or GMMs other than those produced by one or more of the techniques/methods listed below:

1. Mutagenesis.
2. Cell fusion (including protoplast fusion) of prokaryotic species that exchange genetic material by known physiological processes.
3. Cell fusion (including protoplast fusion) of cells of any eukaryotic species, including production of hybridomas and plant cell fusions.
4. Self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism which may or may not be followed by reinsertion of all or part of that nucleic acid (or a synthetic equivalent), with or without prior enzymic or mechanical steps, into cells of the same species or into cells of phylogenetically closely related species which can exchange genetic material by natural physiological processes where the resulting micro-organism is unlikely to cause disease to humans, animals or plants.

   Self-cloning may include the use of recombinant vectors with an extended history of safe use in the particular micro-organisms.

1 **Appendix 4** **Abbreviations**
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3 CA	Competent Authorities
4 DNA	Desoxyribonucleic Acid
5 DSB	Double Strand Break
6 dsRNA	Double Stranded RNA
7 EFSA	European Food Safety Authority
8 EU	European Union
9 GM	Genetically Modified
10 GMM	Genetically Modified Microorganism
11 GMO	Genetically Modified Organism
12 ODM	Oligonucleotide-Directed Mutagenesis
13 ORF	Open Reading Frame
14 PCR	Polymerase Chain reaction
15 RdDM	RNA-dependent DNA Methylation
16 RNA	Ribonucleic Acid
17 RNAi	RNA Interference
18 siRNA	Small Interfering RNA
19 T-DNA	Transfer DNA
20 TFO	triple helix-forming oligonucleotides
21 WG	Working Group
22 ZFN	Zinc Finger Nuclease

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1 **Appendix 5**

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3 **References**

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5 **RdDM /RNA silencing/Transcriptional Gene Silencing**

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