

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

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## New Techniques Working Group

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8

### FINAL REPORT

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#### 101.0 **Introduction**

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12 Directive 90/220/EEC (repealed and replaced by Directive 2001/18/EC) on the  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14 and Directive 90/219/EEC (amended by Directive 98/81/EC and repealed and replaced  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

18

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

24

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

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

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#### 392.0 **Legal context**

40

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

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<sup>1</sup> 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.

<sup>2</sup> Directive 2001/18/EC on the deliberate release into the environment of GMOs, and Directive 2009/41/EC on the contained use of GMMs.

<sup>3</sup> Article 2 of Directive 2001/18/EC

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

3  
4 An organism (*any biological entity*) and a micro-organism (*any microbiological entity,*  
5 *cellular or non-cellular*) must be capable of replication or of transferring genetic  
6 material.

7  
8 Some techniques may include a step in the process where Directive 2009/41/EC  
9 applies. For example:

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

17  
18 Neither of the described steps necessarily affects whether the grown plant is classified  
19 as a GMO or not, if it is released into the environment; its status as a GMO will need  
20 to be considered for deliberate release under Directive 2001/18/EC.

21  
22 Both Directives list techniques that:

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

29  
30 Annex I Part A of Directive 2009/41/EC and Annex IA Part 1 of Directive 2001/18/EC  
31 (techniques giving rise to genetic modification) use the wording '*inter alia*' and  
32 therefore provide indicative lists of techniques leading to genetic modification. The  
33 remaining Annexes provide exhaustive lists. Therefore, the techniques leading to  
34 genetic modification are not limited to those listed under Annex I Part A of Directive  
35 2009/41/EC and Annex IA Part 1 of the Directive 2001/18/EC.

36  
37 The Annexes of both Directives are broadly similar with one exception. Directive  
38 2009/41/EC excludes self-cloning from the scope of the legislation. Self-cloning is  
39 where a nucleic acid is removed from the cell of an organism and all or part of the  
40 nucleic acid is reinserted into cells of the same or a phylogenetically closely related  
41 species (see Appendix 4).

#### 42 43 443.0 **Establishment of the New Techniques Working Group (NTWG)**

45  
46 At the request of the Competent Authorities (CA) under Directive 2001/18/EC, a  
47 working group (WG) was established (October 2007) to analyse a non-exhaustive list  
48 of techniques for which it is unclear whether they would result in a GMO. CAs were  
49 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<sup>4</sup>). 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)<sup>5</sup>. 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

38 - Determine whether there is a need for new guidance or whether the existing  
39 guidance on risk assessment should to be updated or further elaborated, in  
40 anticipation of the placing of products on the market through the application of  
41 the listed techniques.

41

42 - What are the risks in terms of impact on humans, animals and the environment  
43 that the eight techniques listed could pose, irrespective of whether or not they  
44 fall under the GMO legislation? This latter request should consider the most  
45 recent scientific literature and knowledge of plant breeding experts and  
46 compare plants obtained by these new techniques with plants obtained by  
47 conventional plant breeding techniques and secondly with plants obtained with  
currently used genetic modification techniques.

<sup>4</sup> No meeting of the working group took place between January 2010 and January 2011 due to the change of  
3competences within the Commission and the need for delivering on other priorities.

<sup>5</sup> <http://ftp.jrc.es/EURdoc/JRC63971.pdf>.

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**33.1 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<sup>6</sup> 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).

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<sup>6</sup> Annex IA and IB of Directive 2001/18/EC and Annex I and Annex II Part A of Directive 2009/41/EC – see 3section 2.0 of this document for further interpretation.

13.2 **'New techniques' considered by the WG**

2

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

- 5 a. Oligonucleotide Directed Mutagenesis (ODM)
- 6 b. Zinc Finger Nuclease Technology (ZFN) (comprising ZFN-1, ZFN-2 and ZFN-  
7 3 as defined in this report )
- 8 c. Cisgenesis (comprising Cisgenesis and Intragenesis)
- 9 d. Grafting
- 10 e. Agro-infiltration
- 11 f. RNA-dependent DNA methylation (RdDM)
- 12 g. Reverse Breeding
- 13 h. Synthetic Genomics

14

15

164.0 **Terms and issues discussed**

17

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

24

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

27

28 When analysing organisms produced by a particular technique in the light of the  
29 criteria set out in Annex IB of Directive 2001/18/EC and Annex II Part A of Directive  
30 2009/41/EC, a key consideration was whether these techniques involve the use of  
31 recombinant nucleic acid molecules or GMOs/GMMs other than those produced by  
32 mutagenesis and cell fusion (including protoplast fusion) and in the case of GMMs,  
33 self-cloning.

34

35

364.1 **"..altered in a way...."**

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38 The term "*altered in a way*" is used in Article 2(2) of Directive 2001/18/EC and  
39 Article 2(b) of Directive 2009/41/EC which define GMO and GMM respectively as an  
40 "*organism*" or "*micro-organism in which the genetic material has been altered in a*  
41 *way that does not occur naturally by mating and/or natural recombination*". The  
42 techniques ("process") leading to the generation of GMOs are listed under Annex IA  
43 Part 1 of Directive 2001/18/EC and Annex I, Part A of Directive 2009/41/EC, and  
44 under Annex IB of Directive 2001/18/EC and Annex II Part A of Directive  
45 2009/41/EC (Section 2.0 of this document provides an overview of the annexes and  
46 their respective techniques).

47

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

- 1 1. The emphasis is on the technique; the resulting organism is a GMO, even if the  
2 same modification or an identical organism could be obtained by the techniques  
3 listed in Annex I A Part 2 or Annex I B of Directive 2001/18/EC and Annex I Part  
4 B or Annex II Part A of Directive 2009/41/EC.
- 5 2. The emphasis is on the resulting organism; if the resulting organism is  
6 indistinguishable from an organism obtained from natural processes, conventional  
7 breeding or by application of the techniques listed in Annex IA Part 2 or Annex IB  
8 of Directive. 2001/18/EC and Annex I Part B or Annex II Part A of Directive.  
9 2009/41/EC, then it cannot be considered as a GMO and would therefore be  
10 considered out of the scope of the Directives. (Article 2)

11  
12 Experts also discussed the degree of change that should be considered an alteration  
13 according to the Directives, given the plasticity of genomes and the genetic variation  
14 that occurs naturally and through traditional breeding techniques. A minority of  
15 experts considered a single base pair change was sufficient to be captured by the  
16 legislation.

#### 17 18 19 **194.2 Nucleic acids and recombinant nucleic acid**

20  
21 The term "nucleic acid" is used in Annex IA Part 1(1) of Directive 2001/18/EC and  
22 Annex I Part A(1) of Directive 2009/41/EC.

23  
24 Nucleic acid molecules e.g. DNA and RNA can be produced naturally or by synthetic  
25 means. In some of the techniques proposed for consideration, synthetic analogues of  
26 nucleic acids or nucleic acids containing synthetic nucleotide analogues are used, such  
27 as locked nucleic acids (LNA) and peptide nucleic acids (PNA).

28  
29 LNA and PNA have different chemical properties from DNA and RNA and can behave  
30 differently *in vivo* compared to nucleic acid molecules e.g. in replication, transcription  
31 and translation. It was noted that LNA or PNA are used in the same way as nucleic  
32 acids, i.e. as tools to alter the genetic material in cells for example for the purpose of  
33 site-directed mutagenesis.

34  
35 The term "recombinant nucleic acid molecules" is used in Annex IB of Directive  
36 2001/18/EC and Annex II Part A of Directive 2009/41/EC. In line with the  
37 aforementioned Directives, a recombinant nucleic acid molecule is created outside the  
38 cells through the formation of a new combination of genetic material/nucleic acid  
39 molecules. There was a discussion on how many nucleotides could constitute a new  
40 combination of genetic material/nucleic acid molecules in this context. A majority of  
41 experts concluded that in order to form a new combination, a nucleotide sequence of  
42 at least 20 bp is required. A minority of experts were of the opinion that under the  
43 current definition, the replacement of only one nucleotide in a nucleic acid molecule  
44 could be interpreted as producing a recombinant nucleic acid.

45  
46 The definition of a recombinant nucleic acid molecule is important because Annex IB  
47 of Directive 2001/18/EC and Annex II Part A of Directive 2009/41/EC both stipulate  
48 that techniques/methods of genetic modification yielding organisms to be excluded  
49 from the Directive, may only be excluded on the condition that they do not involve the  
50 use of recombinant nucleic acid molecules.

14.3 **Heritable material**

2

3 The term heritable material is used in Annex IA, Part 1(2) and Part 1(3) of Directive  
4 2001/18/EC and Annex I, Part A(2) and Part A(3) of Directive 2009/41/EC. In both  
5 instances the term is used in the context of genetic material, which, when introduced  
6 directly (i.e. without the involvement of a vector system) into an organism (micro-  
7 organism) by procedures including micro-injection, micro-encapsulation or cell  
8 fusion, will result in a "technique of genetic modification" in the sense of the  
9 Directives. Although heritable material is not clearly defined in the Directive, there are  
10 two possible interpretations:

11 i. "heritable material" must be inherited in the case in question. The argument  
12 being that the first indent in the list of Annex IA Part 1<sup>7</sup> involves the use of  
13 vectors and refers to the transfer of genetic material into a host organism and  
14 continued propagation. In order for this to be consistent with the second indent  
15 in the list of Annex IA Part 1<sup>8</sup> heritable material should be interpreted as being  
16 propagated through the host organism and not just being transiently present  
17 (see section 4.4); and,

18 ii. "heritable material" has simply to be capable of being inherited.

19

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

27

28 The consequences for taking these different approaches when considering whether  
29 ZFN and ODM are captured by Annex 1A Part 1 are addressed in the specific sections  
30 (5.1 and 5.2, respectively).

31

32

33 4.4 **GMO offspring**

34

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

37 • In the case of sexual propagation in plants, the offspring are the product  
38 of two parents.

39 • In the case of vegetative propagation in plants, the offspring is the  
40 product of a single parent.

41 • In the case of animals it is essentially the same, but asexual propagation  
42 is rare, e.g. parthenogenesis, or does not happen naturally, e.g. "cloning".

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

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

8

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

8  
9 Some of the techniques discussed in this report involve intermediate organisms or  
10 cells that contain new DNA. Further on in the process, however, some of these  
11 intermediate organisms or cells give rise to offspring that no longer contain the new  
12 DNA. In the NTWG different views were expressed on whether and when the  
13 offspring of a GMO can be classified as a non-GMO. This aspect is not considered in  
14 the Directives.

15  
16 For the techniques described in this report all experts agree that unintended changes in  
17 the DNA sequence due to the (former) presence/insertion of the new DNA may have  
18 taken place, and would probably appear at very low frequency<sup>9</sup>. If such unintended  
19 changes appeared, they would be comparable to natural or induced changes not  
20 involving techniques that lead to GMO. All experts agreed that once it is established  
21 that the 'foreign' genetic material is no longer present in the resulting organism it is no  
22 longer considered a GMO, however, some expert raised concerns reading the  
23 Directives. Clear criteria would be needed to establish whether the 'foreign' genetic  
24 material is no longer present in the resulting organism in order for the offspring to be  
25 designated non-GMO.

26  
27 For other techniques the offspring of a GMO may still be considered a GMO even  
28 when the new DNA is no longer present. The main underlying argument being that  
29 also unintended changes in the DNA sequence due to the (former) presence/insertion  
30 of the new DNA may have taken place.

31  
32  
33 **334.5 Transient Presence (with respect to exogenous nucleic acid molecules in the**  
34 **cell/transient presence of recombinant DNA)**

35  
36 Despite reference in Annex IA Part 1(1) of Directive 2001/18/EC (and Annex I Part  
37 A(1) of Directive 2009/41/EC) to the "*insertion of nucleic acid molecules...into any*  
38 *virus, bacterial plasmid or other vector system and their incorporation into a host*  
39 *organism in which they do not naturally occur but in which they are capable of*  
40 *continued propagation*", the legislation makes no reference to the term transient  
41 presence or effect. However, it does refer to 'capable of continued propagation' and  
42 this is linked to the discussion on heritability. Since this term can have a bearing on  
43 whether the final organism is considered a GMO, it is considered important to discuss  
44 it.

45  
46 Sometimes techniques that introduce genetic material into a cell do not result in a  
47 stable genetic transformation. Instead, the introduction can result in an effect for a

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



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

3  
4 The transient presence of nucleic acid molecules in cells can be utilised to (over)  
5 express a foreign gene (e.g. synthesis of foreign proteins) temporarily or to change the  
6 expression level of a gene in the recipient cell, by modifying its regulation (e.g.  
7 agroinfiltration). In such cases, the trait is not inherited and does not by itself alter the  
8 sequence of the recipient cell's genetic material. However, the transient presence of  
9 nucleic acids in cells can also be used to induce mutations (ZFN) or changes in the  
10 methylation of DNA (RdDM). These changes in the recipient cell's genome are either  
11 permanent genetic alterations (mutations) or can remain in the progeny for some  
12 generations (epigenetic alterations like methylation of DNA).

13  
14 A majority of experts consider that if the 'foreign' genetic material is present but is not  
15 able to replicate the organism should not be considered a GMO. A minority of experts  
16 agreed that as long as this 'foreign' genetic material is present even if it's not able to  
17 replicate the organism should be considered a GMO. All experts agreed that once it is  
18 established that the 'foreign' genetic material is no longer present in the resulting  
19 organism it is no longer considered a GMO.

20  
21 See heritable material (section 4.3)

## 22 23 **4.6 Organism**

24  
25 In accordance with Article 2(1) of Directive 2001/18/EC, *an organism means any*  
26 *biological entity capable of replication or of transferring genetic material.*

27  
28 In accordance with Article 2 (a) of Directive 2009/41/EC, *a micro-organism means*  
29 *any microbiological entity, cellular or non-cellular, capable of replication or of*  
30 *transferring genetic material, including viruses, viroids, and animal and plant cells in*  
31 *culture.*

32  
33 All experts agreed that plasmids as such are not 'organisms' as defined in the  
34 Directives. Instead they are a component of an organism i.e. of the bacterium from  
35 which they have been extracted. As long as the plasmid cannot replicate by itself  
36 outside of bacterial cells and it is not capable of transferring genetic material by itself,  
37 it is not an organism. This contrasts with viruses, which infect cells.

38  
39 With regard to the use of vectors in cell culture, as long as the vectors remain present  
40 in the cells, the technique used will fall under the scope of Directive 2009/41/EC.

41  
42 For the purpose of this report, experts have used the term “resulting organism” to  
43 denote an organism that results after having gone through all the steps of the particular  
44 technique. This could e.g. be a plant or seed intended for deliberate release or placing  
45 on the market or a microorganism intended for contained use.

46  
47 Experts have used the term “intermediate organism” to denote any organism that is  
48 generated in the steps leading to the resulting organism. It should be recognized that  
49 intermediate steps could involve the introduction of heritable material into cells, as  
50 well as the loss of such material. In several cases it was essential for the assessment of

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

5  
6 Synthetic biology and more particularly synthetic genomics, which is based on the  
7 chemical synthesis of an entire genome or of large portions of the genome followed by  
8 its introduction into a cell or cell-free environment where its replication is enabled, has  
9 appeared since the writing of the Directives. Whether or not a synthetic entity will be  
10 considered as an organism should be analysed in the context of the above mentioned  
11 definitions. The definition of an “organism” states that only entities capable of  
12 replication or of transferring genetic material fit under this term. Experts considered  
13 that if a synthetic entity fulfils the condition of capability of replication and/or  
14 transferring genetic material, then it should be defined as an organism in the context of  
15 the Directives.

#### 16 17 **4.7 Similarity**

18  
19 The Directives list techniques that give rise to genetic modification, that are not  
20 considered to result in genetic modification or that yield organisms to be excluded  
21 from the Directives. In order to classify the status of the ‘new techniques’, experts  
22 considered whether they were similar to the listed techniques.

23  
24 Experts also discussed whether the changes (intended and unintended) induced by  
25 these ‘new techniques’ can also be produced by traditional breeding techniques,  
26 natural processes or techniques listed in Annex 1B of Directive 2001/18/EC and  
27 Annex II Part A of Directive 2009/41/EC. These discussions took into account  
28 whether novel combinations of genetic material could be generated and in one case,  
29 whether inserted DNA should be considered heterogenous rather than cisgenic.

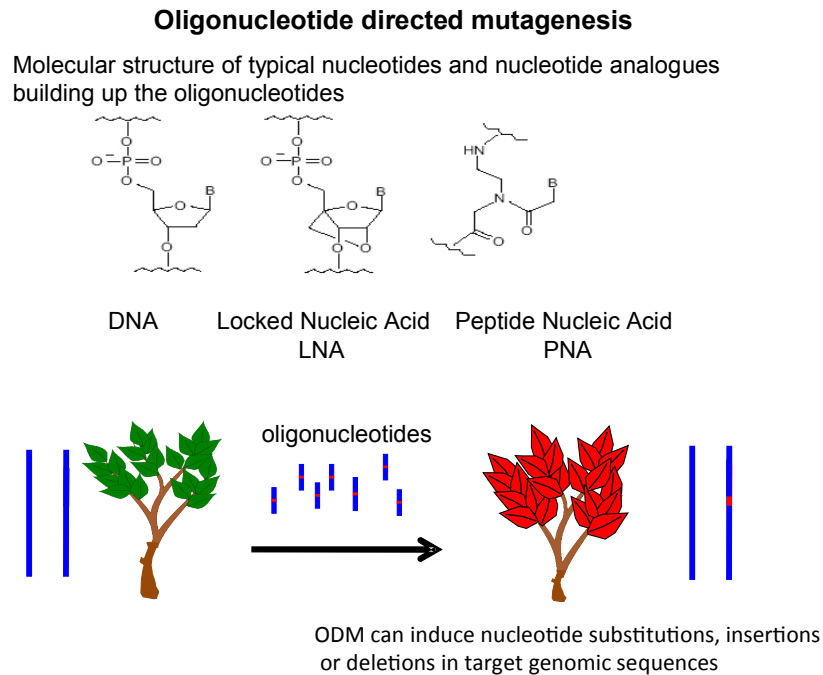
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15.0 **Evaluation of the Techniques**

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35.1 **Oligonucleotide-directed mutagenesis (ODM)**

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**Fig. 1 Oligonucleotide-directed mutagenesis**

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**155.1.1 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<sup>10</sup>.

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:

<sup>10</sup> replacement of one or a few base pairs or introduction of short deletions

- 1 • single-stranded homologous DNA with a mismatch to the target  
2 sequence;
- 3 • chimeric oligonucleotides consisting of RNA stretches within single-  
4 stranded DNA;
- 5 • triple helix-forming oligonucleotides (TFOs) which form relatively  
6 stable associations with duplex DNA via Hoogsteen hydrogenbonds (parallel  
7 and antiparallel)<sup>11</sup>;
- 8 • RNA oligonucleotides were recently investigated to induce RNA-  
9 mediated, targeted DNA nucleotide sequence changes and RNA-templated  
10 DNA repair resulting in point mutations;
- 11 • LNA or PNA or any other nucleic acid analogues could also be used.

### 12 13 **135.1.2 Application of the technique**

14  
15 The induction of point mutations has been successfully performed in agriculturally  
16 important plants including rapeseed, maize, tobacco, rice, and wheat (e.g. herbicide  
17 tolerance) and it is being explored in animals including sheep and cattle (e.g. genetic  
18 improvement of livestock animals). The technique has been used successfully in  
19 bacteria and yeast mainly as a tool to perform basic research on gene expression,  
20 regulation and genetic recombination, and in human cells for gene therapy.

### 21 22 **225.1.3 Other issues**

#### 23 24 **Potential impacts of the technique**

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

### 27 28 **285.1.4 Relevant issues for classification**

#### 29 30 • **Similarity to mutagenesis:**

- 31 - Oligonucleotide-directed mutagenesis (ODM) is a form of  
32 mutagenesis induced by oligonucleotides.
- 33 - Only oligonucleotides with sequence similarity or analogy to the  
34 recipient's genome are used (they may be modified chemically to improve  
35 stability).
- 36 - During the application of ODM, modifications are made to the  
37 organism's genetic material by the hosts own repair mechanisms.
- 38 - The induced point mutations are site-specific. Similar mutations can  
39 occur spontaneously in nature or may be induced by conventional mutagenesis  
40 (chemical or radiation).

- 41 • **Nucleic acids:** A majority of experts were of the view that oligonucleotides  
42 in this technique cannot be considered as recombinant nucleic acids in the sense of

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2<sup>11</sup> 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.

1 Annex I Part B of the Directive 2001/18/EC or Directive 2009/41/EC. For a  
2 minority of experts it is not possible to arrive at this conclusion.

3  
4 **45.1.5 Coverage by GMO legislation**

5  
6 **A Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the**  
7 **technique may be attributed**

8  
9 There are two possible interpretations about the coverage of ODM by the  
10 GMO legislation (see in particular sections 4.2, 4.3 and 4.4):

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

28  
29 **B Concluding remarks for regulation on the status of the technique**

30  
31 **• Directive 2001/18/EC/ Directive 2009/41/EC**

32 All experts agree that ODM results in changes in organism that can be  
33 obtained with other forms of mutagenesis. They also noted that ODM is  
34 expected to generate fewer unintentional changes or effects than those  
35 introduced into organisms by irradiation or chemical mutagenesis,  
36 which is listed under indent 1 of Annex IB/Annex II Part A "as a  
37 technique of genetic modification yielding organisms to be excluded  
38 from the Directives". Therefore, ODM is captured by Annex IB/ Annex  
39 II Part A. This was a majority opinion.

40 **• Detection**

41 Organisms developed through ODM cannot be distinguished at the  
42 molecular level from those developed through "conventional" mutation  
43 techniques (using chemicals or ionizing radiations) or through selection  
44 in natural populations.

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

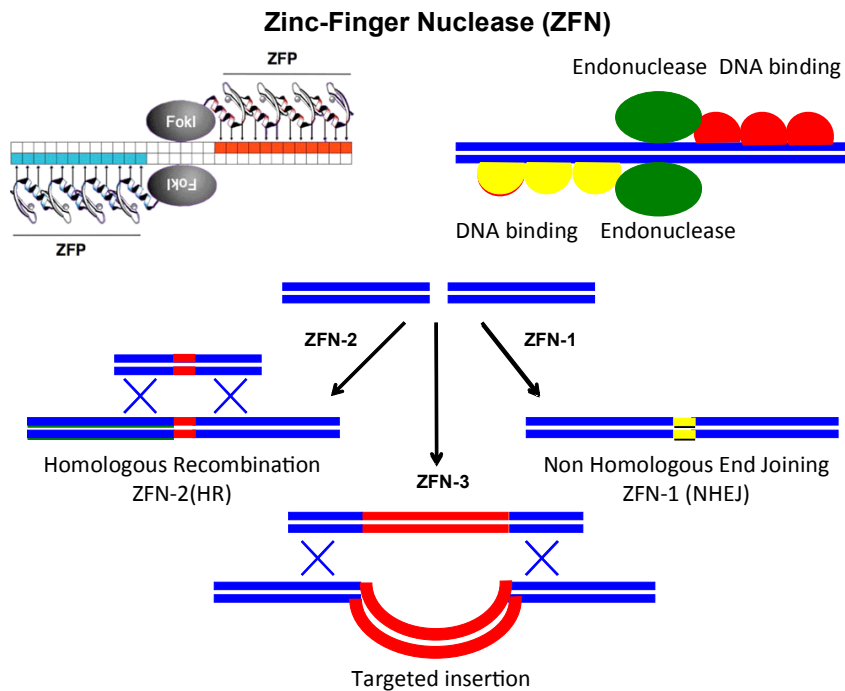
6<sup>13</sup> Indent 2 of Annex I A Part 1: "techniques involving the direct introduction into an organism of heritable  
7 material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation".

1 Final

1

15.2 **Zinc Finger Nuclease and related techniques**

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3

4

5**Fig. 2 Zinc Finger Nuclease technique**

6

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

10

11

12

13**15.2.0 Introduction**

14

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

25

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

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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.

**355.2.1 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.

**405.2.1.1 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)



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

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

21  
22 **225.2.1.2 Application of the technique**

23  
24 ZFN-1 and ZFN-2 can be used for targeted random (ZFN-1) or nucleotide-specific  
25 (ZFN-2) mutagenesis. In principle, ZFN-1/2 can be applied to any cell line or  
26 organism provided there is the possibility to introduce the ZFN proteins or their  
27 coding nucleic acids.

28  
29 **295.2.1.3 Other issues**

30  
31 A **Potential impacts of the technique**  
32 Same as ODM (see 5.1.1.3).

33  
34 **345.2.1.4 Relevant issues for classification**

- 35  
36 • **Transient presence/effect:**  
37 Until recently, the approach has been to insert recombinant nucleic acids  
38 encoding ZFN into a vector. Provided the construct does not replicate or  
39 integrate, its presence in the cell is transient (see section 4.4). In the case of  
40 ZFN-2, the repair template is always provided as a specific fragment of DNA.  
41 Both the construct and the repair template are transiently present in the  
42 recipient cell. The ZFN can also be introduced into the cell as mRNA. It  
43 should be noted that recent technical developments allow the ZFN protein to  
44 be delivered directly into the cells, thus avoiding the need for DNA  
45 recombinant vectors.
- 46 • During the application of ZFN-1/ZFN-2, the ZFN encoding nucleic  
47 acids give rise to a permanent heritable change owing to a change in the DNA  
48 nucleotide sequences. Such modifications to the organism's genetic material  
49 are made by the host's own repair mechanisms.

- 1 • **Heritable material:**  
 2 With regard to ZFN-2, there was a discussion as to whether oligonucleotides  
 3 (in this case, the DNA template) constitute heritable material in the sense of the  
 4 Directives, point on which experts did not agree. (see section 4.3)
- 5 • **Resulting organism/Offspring:**  
 6 There was a discussion whether the technique leads to an organism falling  
 7 within the scope of Directive 2001/18/EC, in particular, if this only depends on  
 8 whether the GMO offspring does not carry the transgenic construct (see section  
 9 4.6).
- 10 • **Nucleic Acids:**  
 11 Currently the technique tends to make use of recombinant nucleic acid  
 12 molecules (vector with genes encoding the two ZFN proteins) or the  
 13 corresponding mRNAs. Therefore, in accordance with the chapeau of Annex  
 14 IB of Directive 2001/18/EC and Annex II Part A of Directive 2009/41/EC, the  
 15 technique can not yield organisms to be excluded from the Directive even if it  
 16 results in a genetic modification that may happen spontaneously or through  
 17 other forms of mutagenesis. This conclusion is not valid when the ZFN  
 18 proteins are delivered directly into the cells (see first bullet under “Transient  
 19 presence/effect).
- 20 • **Similarity to mutagenesis:**  
 21 The same mutations may occur spontaneously or through other forms of  
 22 mutagenesis (chemical or physical).

#### 23 245.2.1.5 Coverage by GMO legislation

- 25
- 26 **A. Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the**  
 27 **technique may be attributed**
- 28
- 29 • All experts agreed that when the ZFN proteins (concerns ZFN-1) are  
 30 introduced directly into the cell as a protein, the technique is fully  
 31 captured by Annex IB (Directive 2001/18/EC) or Annex II Part A  
 32 (Directive 2009/41/EC) (under which organisms resulting by the listed  
 33 techniques are excluded from scope of the Directives under certain  
 34 conditions<sup>14</sup>.
- 35 • A majority considers the intermediate organisms containing non-  
 36 replicative constructs or mRNA are not GMOs. A minority of experts  
 37 agrees that these organisms, containing nucleic acids encoding ZFN  
 38 proteins (concerns both ZFN-1 and -2), irrespective of its heritability  
 39 and its capability of continued propagation, is covered by Annex IA,  
 40 Part 1 of Directive 2001/18/EC and Annex 1 Part A of Directive  
 41 2009/41/EC.
- 42 • A majority of experts agreed that in the case of ZFN technique, when  
 43 ZFN proteins are introduced together with repair template directly into  
 44 cells, the technique is also fully captured by the Annex IB (Directive  
 45 2001/18/EC) or Annex II Part A (Directive 2009/41/EC) and therefore  
 46 the resulting organisms are out of the scope of the Directives through

<sup>14</sup> 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..."

1 the exclusions that these Annexes provide. A majority of experts were  
2 of the view that oligonucleotide templates in this technique cannot be  
3 considered as recombinant nucleic acids in the sense of Directive  
4 2001/18/EC or Directive 2009/41/EC. For a minority of experts it is not  
5 possible to arrive at this conclusion.

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

16  
17 **B Concluding remarks for regulation on the status of the technique**

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

2  
3 **15.2.2 Zinc Finger Nuclease-3**

4 **35.2.2.1 Definition and brief description of the technique based on the current scientific knowledge and current scientific literature**

5  
6 Zinc Finger Nuclease-3 technique (ZFN-3) targets delivery of transgenes (insertions) by homologous recombination.

7  
8  
9 DNA fragments or gene cassettes up to several kbp in length can be inserted precisely to a desired site in the genome or a gene. In practice, a recombinant DNA molecule is constructed in which the DNA fragment or the gene cassette of interest (donor DNA) is sandwiched between stretches of DNA that are homologous with the DNA sequences flanking the DSB site. Donor DNA can come from any species and it is delivered to the cell, along with the ZFN, and it is targeted to the desired site of the genome and inserted into the DSB site.

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16 **175.2.2.2 Application of the technique**

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18  
19 There has been an emphasis to develop and use the technology on human cells although it has also been seen as a powerful tool for genetic engineering of animals. Furthermore, potential applications of the technique include targeted gene addition, replacement and trait stacking in plants. As demonstrated recently (reference), it is a breakthrough to insert DNA into a specific site in the plant (e.g. maize<sup>15</sup>) genome. Previously, this has not been possible.

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21  
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24  
25 **265.2.2.3 Relevant issues for classification**

26  
27  
28 • **Nucleic acids and recombinant nucleic acids:**

29 New combinations of genetic material are introduced into the cell when the donor DNA fragment is integrated into the recipient genome and is continuously propagated.

30  
31  
32 • **Similarity to self-cloning:**

33 The technique may meet the criteria of self-cloning when:

- 34 - the donor DNA (or template of the synthetic DNA) originates from an organism of the same species as the recipient or from a phylogenetically closely related species;
- 35  
36  
37 - the recombinant vector has an extended history of safe use in the particular micro-organism;
- 38  
39 - the resulting micro-organism is unlikely to cause disease to humans, animals or plants, (see also relevant comments on cisgenesis).

40  
41  
42 **425.2.2.4 Coverage by GMO legislation**

43  
44 **Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the technique may be attributed**

45  
46  

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<sup>215</sup> Shukla et al., 2009, Nature 459. 437-441

- 1       •       All experts agreed that ZFN-3 is covered by Annex IA, Part 1 of Directive  
2       2001/18/EC since it is a recombinant nucleic acid technique involving the  
3       formation of new combinations of genetic material. Organisms developed  
4       through this technique therefore fall within the scope of the Directive.  
5       •       The technique may in some cases meet the criteria of self-cloning (see 5.2.2.3  
6       above) as described in Annex II, Part A(4)<sup>16</sup> of Directive 2009/41/EC and when  
7       that is the case it may be considered to fall outside the scope of Directive  
8       2009/41/EC.  
9

#### 10 5.2.2.5 Other issues

11

##### 12       **Potential impacts of the technique**

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

15

##### 16       **B.       Concluding remarks for regulation on the status of the technique**

17

- 18       •       While the ZFN-3 technique generally is within the scope of Directives  
19       2001/18/EC and 2009/41/EC, in some cases it could meet the criteria of  
20       self-cloning as described in Annex II, Part A of Directive 2009/41/EC and  
21       when that is the case it may be considered as falling outside the scope of  
22       Directive 2009/41/EC.

23

##### 23       •       **Detection**

24       Detection and identification of organisms modified by ZFN-3 technology  
25       are possible through the amplification based methods (PCR) currently used  
26       for GMO detection, with the prerequisite that prior adequate DNA  
27       sequence information on the introduced modification is available.  
28

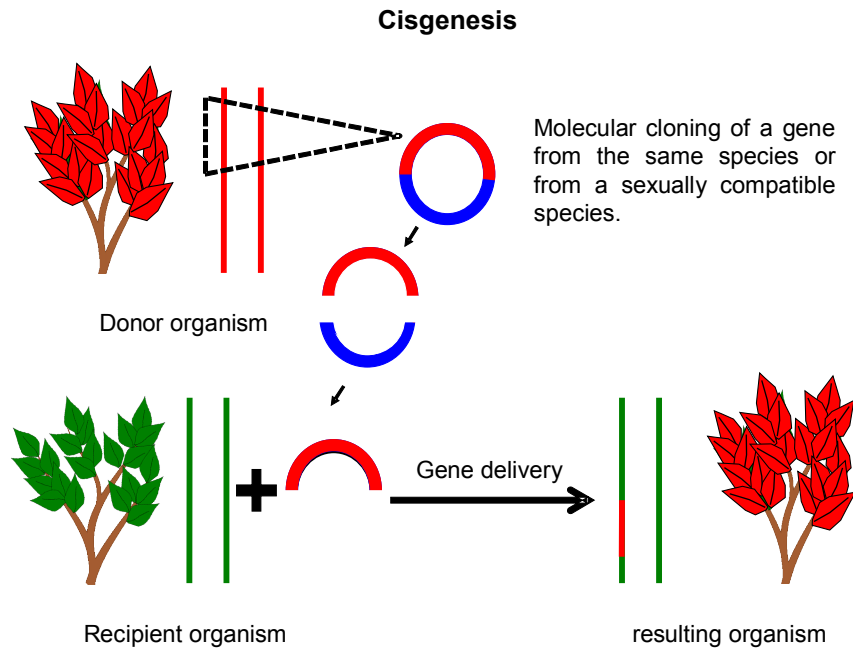
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<sup>16</sup> Self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism which may or may not be followed by reinsertion of all or part of that nucleic acid (or a synthetic equivalent), with or without prior enzymic 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.

15.3 Cisgenesis and Intragenesis

2



3

4

5

6Fig. 3 Cisgenesis

7

8Schematic representation of cisgenesis. Blue: nucleic acid from the vector; red: DNA sequences from the donor  
9plant; green: DNA sequences from the recipient plant. Donor and recipient organism belong to species which are  
10sexually compatible.

11

12

13**5.3.1 Definition and brief description of the technique based on the current scientific  
14 knowledge and current scientific literature**

15

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

20

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

26

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

31

1 Intragenesis is a genetic modification of a recipient organism that involves the  
2 insertion of a reorganised, full or partial coding region of a gene frequently combined  
3 with a promoter and/or terminator from another gene (intragene) of the same species  
4 or a crossable species. These may be arranged in a sense or antisense orientation  
5 compared to their orientation in the donor organism.  
6

### 7 **75.3.2 Application of the technique**

8  
9 Often, the genome of an undomesticated line of a plant species may be a source of a  
10 valuable gene, e.g. in determining disease resistance. In order to transfer such a gene  
11 of interest to a domesticated plant line of that species, e.g. of a high yielding variety, it  
12 is necessary to perform a consecutive line of several crosses and backcrosses. Thus it  
13 may take 10 - 15 years (for annual plants) or more to develop a cultivar possessing the  
14 desired gene from the non-adapted plant by traditional breeding. The application of  
15 cisgenesis would condense this otherwise lengthy process. In addition, cisgenesis and  
16 intragenesis prevents so-called linkage drag of unwanted or undesirable genes, which  
17 is often observed in traditional breeding. Cisgenesis and intragenesis are currently  
18 mainly applied in plants, however the technique may be similarly used in animal  
19 breeding. According to the experts current commercial applications relate more  
20 frequently to intragenesis than to cisgenesis.  
21

### 22 **225.3.3 Other issues**

#### 23 **A. Potential impacts of technique<sup>17</sup>**

24 Cisgenesis may lead to the disruption of existing ORFs or creation of new  
25 one(s), due to random insertion of the gene in any part of the genome, or over  
26 expression of a native (cis-)gene. This could lead to altered biochemical  
27 properties, allergenicity, and/or toxicity. However, similar effects can  
28 sometimes take place in traditional breeding or via normal biological  
29 processes.  
30

### 31 **325.3.4 Relevant issues for classification**

- 32 • **Similarity to currently used techniques of genetic modification:** The  
33 random insertion of a cisgene or intragene could induce alterations in the plant  
34 genome in a similar manner to transgenes, e.g. the creation of new open reading  
35 frames (ORFs) and the disruption of existing genes. There is also the possibility of  
36 multiple insertions and over-expression of the inserted gene(s).  
37
- 38 • **Similarity to traditional breeding:** Cisgenesis yields organisms similar to  
39 those obtained by traditional breeding techniques or via normal biological  
40 processes of reproduction. This is also the case with cisgenesis with T-DNA  
41 borders, where the T-DNA border sequence is identical to or has high similarity  
42 ( $\geq 85\%$  identity) to DNA sequences already present in the genome of the plant  
43 species.  
44

---

<sup>17</sup> 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.

15.3.5 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  
9 regards cisgenesis with T-DNA border sequences, all experts agreed that if  
10 sequences identical to T-DNA border sequences or with high similarity  
11 ( $\geq 85\%$  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  
20 GM organisms equivalent to organisms resulting from self-cloning, and  
21 such organisms cannot be obtained by traditional breeding. Micro-  
22 organisms developed through self-cloning are covered by Annex II, Part  
23 A(4) of 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.<sup>18</sup>  
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 ( $\geq 85\%$  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  
39 Directive 2001/18/EC and in Directive 2009/41/EC. Assuming that the  
40 logic for having excluded self-cloning from Directive 2009/41/EC was that  
41 it yields micro-organisms with modifications that could also be obtained by  
42 traditional breeding, all experts wondered about the justification for not  
43 excluding them from Directive 2001/18/EC.

44 • **Detection**

45 The production of cisgenes and intragenes into plants can be specifically detected if a sufficient part  
46 of the sequence of the insert as well of the adjacent sequence is known.

---

<sup>18</sup> 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"

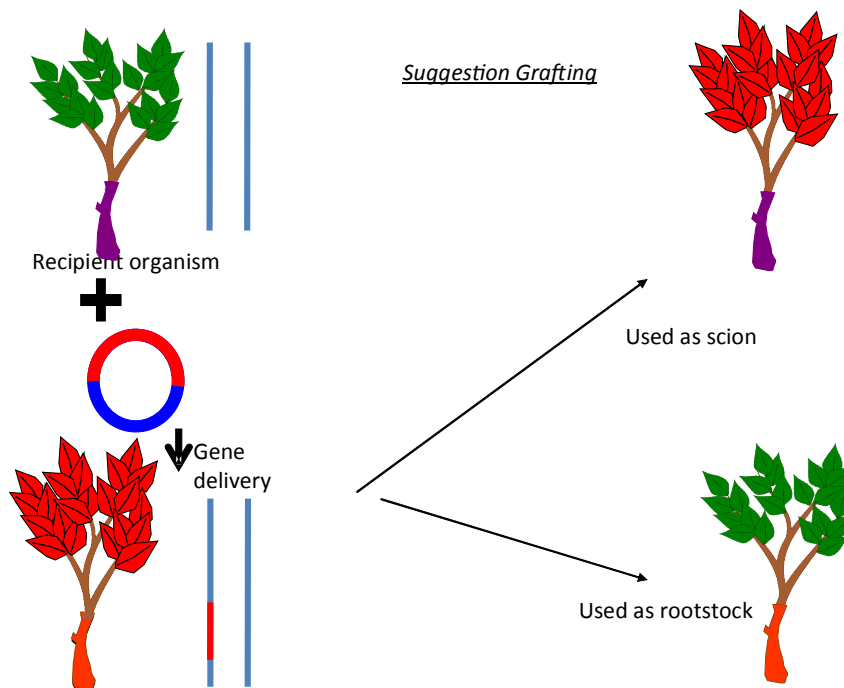


1 Final

1

15.4 **Grafting**

2



3

4**Fig. 4 Grafting**

5Schematic representation of grafting using both conventionally bred and GM plants. Top left: a conventionally  
6bred plant line; bottom left a transgenic plant line derived thereof; right two different chimera, where either the  
7transgenic plant line or the conventionally bred plant line (bottom) are used as root stock or scion, respectively.

8

9

10**105.4.1 Definition and brief description of the technique based on the current scientific  
11 knowledge and current scientific literature**

12

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

21

22 Two possibilities were considered:

- 23 • Grafting a non-GM scion onto a GM rootstock;
- 24 • Grafting a GM scion onto a non-GM rootstock.

25

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

<sup>219</sup> A chimera is an organism consisting of at least two genetically different kinds of tissue.

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

3

#### 45.4.2 **Application of the technique**

5

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

8

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

19

#### 205.4.3 **Other issues**

21

##### 22 **A Potential impacts of technique**

23

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

#### 435.4.4 **Relevant issues for classification**

44

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

- 1 may grow from the rootstock and produce flowers and seeds (fruits). These  
2 seeds or fruits are by definition GM.
- 3 • Where a GM scion is grafted onto a non-GM rootstock, the whole plant is a  
4 chimera in which the flowers seeds, fruits, and parts of the scion like the leaves  
5 are GM.
  - 6 • In some cases, grafting could be used as a tool to obtain an effect through RNA  
7 interference. In this case, the conclusions of the WG for RNA-dependent DNA  
8 methylation should also be taken into account (see point 5.4).
- 9

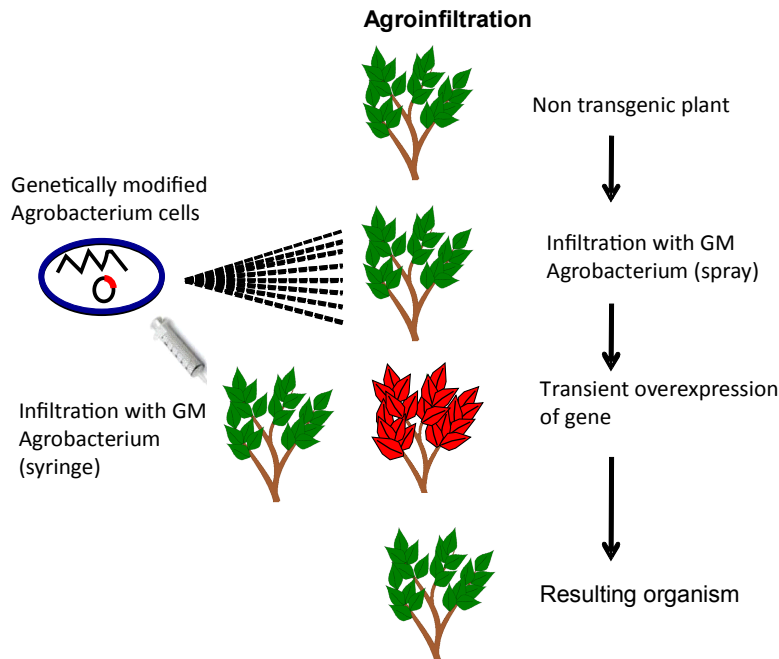
#### 10 105.4.5 Coverage by GMO legislation

- 11
- 12 • There is a general agreement that the whole plant, which is a chimera, falls  
13 within the scope of Directive 2001/18/EC.
  - 14 • Where the fruit/seed/offspring are being considered and where a non-GM scion  
15 is grafted onto a GM rootstock, the resulting fruit/seeds/offspring derived from  
16 the scion do not fall under the scope of Directive 2001/18/EC.
  - 17 • Where a GM scion is grafted onto a non-GM rootstock, the resulting  
18 fruit/seeds/offspring fall under the scope of Directive 2001/18/EC.
  - 19 • **Detection**  
20 The transgenic part of the chimera can be specifically detected if a sufficient  
21 part of the sequence of the insert as well of the adjacent sequence is known. As  
22 the DNA sequence of the non-GM scion is not modified, detection and  
23 identification of the GM rootstock on the basis of the harvested product (part  
24 of the non-GM scion) is currently not possible and is very unlikely to be  
25 developed in the near future.
- 26  
27

15.5. Agro-Infiltration

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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 5.1 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

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

### 3 4 **45.5.2 Application of the technique**

5  
6 Depending on whether or not the plant tissues contain germline cells/tissues, two types  
7 of agro-infiltration can be distinguished:

#### 8 1. **Agro-infiltration “sensu stricto”:**

9 Non-germline tissues (typically, leaf tissues) are agro-infiltrated in order to obtain  
10 localised expression, for instance:

- 11 i. to obtain large amounts of a given protein expressed in plant tissues;
- 12 ii. to test the phenotypic effect of a given gene product in plants (for  
13 instance, when an avirulence<sup>20</sup> gene or similar factor is expressed, the  
14 possibility to rapidly screen plant populations for the presence of the  
15 cognate resistance gene has potential applications in the breeding  
16 industry).

#### 17 2. **“Floral dip”:**

18 Flowers or inflorescences containing germline cells are agro-infiltrated in order to  
19 obtain stable transformation of some embryos that can then be selected at the  
20 germination step. Nowadays this is the technique of choice to transform the model  
21 plant *Arabidopsis thaliana*, and it can also be used for other species in the same  
22 family (including rapeseed, cabbage, mustard etc).

23  
24 Agro-infiltration takes place under conditions of containment. Progeny, if  
25 produced, is grown in a greenhouse and may be grown outdoors in some cases, for  
26 instance when agro-infiltration is used as a tool in the process of selecting  
27 commercial varieties with a given phenotype.

### 28 29 **295.5.3 Relevant Issues for classification of the resulting plant**

- 30  
31 • The agro-infiltrated plant tissues contain *Agrobacterium* cells in their  
32 intercellular space. The *Agrobacterium* cells do not enter the plant cells. Generally  
33 they remain localised in the infiltrated area, where they survive and can even  
34 multiply since this situation is similar to their ecological niche. Some movement of  
35 the bacterial cells to non-infiltrated areas of the plant (including the reproduction  
36 organs) is conceivable but not expected.
- 37 • These *Agrobacterium* cells contain a plasmid with a T-DNA region in which,  
38 typically, foreign DNA has been inserted by biotechnological techniques. The  
39 bacteria actively deliver the T-DNA into the plant cells.
- 40 • The T-DNA accumulates but does not replicate in the plant cell. It undergoes  
41 nuclear transcription and the derived mRNA undergoes translation. Integration into  
42 the genome is a rare event that can occur in a small number of cells in each  
43 infiltrated area: the selection of these rare events to regenerate stable transformants

---

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

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

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

#### 17 185.5.4 Coverage by GMO legislation

##### 19 A Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the 20 technique may be attributed

- 21 • The recombinant *Agrobacterium*. It is a GMM and falls within the  
 22 scope of Directive 2009/41/EC (Annex I, Part A) when it is present.
- 23 • The plants that were subjected to agro-infiltration. Integration of the  
 24 foreign DNA into the genome of a few plant cells of the infiltrated area is a  
 25 rare event that needs to be examined on a case-by-case basis. Furthermore,  
 26 cells in which T-DNA integration into the genome has occurred will not be  
 27 selected for the regeneration of entire plants (even in the case of floral dip,  
 28 this selection process occurs in the following generation). On this basis,  
 29 there was a divergence of opinion among the experts as to whether plants  
 30 subjected to agro-infiltration should formally be considered to fall under  
 31 the scope of the Directives. However all experts agreed that these plants  
 32 fall under the scope of Directive 2009/41/EC since they contain GMMs  
 33 (see above).
- 34 • The progeny of the plants subjected to agro-infiltration “*sensu*  
 35 *stricto*”. Plants derived from lines where the absence of a stably integrated  
 36 event into the genome is supported by relevant data, should be considered  
 37 as falling outside the scope of Directive 2001/18/EC.
- 38 • The progeny of the plants subjected to “floral dip”. Since floral dip is  
 39 actually designed to produce plant lines with stable integration events, the  
 40 progeny lines that are shown to harbour a stable integration event fall  
 41 clearly within Annex IA, Part 1 of Directive 2001/18/EC.  
 42  
 43  
 44

**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**

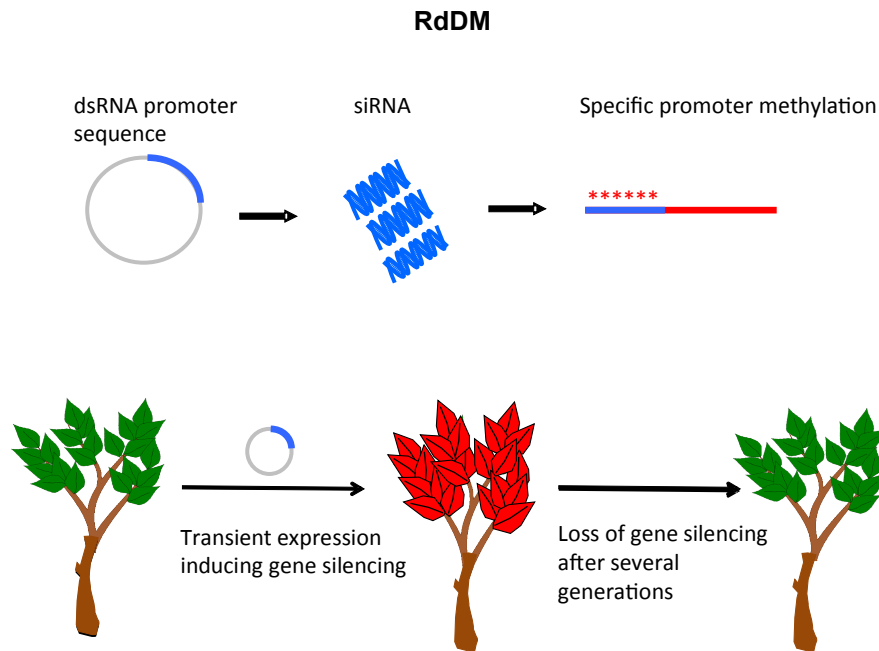
If the constructs introduced into plants by agro-infiltration are not replicated and/or integrated, their presence is transient and can be detected only in the agro-infiltrated plant itself. These DNA fragments will not be transferred to the next generation so they cannot be detected or identified in 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.



15.6 **RNA-dependent DNA methylation (RdDM)**

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3

**4Fig. 6 RNA-dependent DNA methylation**

5Schematic representation of RNA-dependent DNA methylation. SiRNA can be introduced via a vector (top left),  
 6or introduced directly. Specific methylation of plant genes is indicated by asterisks above the DNA sequence.  
 7The red plant in the bottom indicates the (transient) silencing of plant. This does not necessarily mean that the  
 8plant is transgenic.

9

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

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RdDM is a technique that uses the effect of small RNA sequences e.g. micro RNA (miRNA) or small/short interfering RNA (siRNA) to alter gene expression through methylation of specific DNA sequences without changing the nucleotide sequence itself (epigenetic change). The purpose could be to shut down expression of specific genes. This gene silencing obtained by the methylation can be inherited through some generations, but will eventually disappear.

The mechanisms by which the DNA methylation and eventually the gene silencing takes place are part of a complex and naturally occurring mechanisms that organism use in their cell regulation.

Gene silencing through DNA methylation can be accomplished in a cell or in an organism by introducing a gene which, once transcribed, gives rise to the formation of double stranded RNAs (dsRNA) and further into small interfering RNA. If these interfering RNA molecules share homology with a promoter region, they can specifically induce methylation resulting in the silencing of the gene regulated by the promoter. This technique allows for highly targeted gene silencing. DNA methylation patterns can be maintained, even after clearance of the initially new inserted gene e.g.

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

### 3 4 **45.6.2 Application of the technique**

5  
6 In mammalian cells, nematodes and flies, RdDM can be induced by direct introduction  
7 of double stranded RNAs (e.g. by liposomes or naked siRNA covalently conjugated  
8 with protein).

9  
10 In contrast, RdDM in plants is usually established by transformation with a DNA  
11 construct that encodes hairpin RNAs. For the purpose of the further discussion below  
12 certain scenarios can be envisaged:

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

24  
25 A possible limitation of the technique in some applications may be that the epigenetic  
26 changes fade out and in that sense the duration of the effect is unpredictable over time.  
27 Changes over generations are expected to last longer in plants than in animals.

### 28 29 **295.6.3 Relevant Issues for classification**

- 30  
31 • **Similarities to natural processes:** DNA methylation leading to changes in  
32 gene expression and phenotype can also occur naturally.
- 33 • **Intermediate organism and resulting organism:** both the insertion of the  
34 DNA or RNA resulting in RdMD can be accomplished in different ways (e.g.  
35 direct/indirect, with/without vectors involved) and the resulting organisms can  
36 be selected in different ways (e.g. with/without inserted DNA). All these  
37 factors can influence on the interpretation on the GMO status of the  
38 intermediate as well as the resulting organisms and therefore lead to many  
39 different views from the experts (se also 4.6 offspring section).

### 40 41 **415.6.4 Coverage by GMO legislation**

#### 42 43 **A Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the** 44 **technique may be attributed**

45  
46 All experts agreed that the technique used in scenario 1 is covered by Annex  
47 IA, Part 1 of Directive 2001/18/EC and Annex I Part A or Directive  
48 2009/41/EC and the resulting organisms are GMOs.

1 All experts also agree that the new methylation itself is not regulated by the  
2 Directives since methylation of nucleotides is not considered as an alteration of  
3 the genetic material in the sense of the Directives. Therefore, for most of the  
4 experts the resulting organisms in scenario 2 and 3 is not covered by Annex  
5 1A, Part 1 of Directive 2001/18 or Annex I Part A or Directive 2009/41/EC  
6 because neither of these organisms (with the new methylation) contains any  
7 heritable changes in nucleotide sequences. This conclusion is reached even if  
8 the intermediate organisms are considered GMOs. The arguments for this  
9 conclusion are related to the discussion about transient presence and offspring  
10 in chapter 4.4 and 4.5.

11  
12 A majority of experts considered that intermediate organisms (scenarios 2 and  
13 3) do not fall under the definition of GMO of either Directives if the RNA is  
14 directly delivered into the cell without being able to replicate. This was based  
15 on the grounds that the selected offspring organism does not contain the  
16 inserted gene coding for siRNA and no heritable changes in nucleotide  
17 sequences are created. The resulting organisms are thus not GMOs.

18  
19 A few experts considered that if the intermediate organism is considered a  
20 GMO the resulting organism is also a GMO in the sense of the Directive. This  
21 was based on the ground discussed in the section on GMO offspring.

22  
23 All experts agreed that the resulting organism (e.g from scenarios 2 and 3), not  
24 containing the inserted gene coding for RNAi and having no heritable changes  
25 in nucleotide sequences, should logically be outside the scope of the Directives  
26 due to the fact that the resulting organisms are comparable to organisms  
27 obtained with natural processes.

28  
29 **B Additional remarks for regulation on the status of the technique**

30  
31 The current and future status of the technique depends on the interpretation of  
32 the legislation as regards the use of intermediate organisms considered GMOs.

33  
34 **5.6.5 Other issues**

35  
36 **A Potential impacts of the technique**

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

42  
43 A possible limitation of the technique may be envisioned in the fact that the  
44 epigenetic changes are unstable and in that sense unpredictable over time.

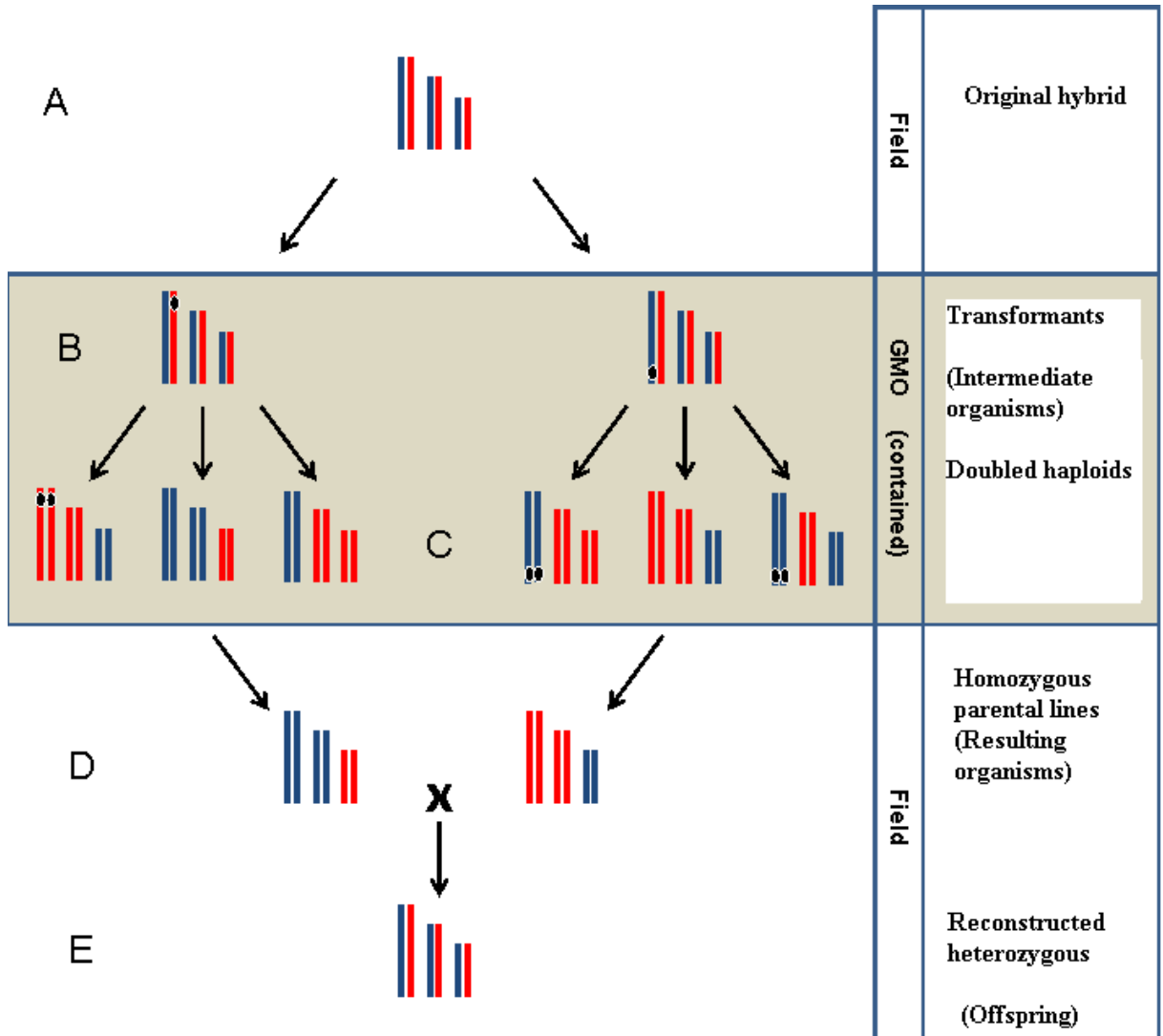
45  
46 **B Concluding remarks**

- 47  
48 • The current and future status of RdDM depends on the interpretation of  
49 the legislation as regards the use of intermediate organisms considered  
50 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.

15.7 **Reverse breeding**  
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**Fig. 7 Reverse Breeding**

Schematic explanation of the steps involved in reverse breeding. For details see text.

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

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

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

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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.

### 225.7.2 **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.

### 285.7.3 **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.

### 375.7.4 **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

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

3

4 **B Concluding remarks for regulation on the status of the technique**

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

9

10 • The genetic material of the resulting organisms and their offspring have  
11 never contained any inserted foreign DNA;

12

13 • the genetic composition of the offspring is the same as the original  
14 organism or plant material; and

15

16 • the resulting organisms and their offspring can be obtained by  
17 traditional breeding techniques.

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

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**15.8 Synthetic Genomics**

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

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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"<sup>21</sup>) 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.

**295.8.2 Application of the technique**

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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 introduced

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



1 into host cells by so-called genome transplantation. As it has been shown, complete  
2 bacterial genomes can be introduced into a living cell where through selection, the  
3 parent genome can be replaced by the introduced one.  
4

### 55.8.3 Relevant Issues for classification

6  
7 The creation of synthetic genomes and their transplantation into recipient hosts  
8 appears to meet the definition of genetic modification as laid out in Directives  
9 2001/18/EC and 2009/41/EC. However, the following elements should be taken into  
10 consideration:

- 11 • Synthetic genomes can be introduced into a recipient such as cell extracts or  
12 protocells. Since these recipients as such, are not capable of replication (in the  
13 meaning of the Directives) or of transferring genetic material they do not meet the  
14 definition of an organism or microorganism.
- 15 • Once synthetic genomes are introduced into a host then the resulting entity meets  
16 the definition of an organism or micro-organism, if it is capable of replication or of  
17 transferring genetic material in the meaning of the Directives.
- 18 • Point 2 of Annex I Part A of Directive 2009/41/EC (and of Annex IA Part 1 of  
19 Directive 2001/18/EC), which refers to techniques involving the direct  
20 introduction of heritable material prepared outside the organism, could be  
21 interpreted as including introduction of full genomes into a recipient structure.  
22

### 235.8.4 Coverage by GMO legislation

#### 24 **A Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the** 25 **technique may be attributed** 26

27  
28 To date most of the work on synthetic genomics has been undertaken under  
29 contained use. However, it could be envisaged that medicinal products (e.g.  
30 vaccines, gene therapy) or environmental sensors could be developed that  
31 would be considered for deliberate release.  
32

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

- 35 (a) the emphasis is on the recipient (cell extracts or protocells), which is  
36 not considered as a (micro-)organism (see above). In this case, the  
37 technique falls outside the scope of the Directives;
- 38 (b) the emphasis is on the resulting entity, which is considered to be a  
39 (micro-)organism. In this case, the technique falls under the scope of  
40 the Directives in the meaning of Point 2 of Annex I Part A of Directive  
41 2009/41/EC (and of Annex IA Part 1 of Directive 2001/18/EC), which  
42 refers to techniques involving the direct introduction of heritable  
43 material prepared outside the organism. When recombinant nucleic acid  
44 molecules are used, Point 1 of the above-mentioned annexes may also  
45 apply.  
46

1 **B Concluding remarks for regulation on the status of the technique**

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

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

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13 **5.8.5 Other issues**

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15 **Potential impact of the technique**

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

23

24

1	<b>Appendix 1</b>	<b>Terms of Reference</b>
2		
3	<b>Appendix 2</b>	<b>Experts nominated by the Competent Authorities for participation in the New Techniques Working Group</b>
4		
5		
6	<b>Appendix 3</b>	<b>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</b>
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10	<b>Appendix 4</b>	<b>Abbreviations</b>
11		
12	<b>Appendix 5</b>	<b>References</b>

1 **Appendix 1**

2 **Terms of Reference**

3



EUROPEAN COMMISSION  
DIRECTORATE-GENERAL  
ENVIRONMENT

Directorate B - Protecting the Natural Environment

4

Brussels,

5

ENV B3/AA/ D(2008)

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**WORKING GROUP ON THE ESTABLISHMENT OF A LIST OF TECHNIQUES**

7

**FALLING UNDER THE SCOPE OF**

8

**DIRECTIVE 2001/18/EC ON THE DELIBERATE RELEASE OF GENETICALLY MODIFIED**

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**ORGANISMS INTO THE ENVIRONMENT**

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**AND DIRECTIVE 90/219/EEC**

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**ON THE CONTAINED USE OF GENETICALLY MODIFIED MICRO-ORGANISMS**

12

**TERMS OF REFERENCE**

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13 **Introduction**

14 At the meeting of the Competent Authorities under Directive 2001/18/EC<sup>22</sup> which was held on  
15 16 April 2007, the Dutch delegation proposed that a Working Group (WG) be set up to  
16 address new techniques which are applied in plant breeding and modification of organisms in  
17 general. The background for this request is that Member States increasingly deal with  
18 questions from stakeholders whether newly applied techniques result in a genetically  
19 modified organism (GMO). These questions have led to different interpretations among  
20 Member States. In order to harmonize the approach of Member States in this issue, the Dutch  
21 delegation proposed that the WG address whether these (new) techniques lead to GMOs as  
22 defined under Directives 2001/18/EC and 90/219/EEC<sup>23</sup>. The proposal was welcomed by BE,  
23 DK, DE, SE, FI, FR, AT, MT, NO and SL.

24 **Terms of reference**

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

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

<sup>23</sup> Directive 90/219/EEC, Council Directive of 23 April 1990 on the contained use of genetically modified  
6 micro-organisms

1excluded from the scope of the Directives. The following parts of Directive 2001/18/EC and  
2Directive 90/219/EEC as amended by Directive 98/81/EC<sup>24</sup> have to be taken into account:

- 3           •           Definition of a GMO : "*genetically modified organism (GMO)*" means  
4           an organism, with the exception of human beings, in which the genetic material  
5           has been altered in a way that does not occur naturally by mating and/or  
6           natural recombination" (Directive 2001/18/EC Article 2)
- 7           •           Definition of a GMM: ""*genetically modified micro-organism (GMM)*"  
8           shall mean a micro-organism in which the genetic material has been altered in  
9           a way that does not occur naturally by mating and/or natural recombination"  
10          (Directive 90/219/EEC, Article 2)
- 11          •           List of the techniques leading to genetic modification (Annex I Part A  
12          of Directive 90/219/EEC and Annex IA, Part 1 of Directive 2001/18/EC)
- 13          •           List of the techniques which are not considered to result in genetic  
14          modification (Annex I, Part B of Directive 90/219/EEC and Annex IA Part 2 of  
15          Directive 2001/18/EC)
- 16          •           List of the techniques of genetic modification yielding organisms to be  
17          excluded from the Directives (Annex II Part A of Directive 90/219/EEC and  
18          Annex IB of Directive 2001/18/EC)
- 19          •           The objective of the legislation is to protect human health and the  
20          environment (Directive 2001/18/EC, Article 1; Directive 90/219/EEC Article  
21          1)

22The techniques listed in the Annexes (Annex IA Part 1, Annex IA Part 2 and Annex IB of  
23Directive 2001/18/EC and Annex I Part A, Annex I Part B and Annex II Part A of Directive  
2490/219/EEC) could be complemented with relevant new techniques, if that is considered  
25necessary, and according to the appropriate procedures foreseen for a possible review of  
26Directive 90/219/EEC and Directive 2001/18/EC.

27As stipulated by Article 31(1) of Directive 2001/18/EC, Member States and the Commission  
28shall meet regularly and exchange information on the experience acquired with regard to the  
29prevention of risks related to the release and placing on the market of GMOs. The  
30establishment of a specialised WG focusing on techniques leading to genetic modification can  
31serve the purpose of risk prevention in the sense of the above provision.

### 32 **Objective(s)**

33The WG shall evaluate a list of new techniques for which it is unclear whether they result in  
34genetic modification. These techniques will be discussed in the light of:

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

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<sup>24</sup> Council Directive 98/81/EC of 26 October 1998 amending Directive 90/219/EEC on the contained use  
3of genetically modified micro-organisms.

## 1 Final

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 under  
31       the two Directives. It shall be reviewed and updated in the light of new scientific  
32       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 WG  
35 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.

## 1 Final

1 Further information as regards the structure and the substance of the work progress will be  
2 provided on a regular basis to Member States.

### 3 **Proposed Work Plan and Agenda**

4 During the first meeting of the New Techniques WG, the experts will compile and  
5 complement the list of techniques to be analysed. A starting point will be the list of techniques  
6 already provided by the Member States in 2007. A prioritisation of the tasks shall be defined.

7 It is planned to work in sub-groups dealing with different categories of techniques, if suitable.

8 Each sub-group will nominate a rapporteur. The outcome of the first meeting shall be

9 submitted to and agreed by the Competent Authorities under Directive 2001/18/EC and

10 Directive 90/219/EEC.

11 The sub-groups will work via e-mail co-ordination. The results will be presented at the

12 subsequent meeting of the WG. It is planned to commence the activity of the group in

13 December 2008. A document outlining the state of play of group's work will be presented to

14 the Competent Authorities on a regular basis.

1 **Appendix 2**

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3 **Experts nominated by the Competent Authorities for participation in the New**4 **Techniques Working Group**

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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
Dr. Philippe Herman	Scientific Institute of Public Health - Division of Biosafety and Biotechnology (SBB)	Belgium
Dr. Katia Pauwels	Scientific Institute of Public Health - Division of Biosafety and Biotechnology (SBB)	Belgium
Dr. Genoveva Nacheva	Bulgarian Academy of Science	Bulgaria
Prof. Dr. Milan Bartos, Ph.D.	Genex CZ, Ltd.	Czech Republic
Dr. Jaroslava Ovesna	Crop Research Institute	Czech Republic
Mr. Jan Pedersen	National Food Institute	Denmark
Dr Hannes Kollist	University of Tartu	Estonia
Dr. Kirsi Törmäkangas	Ministry of Social Affairs and Health Board for Gene Technology	Finland
Prof. Matti Sarvas	National Institute for Health and Welfare & National Board for Genetechnology	Finland
Mr. Olivier Le Gall	INRA de Bordeaux	France
Prof. Jean-Christophe Pages	universités – praticien hospitalier en biochimie biologie moléculaire	France
Prof. Dr. Detlef Bartsch	Federal Office of Consumers Protection and Food Safety	Germany
Prof. Dr. Hans-Jörg Buhk	Federal Office of Consumers Protection and Food Safety	Germany
Prof. Wilfried Wackernagel	Universität Oldenburg	Germany
Dr. Tom McLoughlin	Environmental Protection Agency	Republic of Ireland
Ms. Bernadette Murray	Environmental Protection Agency	Republic of Ireland
Mr. Donal Grant	Environmental Protection Agency	Republic of Ireland
Ms. Elena Sturchio	Istituto Superiore per la Prevenzione e la Sicurezza del Lavoro (ISPESL)	Italy
Prof. Isaak Rashal	University of Latvia	Latvia
Mrs. Odeta Pivoriene	Ministry of the Environment	Lithuania
Dr. Boet Glandorf	National Institute of Public Health and the Environment	The Netherlands



1 Final

Ms. Hanneke Bresser	Ministry of Infrastructure and Environment	The Netherlands
Dr. Eirik Biering	Directorate for Nature Management	Norway
Dr. Casper Linnestad	The Norwegian Ministry of the Environment	Norway
Ms. Tove Loken	Ministry of Health and Care Services	Norway
Dr. Teresa Borges	General Health Directorate	Portugal
Clara Fernandes	Instituto Nacional dos Recursos Biológicos, I.P.	Portugal
João Lavinha	National Institute of Health	Portugal
Prof. Călina Petruța Cornea	Romanian Biosafety Commission	Romania
Ms. Zdenka Balatova	Ministry of the Environment	Slovakia
Mr. Piet van der Meer	HORIZONS sprl	Slovakia
Dr. Borut Bohanec	Biotechnical Faculty Ljubljana	Slovenia
Dr. Marko Dolinar	Faculty of Chemistry and Chemical Technology	Slovenia
Mr. D. Rafael Pérez Mellado	Centro Nacional de. Biotecnologia, Madrid	Spain
Ms. Katarina Eskils	Swedish Work Environment Authority (SWEA)	Sweden
Dr Marie Nyman	Swedish Gene Technology Advisory Board; The	Sweden
Ms. Louise Ball	DEFRA	United Kingdom
Mr. Michael Paton	HSE	United Kingdom

1

1 **Appendix 3**

2 **GMO Definitions in Directives 2001/18/EC and 2009/41/EC**

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4

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

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Article 2 (Definitions)

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For the purpose of this Directive:

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(1) "organism" means any biological entity capable of replication or of transferring genetic material;

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(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;

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Within the terms of this definition:

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(a) genetic modification occurs at least through the use of the techniques listed in Annex I A, part 1;

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(b) the techniques listed in Annex I A, part 2, are not considered to result in genetic modification.

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(3) ... (8)

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Annex I A

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Techniques referred to in Article 2 (2)

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Part 1

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

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(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;

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(2) techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation;

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(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.

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Part 2

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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.

**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) ....

1 Annex I

2 Part A

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

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

17  
18 Part B

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

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

27  
28 Annex II

29 Part A

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

- 34 1. Mutagenesis.
- 35 2. Cell fusion (including protoplast fusion) of prokaryotic species that exchange  
36 genetic material by known physiological processes.
- 37 3. Cell fusion (including protoplast fusion) of cells of any eukaryotic species,  
38 including production of hybridomas and plant cell fusions.
- 39 4. Self-cloning consisting in the removal of nucleic acid sequences from a cell of  
40 an organism which may or may not be followed by reinsertion of all or part of  
41 that nucleic acid (or a synthetic equivalent), with or without prior enzymic or  
42 mechanical steps, into cells of the same species or into cells of  
43 phylogenetically closely related species which can exchange genetic material  
44 by natural physiological processes where the resulting micro-organism is  
45 unlikely to cause disease to humans, animals or plants.  
46 Self-cloning may include the use of recombinant vectors with an extended  
47 history of safe use in the particular micro-organisms.

1Appendix 4

Abbreviations

2

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

## 1Appendix 5

2

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4

## 5RdDM /RNA silencing/Transcriptional Gene Silencing

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