ACRE advice: New techniques used in plant breeding

Executive summary

Over recent years the number of enquiries has increased as to whether organisms produced by certain techniques are captured by the EU's GMO legislation. In this advice, ACRE considers techniques that were identified by an EU Commission working group as posing particular challenges (these are listed in the first column of Table 1).

ACRE has discussed these techniques in the context of plant breeding although many of them (or equivalents) are used in the modification of other organisms such as animals and microorganisms. We have come to our conclusions about the status of these techniques and the organisms that they generate by determining whether the scientific terms in the definition apply in each case. However, as the legal significance of many of these terms is not clear, Ministers will need to seek a legal opinion on our conclusions. Our considerations have highlighted three main issues where legal clarification is required. These issues are as follows.

A. **Are the offspring of GMOs necessarily GMOs? The answer may affect our conclusion on the status of organisms produced by reverse breeding.**
B. **It is not clear what '<u>altering</u> the genetic material of an organism in a way that does not occur naturally by mating or natural recombination' means in the context of this legislation. Does it require the formation of new combinations of genetic material that do not occur naturally, as may be implied by the examples of GM techniques in Part 1 of Annex 1A in Directive 2001/18/EC? In which case, it would <u>not</u> include modifications that do not alter the nucleotide sequence of the genetic material, or changes that will result in nucleotide sequences that are likely to be present in nature or as a result of conventional breeding. This fundamental question has ramifications for other issues linked to interpreting the definition. In particular, the answer may affect our conclusions on cisgenics and RNA-dependent DNA methylation.**
C. **ACRE contests the relevance of referring to 'recombinant nucleic acid molecules' where these molecules are not inserted into the genome of the host organism i.e. in Annex 1B of Directive 2001/18/EC, which lists GM techniques that produce**

organisms that are excluded from the legislation. This point is relevant to our conclusion on the status of organisms produced by mutation, such as through the use of zinc finger nucleases or oligonucleotides.

Table 1 summarises ACRE's conclusions on the status of these techniques and the organisms generated by them. We note that a combination of these techniques may be used in the generation of a new plant variety. The asterisks in Table 1 indicate where there is flexibility in the legal interpretation of the definition that could affect ACRE's conclusions. The three issues listed above are the source of this uncertainty; the letters in brackets in Table 1 relate to this list. This uncertainty is discussed in more detail in the respective sections of the report.

The techniques that require a decision as to the regulatory status of their products most urgently are cisgenesis and oligo-directed mutagenesis.

Techniques	**Involves a GM technique?**	**Produces an intermediate product that is a GMO?**	**Offspring [1] are GMOs?**
Cisgenesis/intragenesis	Yes*(B)/Yes	-	Yes*(B)/Yes
Reverse breeding	Yes	Yes	No[2]* (A)
Agroinfiltration	Yes	questionable[3]	No[2]
Grafting (non-GM scion/GM rootstock)	No	Yes	No
RNA-dependent DNA methylation	No - nucleic acid molecules not inserted into genome.	No	No*(B)
	Yes – nucleic acid	Yes	No[2]*(B)

[1] Produced by sexual reproduction.
[2] Generally a technique that involves the insertion of nucleic acid molecules into the host plant's genome would be considered a GM technique (noting our question about the need to form new combinations of genetic material). Intermediate products containing this genetic material would be considered GMOs. However, offspring that do not contain this inserted material would not be considered GMOs.
[3] Questionable but not significant to regulators in this context because the plants are containers for GM *Agrobacterium* and this will need to be regulated anyway. It is significant in the context of animals vaccinated with DNA vaccines – but this is not discussed here because it raises further issues about continued propagation/ heritability of the genetic modification.

	molecules inserted into genome		
Oligo-directed mutagenesis	Yes	No	No
Zinc finger nucleases (mutagenesis)	Yes	No	No*(C)

Table 1, summarising ACRE's conclusions on the individual techniques described in this advice and the organisms produced by them. The third column shows that organisms generated during intermediate steps in the process/technique may have a different status to organisms generated at the end of the process. The latter are dealt with in the final column and are organisms that regulators, responsible for the deliberate release of GMOs, are likely to have to take a view on. Intermediate organisms may have a different status to their offspring in cases where the intermediate organism contains a transgene but selected offspring do not (e.g. reverse breeding and RNA-dependent DNA methylation).

* the asterisk shows where there is uncertainty about the legal interpretation of the definition, which affects ACRE's conclusion on the status of the technique and/or the organisms produced by a technique. The bracketed letters (A, B or C) link this uncertainty to one of the three issues identified earlier in this summary.

ACRE is concerned by the extent to which the definition of a GMO is open to interpretation. We advise that a transparent, scientifically robust interpretation be adopted if the EU continues to employ the current definition. In particular, ACRE advises that the changes conferred by these techniques are considered in the context of the extensive genetic and epigenetic variation that is present in organisms of the same species. The extent of the plasticity of plant genomes and epigenomes (and the epi/genomes of other organisms) is becoming increasingly apparent as the analytical technology in this area advances.

ACRE has not been asked to consider definitions adopted in other legislative instruments and protocols. Different definitions and approaches to regulation will lead to different outcomes as to whether an organism is considered GM or not. ACRE advises that this should be taken into account when assessing the consequences of including or exempting a technique or excluding the products of a GM technique.

Structure of the advice:

1. Introduction

2. Legislation

3. Cisgenics

4. Reverse Breeding

5. Agroinfiltration

6. Grafting on genetically modified rootstock

7. RNA-dependent DNA methylation via RNAi/siRNA

8. Oligonucleotide-Directed Mutagenesis

9. Zinc finger nucleases

10. Conclusions

References

1. Introduction

In 2007 the EU Commission and Member States agreed to establish an expert working group on 'New Techniques'. This was in response to an increasing number of enquiries concerning the status of products generated by techniques that regulators had not considered previously. The group finalised its report at the beginning of 2012[4].

The EU working group was mandated to consider the status of these techniques in line with the definitions used in EU Directives dealing with the regulation of the deliberate release (Directive 2001/18 EC) and contained use (Directive 2009/41/EC) of GMOs and GMMs respectively. It was not asked to consider equivalent definitions adopted in other legislative instruments and protocols. Different definitions and approaches to regulation will lead to different outcomes as to whether an organism is considered GM or not. This should be taken into account when assessing the consequences of including or exempting a technique or excluding the products of a GM technique. For example, the UK is a signatory to the Cartegena Protocol on Biodiversity, which has a different definition of a GMO (referred to as a Living Modified Organism in this context) to that of Directive 2001/18/EC.

ACRE has been requested to provide advice that will prepare UK Ministers for discussions that will ensue at EU-level. ACRE has been asked to consider the techniques that the working group is discussing and to advise Ministers on the science surrounding whether the techniques (and the organisms they generate) are captured, and on arguments for and against their regulation under the GMO legislation. While it is the role of decision-makers to interpret the legislation, it is important that ACRE advises, where appropriate, on the scientific terms used

[4] This is document will not be published until it has been discussed by competent authorities under the GMO contained use and deliberate release Directives.

in the legislation, not only where they apply to a technique, but also where there is uncertainty and scientifically credible options open to regulators.

ACRE has focused its attention on the use of the techniques as tools in plant breeding. The EU working group is also considering techniques that might fall under the scope of Directive 2009/41/EC (which concerns the contained use of GM microorganisms). The definitions and examples of GM and non-GM techniques in this legislation are very similar to those in Directive 2001/18/EC. However, there are differences: in particular, the contained use legislation excludes microorganisms produced by self-cloning (cisgenesis) as long as the resulting microorganisms are unlikely to cause disease[5], whereas self-cloning is not excluded in the deliberate release legislation.

The aim of plant breeding is to take advantage of existing genetic variation or to generate variation from which desirable characteristics can be selected. As the technology has advanced, the tendency is for breeders to introduce more targeted/precise changes to plant genomes and to be able to identify desirable genotypes more rapidly. Breeding techniques involving recombinant nucleic acids have also advanced since the EU's GMO legislation was drafted in the late 1980s (and came into force in 1990). Heritable changes in plants may be conferred by techniques other than those that involve the stable integration of foreign DNA into the genome. For example, a relatively short nucleic acid molecule, that is not necessarily recombinant and that is not inserted into the plant's genome may be used to trigger the plant to make changes to its own genome either in the form of short additions or deletions of DNA or chemical changes that result in altered expression of a particular gene. Recombinant nucleic acid techniques may be used at an intermediate stage in the production of a new breeding line without transgenic material being present in the final product. As knowledge and associated technology develop, regulators should assess whether the legislation continues to serve the purpose for which it was intended.

[5] Annex II Part A of the Contained Use Directive defines self-cloning as 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.

It is important to understand the nature of the changes conferred by these new techniques in context, i.e. to compare techniques that are currently considered to create GMOs with those techniques that are not captured by the legislation and with changes that occur naturally.

With respect to the latter, the plasticity that plants exhibit should be highlighted – both in terms of the characteristics that individual plants show under different conditions as well as those shown by different plants of the same species (e.g. ecotypes or commercial varieties). It allows plants from the same species to survive in different ecological niches and to adapt *in situ* to changing environmental conditions such as temperature changes or pathogens.

Plasticity may derive from variations in the genetic code (between plants) such as single nucleotide polymorphisms, deletions, duplications or differences in the copy number of certain genes etc. Differences in genetic sequence result from an array of natural processes such as recombination, mutation, changes in ploidy and the introgression of novel alleles through crossing with sexually compatible plants. However, differences in DNA sequence may not be the basis of all of the molecular changes that underpin the plasticity of plants. Altered cytosine methylation patterns, chromatin modifications and changes to the populations of small regulatory RNA molecules may also result in altered gene expression and in modifications to other genomic processes such as recombination and replication. These changes may be reversible such that they allow the plant to react to fluctuating environmental conditions. Some may be more stable, and in some cases, can be inherited by future generations.

Recently, new sequencing technologies have been used to explore the diversity that exists in the genomes and epigenomes of plants. To date, most of these studies have been carried out in the commonly found weed, *Arabidopsis thaliana* (Bevan, 2011; Cao et al., 2011). However, similar results have been obtained from crops including maize (Eichten et al., 2011; Hansey et al., 2012; Springer et al., 2009), wheat (Saintenac et al., 2011) and other grasses (Xu et al., 2012). The results of these analyses highlight the variation in DNA sequence, the differing copy numbers of particular sequences (Schrider and Hahn, 2010) and the variation in the methylation pattern of the DNA of individual plants within a species. Such variation underlies the traditional processes of plant breeding.

Against this background variation, the EU regulatory system requires the detection and traceability of GMOs (EC, 2003; Holst-Jensen et al., 2012). To date, this has been achieved by mapping sections of DNA that bridge the junction between the host genome and the inserted DNA. Emerging techniques that do not involve the introduction into the genome of heterologous DNA fragments from other species could pose challenges for unambiguous detection and testing, and ultimately enforcement of the EU regulatory system (Lusser et al., 2012).

The chapters dealing with the new techniques follow the structure:

- Background on the technique (in particular, how different it is from conventional breeding and established GM techniques);
- Significance of the technique (i.e. its potential to contribute to plant breeding and whether there are products near market)[6].
- Status of the techniques and their products, describing different perspectives;
- ACRE's conclusions.

The organisms (or products derived from them) generated by these techniques are likely to be captured by other regulatory controls such as the EU's Novel Foods Regulation and/or Plant Varieties and Seeds legislation. ACRE did not take these other legal instruments into account during its deliberations.

2. Legislation

ACRE's remit is to advise UK Ministers on the environmental safety of GMOs in accordance with the Environmental Protection Act (1990) and European Deliberate Release Directive 2001/18/EC (EC, 2001).

The Deliberate Release Directive provides a general definition of a GMO. Annexes supply additional information regarding the techniques that (i) result in genetic modification, (ii) are not considered to result in genetic modification, or that (iii) result in genetic modification but yield organisms that are excluded from the scope of the Directive i.e. that are not GMOs (see Table. 2).

[6] Please refer to Lusser et al (2012), for more detailed information on the commercial pipeline including the drivers and constraints for adoption of these new breeding techniques.

Table 2. The definition of a GMO according to Directive 2001/18/EC

Directive 2001/18/EC
Article 2 (1) "organism" means any biological entity capable of replication or of transferring genetic material; (2) "genetically modified organism (GMO)" means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. Within the terms of this definition: (a) genetic modification occurs at least through the use of the techniques listed in Annex I A, Part 1; (b) the techniques listed in Annex I A, Part 2, are not considered to result in genetic modification. ***Article 3.1*** This Directive shall not apply to organisms obtained through the techniques of genetic modification listed in Annex I B.
Annex I A ***Techniques referred to in Article 2(2)*** ***Part 1*** Techniques of genetic modification referred to in Article 2(2)(a) are *inter alia*: (1) Recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation; (2) Techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation; (3) Cell fusion (including protoplast fusion) or hybridization 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.
Annex I A ***Techniques referred to in Article 2(2)*** ***Part 2*** Techniques referred to in Article 2(2)(b) which are not considered to result in genetic modification, on condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms made by techniques/methods other than those excluded by Annex I B: (1) in vitro fertilization; (2) natural processes such as: conjugation, transduction, transformation; (3) polyploidy induction.
Annex I B ***Techniques referred to in Article 3*** 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.

In the European Union a novel organism will be regulated under the GMO regulatory framework if it has been developed using a technique that results in the organism being 'altered in a way that does not occur naturally by mating or natural recombination' (and it is

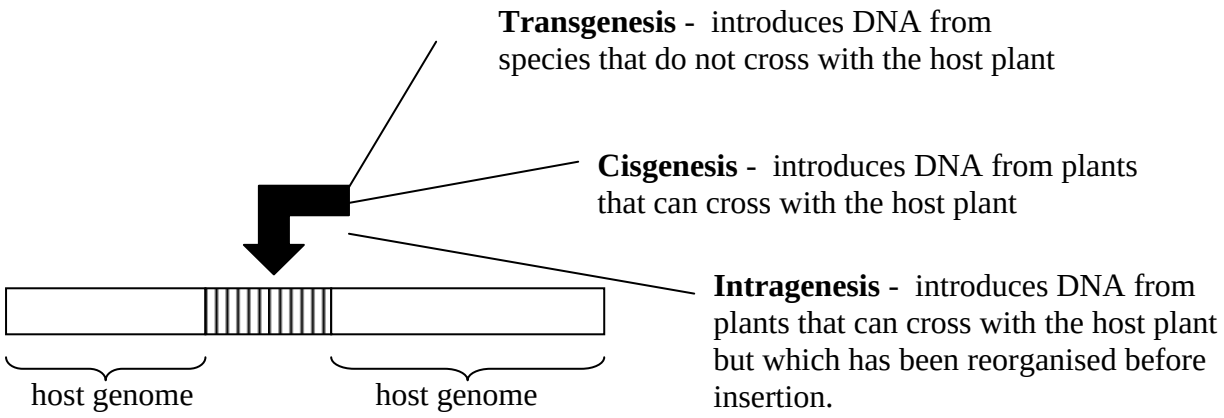
not produced using one of the two GM techniques listed in Annex 1B of the Directive – see Table 2).

With the advance of technology, new techniques have emerged, such as those allowing introduction of DNA from the same species (*e.g.* cisgenesis), modification of expression of existing genes (*e.g.* RNA interference), or introduction of targeted changes to nucleotides in the genome (*e.g.* oligonucleotide-mediated mutagenesis). These techniques may challenge the current regulatory definition of a GMO because it is not always clear whether the products obtained through these techniques are subject to the prevailing European GMO legislation or not. Answering this question is of utmost importance for developers and regulators of novel organisms, given the complexity and associated costs of applying the GMO legislation (Devos et al., 2010).

The Dutch Commission on Genetic Modification (COGEM) has played a leading role in the discussion on new plant breeding techniques and their application (COGEM, 2006, 2009 and 2010).

A number of other groups have questioned the status of particular techniques or the organisms that are derived from them. There have been a number of scientific papers arguing for the exemption of cisgenic plants from the scope of the EU Directives (see *e.g.* Jacobsen and Schouten, 2008). When considering oligonucleotide-directed mutagenesis, Breyer *et al.* (2009) concluded 'that any political decision on this issue should be taken on the basis of a broad reflection at EU level, while avoiding discrepancies at international level'.

3. Cisgenesis and cisgenic organisms / (intragenesis and intragenic organisms)



Background:

Amongst the broad array of newly developed plant breeding techniques, cisgenesis and the development of cisgenic plants represent an interesting case (Jacobson and Schouten 2009; Rommens et al. 2007). Whilst cisgenic plants have been modified using the same technique as transgenic plants, the introduced DNA derives from the plant itself or from a close, sexually compatible relative (see below).

This contrasts with the gene revolution in GM-plant breeding to date that has focused on the transfer of transgenes (DNA sequences, including a gene, from one organism that are inserted into the genome of another organism). For plant breeding, such transgenes represent a major advance in generating a new gene pool (Table 2), whereas cisgenes are available to breeders using traditional breeding techniques.

In cisgenic plants, the DNA inserted is a copy of that found in the donor organism i.e. the gene of interest is in its normal orientation and flanked by its native regulatory elements such that new combinations of DNA are not engineered. The genetic elements (the gene(s) of interested and associated regulatory elements such as promoters) inserted into intragenic plants can be from different sources, i.e. are combinations of genetic elements that are found in a specific plant species and/or species that are able to cross-hybridise with it. These combinations may not otherwise be found together in nature. This technique allows for vectors, including T-DNA borders, that deliver the DNA into the donor organism to be engineered from DNA present in the species being modified or from a sexually compatible species. This technique could be used to introduce small ('back to front') fragments of DNA into plants that would silence targeted native genes.

In response to consumer concerns about the insertion of 'foreign DNA' in transgenic plants, the Simplot Company developed marker-free and all-native DNA transformation methods (Rommens *et al*., 2004, 2005). The genetic elements incorporated into a plant's genome are derived from either the targeted plant species or a plant that is sexually compatible with that species. The transformation vectors contain a cytokinin biosynthesis gene inserted in their backbone to facilitate selection against the inadvertent transfer of superfluous vector DNA. The use of these vectors makes it unnecessary to supplement tissue culture media with phytohormones and selection agents, and yields marker-free and backbone-free 'intragenic'

plants at frequencies between 1.8% and 9.9%, dependent on the plant species (Richael et al., 2008).

Cisgenesis and intragenesis both involve the insertion of DNA at random sites into the recipient genome (as is the case with transgenesis).

For more precise definitions of cisgenics and intragenics, please refer to Table 3. Note that some authors consider the term intragenesis to capture those plants in which the inserted DNA derives from any plant and do not restrict this to DNA from plants of the same or a sexually compatible species as the recipient.

Table 3. Description of transgenes, intragenes and cisgenes in plants. (Jacobsen and Schouten 2009)

Type	Definition
Transgene	A transgene is a (synthetic) gene with some or all regulatory sequences and coding sequences from donors other than crossable plants, including micro-organisms and animals. These genes belong to a new gene pool for plant breeding
Intragene	An intragene is a gene comprising of natural functional elements, such as coding part, promoter and terminator originating from different genes from the crop plant itself or from crossable species. All natural gene elements belong to the traditional breeders' gene pool
Cisgene	A cisgene is an existing natural gene from the crop plant itself or from crossable species. It contains its native promoter and terminator. The gene belongs to the traditional breeders' gene pool and is the already existing result of natural evolution.

Significance of this technology

Cisgenesis can speed up the breeding process significantly, particularly in self-incompatible, vegetative crops such as potato and apple. In these crops, a new variety could be developed in approximately 5 years using cisgenics (if the gene(s) of interest have been isolated) whereas this might take 25 years or more using conventional breeding. This is because unwanted genes (or alleles of genes) that were also introduced have to be removed via a series of backcrosses to the elite parental variety. In addition, it is also not possible to restore completely the elite variety with the new characteristic(s) encoded by the introduced genes, which is the ultimate objective. It is claimed that the first true example of this method is that of apple that contains a scab resistance gene under the control of its own promoter (Vanblare

et al., 2011) with other recent examples being reported in poplar (Han et al., 2012) and barley (Holme et al., 2012).

At present, the production of cisgenic and intragenic plants is at the research stage, though field trials have been conducted. Developments in gene isolation/characterisation techniques will provide increasing numbers of cisgenes for use in plant breeding. Aligned with developments in genetic modification techniques, cisgenesis has the potential to make a significant contribution to crop improvement programmes.

Status of the techniques and their products

Since only genetic elements (including genes and the DNA that regulates them) from the same species or a sexually compatible species (i.e. genetic material that would be available to conventional breeders) are introduced, COGEM argues that cisgenic plants should be regulated less strictly than transgenic plants in some cases.

Other authors have requested the complete exemption of cisgenic plants from current GMO regulatory oversight (Myskja 2006; Schouten et al. 2006a, b; Rommens et al. 2007). In Australia, cisgenic plants are excluded from GM legislation. Under Canadian and US law they are considered in a similar way to any other new plant variety. However, some authors have argued that these plants should have the same standard of regulatory oversight as transgenic plants. For example, Russell and Sparrow (2008) strongly recommended cisgenesis to be included in the GMO legislation's scope by broadening the GMO definition to include cisgenesis, as is currently the case in New Zealand.

It is interesting to note that Directive 2009/41/EC dealing with the contained use of genetically modified microorganisms (GMMs) excludes self-cloning (and consequently cisgenic organisms), as long as the resulting GMM is unlikely to cause disease. This exception is not made in the Deliberate Release legislation. The Directives dealing with the deliberate release and contained use of GMOs/ GMMs respectively were drafted to be comparable. This implies the difference was intended and it would be difficult to provide a legally sound argument that cisgenic plants are not GMOs under the Deliberate Release Directive. However, the definition of a Living Modified Organism under the Cartegena Protocol on Biodiversity may not capture a cisgenic plant (Jacobsen and Schouten 2009).

Jacobsen and Schouten (2008) consider that derogation from the GM legislation should be considered and that this could be approached by:

(1) Application and approval of a crop-gene-specific derogation, based on risk assessment.

(2) Phenotypic and molecular selection of cisgenic plants that are backbone-free (i.e. lacking vector DNA from outside the T-DNA borders) in the glasshouse and gauzehouse. Specific monitoring and surveillance of selected plants can be carried out in extended field experiments.

(3) Exemption of this crop-gene combination in Annex 1B of the Directive as a first step to general exemption of cisgenesis.

(4) Monitoring of more examples of crop-gene-specific derogations, such as apple. Positive results from such monitoring could lead to general exemption of these cisgenic plants.

(5) Ultimately the decision whether or not cisgenesis of plants in general can be added to Annex 1B of the Directive.

The EU Commission's New Techniques working group concluded that both cisgenesis and intragenesis are GM techniques, in that they are captured by the examples of GM techniques described in Part 1 of Annex 1A in Directive 2001/18/EC (please refer to Table 1).

Conclusions.

Cisgenesis and intragenesis are not included in the list of techniques in Part 2 of Annex 1A, which includes methods that are considered not to result in genetic modification. It is arguable whether example 2 in Part 1 of Annex 1A applies to cisgenesis, as it describes '*recombinant nucleic acid techniques involving the formation of new combinations of genetic material*...' '*and their incorporation into a host organism in which they do not naturally occur*'. Cisgenesis involves the use of unmodified genetic material from the same species, so products may be indistinguishable from plants generated by naturally occurring genetic variation. In contrast, intragenesis is more likely[7] (though not inevitably) to generate new combinations of genetic material that do not occur naturally. However, these examples in Part 1 of Annex 1A are *inter alia* and as such are not comprehensive. In the absence of any more information in the definition or any supplementary guidance, it is not possible to resolve further the question of whether cisgenic plants are captured by the GMO legislation. As discussed previously, some would argue that cisgenesis is a GM technique. If this is the case, a further question that

[7] Due to the genetic variability of genomes, it is possible , in some cases, that the sequence of the inserted DNA will be present naturally.

should be addressed is whether organisms generated by cisgenesis could then be excluded under Annex 1B of the Directive. As this Annex provides a comprehensive list of GM techniques that generate organisms that can be excluded from the legislation, cisgenics would need to be added. In ACRE's view, this should be considered.

Points for regulators to consider in determining whether to include cisgenic plants in Annex 1B are:

- The cisgenic phenotype could be produced using conventional techniques – although this would take longer and linkage drag would occur.
- Where cisgenic plants are produced using *Agrobacterium*-mediated transformation, T-DNA borders from the bacterium are likely to be present in the host plant (EFSA, 2012).
- DNA is inserted into a recipient plant's genome. Applicants are expected to consider insertion a potential hazard in transgenesis, i.e. whether the following could lead to adverse effects: the formation of novel open reading frames, positional effects on gene expression and disruption of native genes. However, these phenomena could occur during introgression of genes using non-GM techniques and plant breeders will select plants (cisgenic or otherwise) with a stable desired phenotype.
- Cisgenic plant lines could be distinguished using event specific tests (i.e. PCR across the regions flanking the insertion site; Holst-Jensen et al., 2012).

Alternatively,

- Regulators may want to consider COGEM's conclusions about a lighter touch in regulating cisgenics in some cases.

4. Reverse Breeding (involving double haploids)

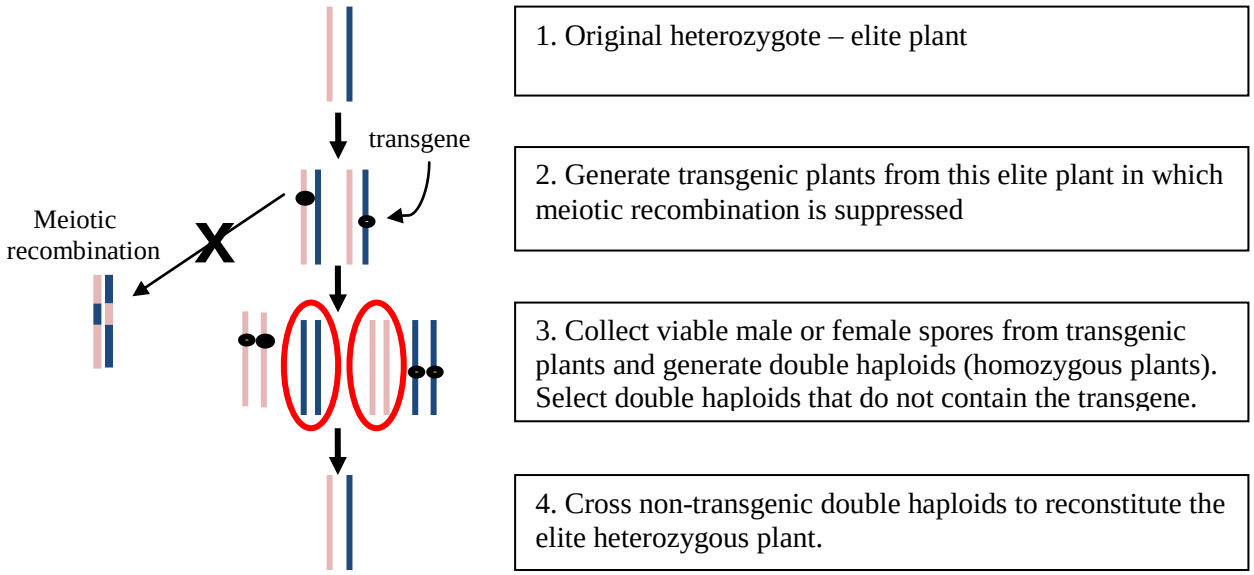
Background

Double haploids are plants in which both members of each chromosome pair are identical. Two different double haploids, when crossed, will show pronounced heterosis[8] and this makes the approach potentially valuable in breeding species where F1 hybrids[9] cannot be produced using for example cytoplasmic male sterility.

[8] Heterosis also referred to as hybrid vigour or outbreeding enhancement.
[9] F1 hybrid: the first generation resulting from cross-hybridisation.

Double haploids (DHs) can be produced by a variety of methods (including reverse breeding). In all cases, however, the phenotype is the same and there is no way of telling via genome analysis which method was used. In some species, crossing with wild relatives (e.g. barley crossed with *Hordeum bulbosum*) yields seed in which the paternal chromosome complement can be eliminated, where the embryo is derived only from the maternal chromosome set. Such haploid embryos may undergo spontaneous chromosome-doubling and the DH progeny can be detected easily and multiplied by selfing. In other species, treatment of haploid cells with chemicals such as colchicine induces chromosome doubling to form double haploids, which again can be detected by cytological techniques. It has been suggested also that the propensity to form double haploids could, in itself, be bred for or even incorporated by wide crossing (a form of conventional breeding).

In all cases, however, the end result differs from "wild type" plants only in the absence of heterologous pairs, a difference that is restored during the formation of the hybrid seed, from which the crop is grown.



Reverse breeding (Wijnker et al., 2012) uses genetic modification to facilitate the production of perfectly complementing homozygous parental lines (double haploids), which can be crossed to generate elite heterozygous plants. The method is based on reducing genetic recombination in the elite heterozygote (step 2 in Figure 1) by inserting transgenes that suppress meiotic crossing over. Once this has been achieved, the transgene is no longer

necessary. Male or female spores (i.e. pollen and embryo sacs containing haploid cells) produced by the GM heterozygotes can be cultured in the laboratory to generate homozygous, double haploid (DH) plants (step 3 in Figure 1). Some of these DHs will be GMOs, whereas others will not contain the transgene. From these DHs, complementary parents that are not GMOs are selected and used to reconstitute the heterozygote *in perpetuity* (step 4 in Figure 1). Since the fixation of unknown heterozygous genotypes is impossible by traditional breeding methods, reverse breeding could fundamentally change future plant breeding.

Significance of this technique

There is growing interest in the development of plant breeding techniques that are based on modifications of meiosis (Wijnker and de Jong, 2008). However, most techniques are extensions of the 'classic' plant breeding practice aimed at more efficient introgression of traits from alien backgrounds into crops. As a plant breeding tool, reverse breeding may be regarded as more versatile than alternative techniques as its controlled deconstruction of complex genotypes into homozygous parental lines allows the further improvement of these lines by classic breeding methods.

An interesting supplement to existing methods to generate haploids (Dunwell, 2010) is the discovery of a process dependent on the manipulation of centromeric proteins (Maruthachalam and Chan, 2010). Although transgenic techniques may be involved in the procedure, the haploids and doubled haploids produced do not contain any transgene.

Status of the technique and its products

Figure 2 summarises COGEM's assessment of organisms generated by this technique. The committee's conclusions differ depending on whether they refer to the end product or to intermediates generated during the breeding process. COGEM's view is that plants produced in intermediate stages (i.e. step 2 of Figure 1) are GMOs because they contain transgenes that alter the plants' ability to recombine DNA during meiosis, whereas the end products may not be GMOs. This will depend on whether they retain any inserted DNA.

The fundamental question of whether the offspring of GMOs are necessarily GMOs is not addressed specifically in the legislation. Some consider that this is the case. However, others argue that, for it to be captured by this legislation, the organism should be 'altered in a way

that does not occur naturally...' in line with Article 2 of the Directive – and do not consider that this applies to the end products of reverse breeding.

The Commission's New Techniques working group has concluded that the end products of reverse breeding (which have been selected for because they do not contain the transgene(s)) are not GMOs as long as they: (i) 'have never contained any inserted foreign DNA', (ii) 'the genetic composition is the same as the original organism' and (iii) 'can be obtained by traditional breeding techniques'.

Figure 2. COGEM conclusion on reverse breeding (Schouten, Slovenia conf.)

	Intermediate*	End product
• Genetic change?	Yes	No
• Phenotypic change?	Yes	No
• Detectable with DNA-test?	Yes	No
• Extra risk?	?	No
• Under GMO regulation?	Yes	No

(*plants produced in step 2 of Fig.2)

Conclusions

Part 1 of Annex 1A applies to plants containing inserted DNA and as such, the heterozygous plants that are produced as intermediate products in this process are GMOs. The question is whether the non-transgenic offspring of these plants should also be classified as GMOs. ACRE's view is that Article 2 of Directive 2001/18/EC (see Table 2) does not capture these plants because they are not 'altered in a way that does not occur naturally by mating and/ or natural recombination' – the objective of reverse breeding is to reconstitute the genotype of the original heterozygotes (which have been produced by conventional breeding).

Therefore, ACRE is in agreement with COGEM. It is difficult to see any justification for suggesting that plants generated by this approach are GMOs (including where reverse breeding techniques have been employed). It is the exploitation of a rare, but natural tendency for homologous pairs to segregate under certain defined conditions.

5. Agroinfiltration

Background:

Agroinfiltration is a method in which gene sequences are expressed in a transient fashion in plant tissue, normally with the purpose either of expressing a protein at high level (Sainsbury et al., 2009) or suppressing the expression of an endogenous gene through RNA silencing (Schob et al., 1997). Technically, the method is derived from much older procedures developed to test the pathogenicity of bacterial strains (Klement, 1963) or the effectiveness of resistance-inducing chemicals (e.g. White, 1979) in plant tissue by introducing cultures and solutions into the apoplastic compartment of leaves using a syringe and hypodermic needle. Entry of the solution into the leaf occurs through the stomata or a small tear created in the epidermis by contact with the syringe tip. For agroinfiltration on larger scales, for example to produce commercially significant amounts of material, infiltration may be driven by application of a vacuum (Rybicki, 2009).

Agroinfiltration provides a more rapid method than stable transformation for examining the effects of expression of a specific DNA sequence *in planta*. The sequence of interest is placed under the control of a promoter, such as the cauliflower mosaic virus 35S promoter, and the cassette inserted into the T-DNA of a Ti plasmid, which is introduced into a disarmed strain of *Agrobacterium tumefaciens*. Cultures of *A. tumefaciens* containing this Ti plasmid are treated with acetosyringone, which mobilizes the transfer of the T-DNA, and after 24 h the bacterial cells are infiltrated into the leaf. Experiments in tobacco (*Nicotiana tabacum*) in which plantlets were regenerated in the absence of any selection, following agroinfiltration using a T-DNA carrying a marker gene (ß-glucuronidase), indicated that no more than 15% of the cells in the infiltrated leaves are transformed (Jia et al., 2007).

The T-DNA itself, its primary RNA transcripts and any protein that it encodes remain localised in the infiltrated zone of leaf tissue. This is illustrated by experiments in which suppressors of RNA silencing, introduced by agroinfiltration into leaf tissue of plants silenced for *GFP* (green fluorescent protein) gene expression, restore fluorescence only in the directly infiltrated tissue (for example see Li et al., 2002).

There are two ways in which agroinfiltration may influence plant cells and tissues beyond the infiltration zone. Firstly, if an infectious clone of a virus is inserted into the T-DNA present

on the Ti plasmid, the T-DNA itself (in the case of DNA viruses such as geminiviruses) or its RNA transcript (in the case of RNA viruses) may give rise to replicating virus, resulting in infection of the transfected cell and potentially other cells in the plant. This technique, called agroinoculation, is widely used in plant virology and functional geneomics (Palukaitis *et al*., 2008; Kurth et al., 2012). Secondly, the RNA transcript of the T-DNA may stimulate the production of sequence-specific small-interfering (si)RNAs that can direct the destruction of RNA molecules containing homologous sequences. This is RNA silencing. The cell-to-cell movement of siRNAs and their amplification by the plant's RNA-directed RNA polymerase can result in systemic RNA silencing (Himber et al., 2003). Systemic RNA silencing can be induced by agroinfiltration of a small patch of leaf tissue, even when the introduced T-DNA encodes a non-viral sequence (for example see Kościańska et al., 2005).

Significance of the technique

Currently this technique is used in research and is being developed for protein production in plants; it is not used directly in the production of new stable GM plant varieties.

Status of the technique and its products

COGEM has concluded that the offspring produced from the seeds of agroinfiltrated plants are not GMOs as long as they do not contain DNA from the *Agrobacterium* vector. However, these progeny may have an epigenetically 'silenced phenotype', which may be inherited for several generations after the transgene has been lost.

The EU Commission's working group did not reach a consensus on the status of agro-infiltrated plants. However, it agreed that the offspring of these plants would not be captured by the legislation 'once the absence of a stable integration event is shown'.

Conclusions:

The *Agrobacterium* used to infiltrate the plant is clearly a GMO and whilst it is present in the plant, the plant will have to be treated as a container for the GMO. In addition, around 15% of the plant cells in the infiltration zone are likely to be transformed with DNA from the *Agrobacterium*. However, no other parts of the plant will have been genetically modified and if the infiltration area is physically removed or is lost through natural senescence there will be no T-DNA remaining in the plant.

In cases where viruses are not released from the T-DNA but where systemic silencing against expression of a plant gene has been initiated, the silencing is mediated by a class of molecules (siRNAs) that occur naturally in the plant and these will have been generated by the plant's own silencing machinery. Furthermore, neither local nor systemic RNA silencing results in permanent modification of the plant genome.

Thus ACRE is in agreement with COGEM that offspring growing from seed should not be considered GMOs, as long as the GM *Agrobacterium* and its T- DNA are absent.

6. Grafting (non-GM scion onto a GM rootstock)

Background

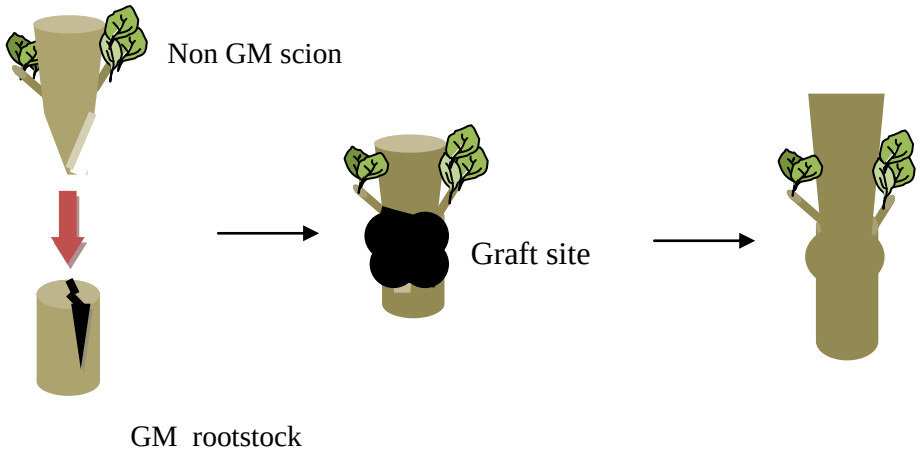


Figure 3. Principle of grafting

There is much interest in methods to protect perennial and grafted crop plants from pests and disease by using transgenic rootstocks with non-transgenic scions (Youk et al., 2009; Haroldsen et al. 2012). The transgenic rootstock of a GM plant that has been genetically modified for resistance to a plant pathogen (e.g. a viral pathogen) is grafted onto a compatible non-transgenic plant tissue, e.g., a scion compatible with the rootstock. The non-transgenic portion of the grafted plant is thereby provided with resistance to the plant pathogen.

Over the past decades, there have been several investigations on the existence of graft hybrids (Ohta 1991; Taller et al., 1998; Stegemann and Bock 2009). Grafting experiments have proved that endogenous mRNA enters and moves along the phloem long-distance

translocation system (Lucas et al., 2001; Ruiz-Medrano et al., 2012). The finding that mRNA or siRNA species may move between cells and around the plant, and the ability of retroviruses or retrotransposons to reverse transcribe mRNA into cDNA potentially capable of being integrated into the genome, indicate that mechanisms exist for the horizontal gene transfer from stock to scion and *vice versa* by grafting (Liu, 2006).

More recently, it has been shown that showed that genetic material is transferred between plants across graft junctions (Stegemann and Bock, 2009; Stegemann et al., 2012). These authors examined grafts between tobacco plants that express either a transgene encoded by nuclear DNA or a transgene encoded by plastid DNA, and identified resulting cells that express both sets of transgenic markers. They showed that genetic exchange can occur in both directions, but only involves the transfer of plastid DNA between cells and is limited to the site around the graft. They emphasized that their data do not lend support to the doctrine of Lysenkoism that 'graft hybridization' would be analogous to sexual hybridization.

In plants and some animals, posttranscriptional RNA silencing can be manifested beyond its sites of initiation, because of the movement of signalling molecules that must have RNA components to account for the nucleotide sequence specificity of their effects (e.g. siRNAs). In a recent study carried out in *Arabidopsis thaliana*, interesting clues were provided that suggest mechanisms by which systemic RNA silencing signals might be produced and perceived between distant plant organs (Fig. 4; reviewed in Dunoyer and Voinnet, 2008).

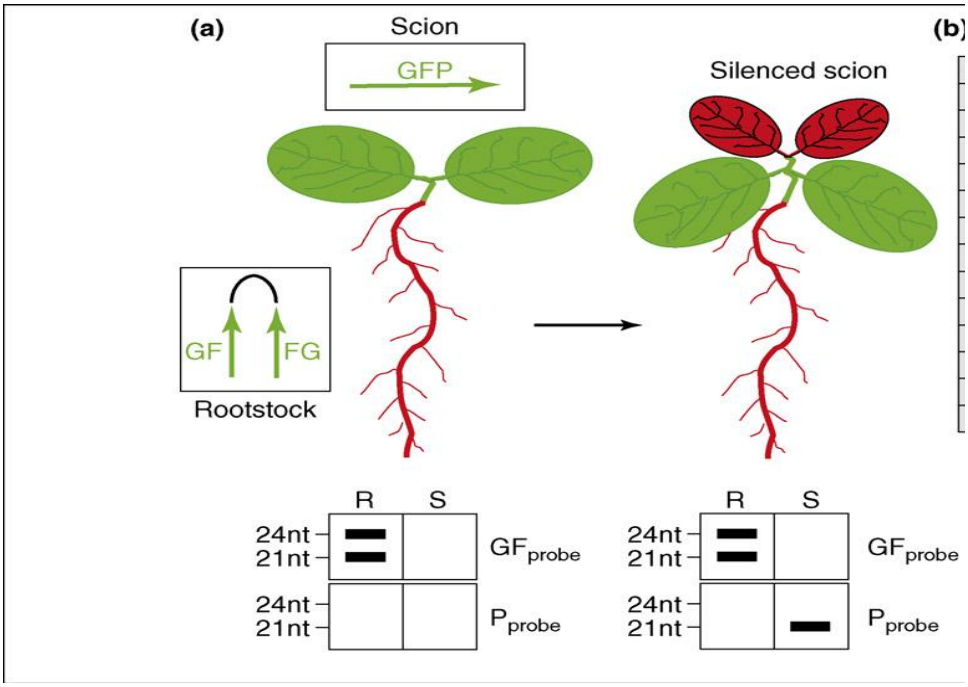


Figure 4. Graft-transmitted long-distance silencing in *Arabidopsis thaliana*. This figure illustrates the model of graft-transmitted long-distance silencing described by Brosnan *et al*. (2007) (a) Transgenic seedlings carrying a non-silenced green fluorescent protein (GFP) transgene (green) are grafted onto transgenic rootstocks expressing an inverted-repeat (IR) construct corresponding to the 50 portion ('GF') of GFP. Graft-transmission of RNA silencing was diagnosed through the loss of GFP expression in newly emerging scion leaves (depicted in red). The drawings below each plant provide a representative depiction of what would be seen on an RNA gel resolving the GF- or P- specific RNA species produced by Dicer-like 4 (DCL4; 21 nucleotides) and DCL3 (24 nucleotides). No GF-specific siRNA were detected in silenced scions (S) despite their abundance in the rootstocks (R). Only 21-nucleotide-long siRNAs homologous to the 30 non-overlapping portion of the transgene ('P') accumulate in these tissues (from Dunoyer and Voinnet, 2008).

Other relevant studies include evidence of gene silencing across the graft between parasitic plant and host (Tomilov et al., 2008), demonstrating that interfering hairpin constructs transformed into host plants can silence expression of the targeted genes in the parasite. Transgenic roots of the hemi-parasitic plant *Triphysaria versicolor* expressing the GUS reporter gene were allowed to parasitize transgenic lettuce roots expressing a hairpin RNA containing a fragment of the GUS gene (hpGUS).

It has also recently been demonstrated that the Bt Cry1Ac protein can be transported from transgenic rootstock to scion in poplar (Wang et al., 2012).

Significance

Grafting is a common practice in the commercial cultivation of fruit trees and ornamental shrubs. There is a great deal of interest in protecting these plants from pests and diseases through the use of GM rootstocks. Field trials of vines grafted onto virus resistant rootstocks have taken place in France.

Status of the technique and its products

COGEM has questioned whether the whole plant should be considered a GMO when it is chimeric. It suggests a case by case approach or regulating the use of the GM rootstock only.

The EU Commission's working group concluded that the whole plant is captured by the legislation. However, the fruit/seeds/offspring produced by a non-GM scion would not fall under the scope of Directive 2001/18/EC.

Conclusions:

ACRE agrees with the working group because Directive 2001/18/EC deals with organisms. In the case of a plant that has a GM rootstock and a non-GM scion, the risk assessment should take this into consideration i.e. focus on the roots but take into account that molecules can move across the graft site.

Seed produced from the non-GM scion would not contain the genetic modification. However, in certain cases e.g. where the genetic modification was directed at causing gene silencing, this characteristic could be inherited. Whether organisms that do not contain inserted DNA but which have engineered chemical modifications (that are heritable) are captured by the GMO legislation is discussed in the section below.

7. RNA-dependent DNA methylation

Background:

Epigenetic mechanisms such as DNA methylation[10] alter gene expression (and other genomic processes) without changing the nucleotide sequence of the plant's genome. Given the sessile nature of plants, it is crucial that they have mechanisms that allow them to react and acclimate to environmental stress. In recent years, scientists have become increasingly interested in environmentally induced epigenetic states that can be passed onto future generations ('transgenerational epigenetic inheritance'). Different epigenetic states of loci in genomes are referred to as epialleles. Epialleles have been identified as the source of altered reproduction characteristics in a number of plants, including crop species (review by Paszkowki and Grossniklaus, 2011). A paper by Hauben et al. (2009) describes the selection of oilseed rape populations with particular epigenetic characteristics that confer increased yield potential.

RNA-dependent DNA methylation is an example of an epigenetic mechanism. Methyl groups are thought to be directed to specific sequences in the genetic code by short, double-stranded RNAs (dsRNA) such as micro RNAs (miRNAs) or small/short interfering RNAs (siRNAs). Gene silencing may result if the sequence of these dsRNA molecules is identical to that of the DNA sequence in the promoter region of a gene or in the gene itself.

As well as selecting for plant lines with particular epigenetic characteristics, plant breeders may also induce them by introducing dsRNA into plant cells to trigger RNA-dependent methylation. dsRNA that is targeted to a particular DNA sequence may be introduced directly into cells. Alternatively, a gene that encodes these molecules (i.e. a template) can be introduced e.g. by direct or vector-mediated techniques. In some cases, the intention is not to insert this DNA template into the genome of the plant. In such cases, the template would be degraded by the cell within a short period (hours, or at most, a few days), but enough time to allow transcription of the silencing RNA. At present, gene silencing through RNA interference is generally achieved through inserting the DNA template encoding dsRNA into the genome (i.e. transformation).

DNA methylation patterns can persist even after the inserted template is no longer present (e.g. because the template was removed from the breeding line by segregating out progeny

[10] DNA methylation suppresses/silences the expression of target genes through the addition of methyl groups to specific nucleotide sequences.

that did not contain it). In some cases, the silencing will be transient (i.e. mitotic), while in other cases it may be inherited over a number of generations (transgenerational; Kanazawa et al., 2011).

The silencing RNA molecules can move between cells to distant parts of the plant, resulting in systemic gene silencing. For example, a transgenic root stock could be grafted onto a non-GM scion to influence gene expression in the aerial parts of the plant (see section on grafting above).

Significance

Plants that have been transformed with a DNA construct designed to encode silencing RNA molecules have been developed for commercial use (e.g. the first GMO that was authorised for food use - Flavr Savr tomato in 1992). These organisms are clearly GMOs because they are transgenic. Commercial varieties of plants in which the methylation pattern has been altered but which do not contain inserted DNA are not available. Currently, such plants are used in research into the initiation and exploitation of epigenetic effects e.g. into imprinting during the process of heterosis (Köhler et al., 2012).

Status of the technique and its products

In 2006, COGEM concluded that 'at this time, it is unclear to what degree the application of epigenetic effects is subject to GMO legislation. If a transgene is present in the plant to induce the effect, there is no doubt that the GMO legislation is applicable. If one of the parent lines was genetically modified and one of its offspring carries the traits in question, it can be said that GMO legislation applies here. However, in other forms of induction of epigenetic effects, GMO legislation appears not to apply even though it concerns a (temporary) heritable effect. COGEM observes that it is still too early at this time to make judgements on any environmental risks of epigenetic mutants'.

The EU Commission's working group concluded that the methylation of DNA is not an alteration of the genetic material in the context of the EU's GMO legislation. Therefore, plants that do not contain inserted DNA but which have an altered methylation status should not be captured by the legislation because they are comparable with organisms obtained through natural processes.

Conclusions:

The key question is whether 'engineered' methylation constitutes an alteration to the genetic material of an organism in the context of Article 2 of Directive 2001/18/EC. This is not clear from the definition. However, all the examples of GM techniques referred to in Part 1 of Annex 1A relate to the formation of new combinations of genetic material and/or the introduction of material into a host organism. As discussed above, there is no alteration in the nucleotide sequence of the host's genome and such chemical changes represent a phenomenon that occurs naturally.

There are methods (DNA sequencing, PCR, restriction analysis) that distinguish between methylated and un-methylated DNA. However, these cannot distinguish between a natural methylation event and one that was conferred by the technique described above.

Directed mutagenesis

Sections 8 and 9 are forms of mutagenesis.

Traditionally, plant breeding relies on natural recombination, or natural or induced mutagenesis or polyploidy, followed by trait selection. Crop plants modified by these methods do not contain transgenes and are thus distinguishable from GM plants, for which the transgene insertion event provides a fingerprint that allows recognition and detection. While mutation breeding generates new genetic variation, the key difference with genetically modified plants produced by recombinant DNA technology can be considered to be the presence of 'foreign' (ie non-host) DNA in the genome of the GMO; while the product of mutation (with the exception of T-DNA insertional mutants) exhibits a rearrangement, small repair insertion or deletion, or in the case of polyploidy, duplication, of existing genomic sequence.

Examples of approaches to directed mutagenesis include the use of oligonucleotides, zinc finger nucleases, transcription activator-like (TAL) protein nucleases (Bogdanove and Voytas, 2011; Deng et al., 2012) and meganucleases. Their use in plants is reviewed in Tzfira et al. (2012).

8. Oligonucleotide-directed mutagenesis

Background

Oligonucleotide-directed mutagenesis (ODM) is a technique used to correct or to introduce specific mutations at defined sites of the genome. ODM is a generic term covering several approaches and applications. It is referenced in the literature under other names such as targeted nucleotide exchange, chimeraplasty, oligonucleotide-mediated gene editing, chimeric oligonucleotide-dependent mismatch repair, oligonucleotide-mediated gene repair, triplex-forming oligonucleotide-induced recombination, oligodeoxynucleotide-directed gene modification, therapeutic nucleic acid repair approach and targeted gene repair (see *e.g.* Andersen et al., 2002; Christensen et al., 2006; Cole-Strauss et al., 1999; de Semir and Aran, 2006; Hutchison et al. 1978; Igoucheva et al., 2006; Kuzma and Kokotovich 2011; Zhang et al., 1998).

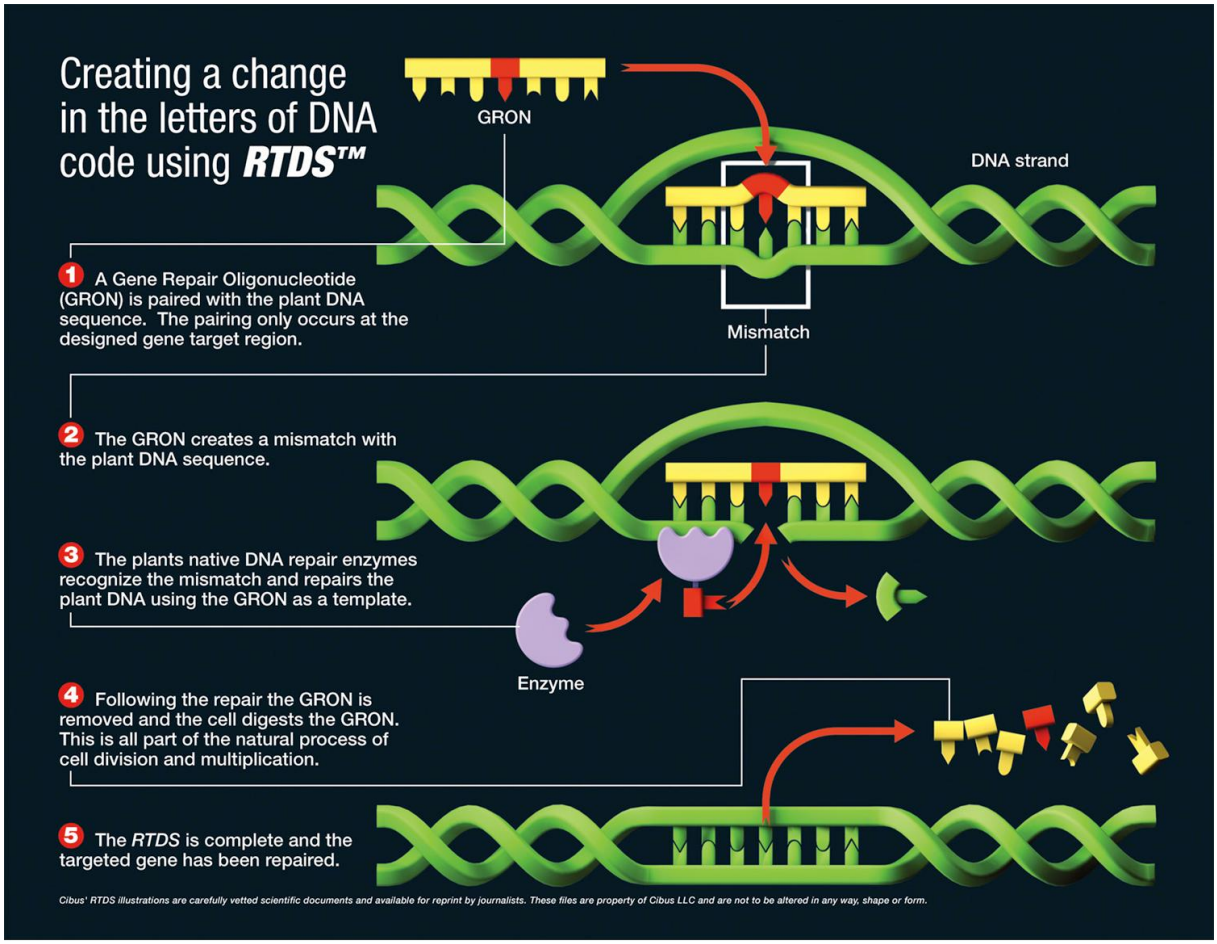


Figure 5. Process of directed gene modification (from Cibus.com)

All these techniques are based on the site-specific correction or directed mutation (base substitution, addition or deletion) of an episomal or chromosomal target gene after introduction of a chemically synthesized oligonucleotide with homology to that target gene (except for the nucleotide(s) to be changed). In all cases, the gene modification is induced directly and exclusively *via* the effect of the oligonucleotide itself, *i.e.* independent of any delivery vector system. The above-mentioned definitions do not cover cases where the oligonucleotide is chemically modified to incorporate a mutagen (the oligonucleotide is used as a vector to deliver the mutagenic agent in a DNA site-specific manner) (Kalish and Glazer, 2005), nor cases where the oligonucleotide is used together with zinc-finger nucleases (ZFNs) to generate double-strand breaks at specific genomic sites (Wright et al., 2005).

Although the usefulness of the technique was first demonstrated in mammalian cells, preliminary studies at the end of the nineties demonstrated that oligonucleotide-mediated mutagenesis is applicable to plants and can induce target gene mutations (Beetham et al., 1999; Gamper et al., 2000; Hohn and Puchta, 1999; Zhu et al., 1999). Successful *in vivo* gene modification has been demonstrated notably in maize, rice, tobacco and wheat, *e.g.* to create plants insensitive to the action of a specific herbicide (Dong et al., 2006; Iida and Terada, 2005; Kochevenko and Willmitzer, 2003; Okuzaki and Toriyama, 2004; Zhu et al., 2000). Altered genes have been shown to be stably maintained during mitosis (Beetham et al., 1999; Kochevenko and Willmitzer, 2003), and transmitted in a Mendelian fashion to subsequent generations (Zhu et al., 1999, 2000). Recently a gene-targeting approach was used successfully to develop a high-tryptophan rice (Saika et al., 2011).

Significance

There are drawbacks to this technique that include the low frequency of the gene modification, the difficulty to further select and regenerate plants bearing mutations (in cases lacking a selective marker), as well as the spontaneous occurrence of somatic mutations which may obscure the effect of the oligonucleotide-directed approach (Li et al., 2007; Reiss, 2003; Ruiter et al., 2003).

Most organisms developed through the technique are used in the laboratory for research and development. However, organisms produced through ODM could soon reach the commercial stage, and some patents have already been filed (Davis et al., 2004; May et al., 2001), which makes it also relevant in the context of environmental releases or marketing applications.

Defra Ministers sought advice from ACRE on herbicide tolerant oilseed rape plants that had been developed using ODM. Our conclusions are published at http://www.google.co.uk/search?hl=en&q=ACRE+advice+Cibus+oilseed+rape+odm&meta=.

Status of the technique and its products

Breyer et al. (2009) concluded that organisms developed through ODM should not fall within the scope of the EU legislation. COGEM is in agreement because it considers that the technique is a form of mutagenesis that does not pose additional risks to conventional mutagenesis (which generates organisms that are excluded from the legislation). However, others argue that oligonucleotides are 'recombinant nucleic acid molecules', which prevents the exclusion of organisms produced using this technology under Annex 1B of Directive 2001/18/EC. COGEM's opinion is that an oligonucleotide used for site-directed mutagenesis should not be considered a recombinant nucleic acid (COGEM, 2010).

Conclusions:

When assessing to what extent ODM can be compared to techniques already listed in the annexes of the Directive, ACRE considers that the following observations can be made: the ODM technique does not fall in the category of techniques that are not considered to result in genetic modification (Annex I A, Part 2 of Directive 2001/18/EC). It is not captured by Part 1 of Annex 1A in that it is not a recombinant nucleic acid technique (the oligonucleotide is a synthetic molecule not generated by recombinant techniques, and does not involve the insertion of foreign DNA into a genome).

However, Part 1 of Annex 1A is not comprehensive and ODM is a form of mutagenesis, which is referred to in Annex 1B. ACRE's view is that if ODM is defined as a GM technique, then the organisms produced should be excluded from the legislation. ACRE advises that oligonucleotides that are used in site-directed mutagenesis should not be considered as being recombinant nucleic acid molecules and thus ODM is captured by Annex 1B. As discussed above, the term 'recombinant nucleic acid molecules' has been debated with respect to molecules that are used as mutagens in techniques such as ODM and zinc finger nuclease (ZFN)-induced mutations. Where these molecules are transiently present in host cells and do not integrate into the host's genome, ACRE considers that their classification as recombinant nucleic acid molecules is not relevant from a scientific point of view. This is particularly apparent when discussing zinc finger nuclease-induced mutations (see below), where the

classification of the resulting organism may depend on whether the mutation was introduced via the introduction of a ZFN protein (which is clearly not a recombinant nucleic acid) as compared to its nucleic acid precursor. This debate about what constitutes a recombinant nucleic acid molecule may also result in the artificial distinguish between organisms produced by different site-direct mutagenic techniques (e.g. ODM and ZFN). ACRE considers that these molecules are mutagens.

Another important point to consider is that organisms developed through ODM could in many cases not be distinguished at the molecular level from those developed through "traditional" mutation techniques (using chemicals or ionizing radiations) or from wild-type organisms (when the introduced change results in the restoration of the wild-type sequence).

9. Zinc Finger Nucleases

Background

Zinc finger nuclease (ZFN) technology provides a means of generating mutation in a targeted (i.e. sequence-specific) manner, and is therefore more precise than the random mutagenesis induced by conventional techniques such as radiation, chemical mutagens or insertional mutagenesis (transposons, T-DNAs). ZFNs are fusion proteins, comprising zinc-finger-based DNA recognition modules linked to an endonuclease domain (Figure 6). ZFNs act by making use of the natural recombinogenic repair potential at double-stranded break (DSB) sites in genomic DNA, and when imprecise repair of the break site occurs by non-homologous end-joining, a mutation is generated (Figure 6). ZFN-induced DSBs also enhance gene targeting at specific loci in human cells and in plants. Therefore the novelty is that zinc-finger domains can be engineered to recognize specific DNA sequences, and at the cleavage site either mutations or DNA insertions can be induced to occur. ZFN technology has been exploited for genome modification in both animals and plants (Zeevi et al., 2012).

Crucially, it is possible to utilize ZFN technology to generate mutant plants that contain no transgene sequences. This can be achieved either by the transient expression of ZFN genes/proteins in cultured plant tissues prior to regeneration and selection (this is feasible if the desired mutant trait, such as herbicide tolerance, is itself selectable), or by removal of transgene sequences following backcrossing, leaving only the mutant sequence which can be at a distinct locus from the original transgene insertion site. Transient expression of

transgenes, for example using viruses as a transient delivery system (Vainstein et al., 2011), will lead to the production of the protein in the plant cells, but not necessarily the integration of the transgene. Backcrossing will breed out integrated transgene sequences in stably transformed lines, leaving mutant plants but no foreign DNA. In either case, the mutant plant would be indistinguishable from a plant generated by more conventional mutagenesis, or by naturally occurring mutation, or by recombination with another mutant variety.

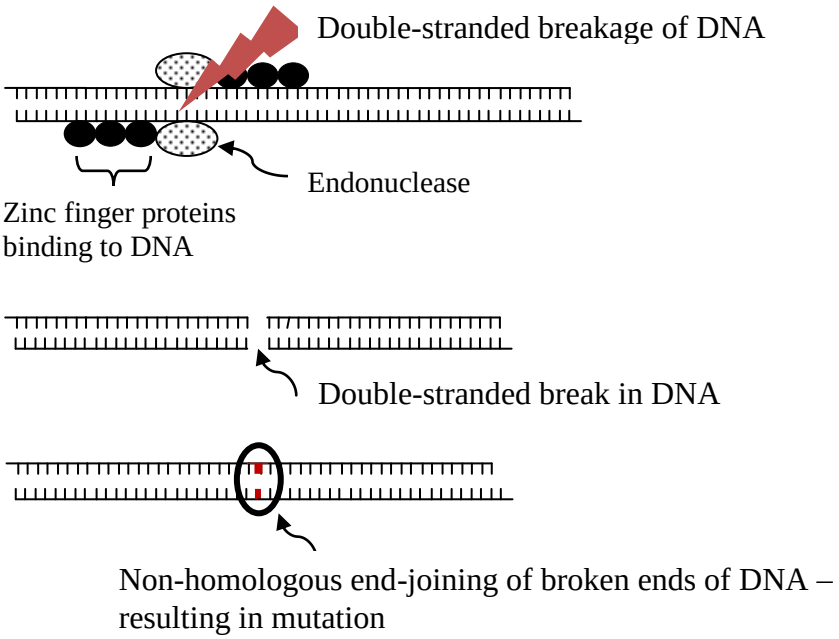


Figure 6. Principle of mode of action of zinc finger nucleases 1.

Significance

Many organisms, including humans[11], are amenable to 'genome editing' through the use of engineered nucleases. However, there are significant challenges that need to be overcome before engineered nucleases become robust tools that can be used to edit genomes.

Status of the technique and its products

The EU Commission's working group divided ZFNs into three categories. The third category (ZFN-3) involves the use of ZFNs to make a double stranded break in a host's DNA into which genetic material prepared outside of the cell can be inserted by homologous recombination. The working group considered that this technique was captured by Annex 1A, Part 1 of Directive 2001/18/EC and that therefore, that the resulting organisms were GMOs.

[11] Article 2 of Directive 2001/18/EC establishes that humans cannot be GMOs.

The working group did not reach a consensus on the other two categories – ZFN-1 and ZFN-2. ZFN-1 refers to the technique described above whereby mutations in the host DNA result from non-homologous joining of the ends created by double-stranded breaks in the DNA. With ZFN-2, a template is introduced into the cell along with the ZFN. This template is an oligonucleotide with homology to the DNA flanking the break-site. However, it contains an alteration at the break site. When the host cell repairs the double-stranded break it uses the oligonucleotide or its own DNA to act as a template. In the cases where the introduced oligonucleotide provides the template, the altered base pair will be incorporated.

The working group was of the view that ZFN-1 and ZFN-2 result in changes in organism that can be obtained with other forms of mutagenesis. Consequently, a majority considered that the organisms produced by these techniques should be excluded in line with Annex IB of Directive 2001/18/EC. However, a minority considered that recombinant nucleic acid molecules had been used and as such the criteria for exclusion had not been met.

Conclusions

The technique is not captured by Part 1 of Annex 1A as long as there is no integration of novel DNA at the break site through homologous recombination.

However, Part 1 of Annex 1A is not comprehensive and ACRE considers this technique a form of mutagenesis, which is referred to in Annex 1B. ACRE's view is that if the use of ZFNs to create site-specific mutations is considered a GM technique, the organisms produced should be excluded from the legislation. This is contentious as some will argue that in certain situations, the technique involves the use of recombinant nucleic acid molecules. Where ZFN protein is delivered directly into the plant cell, this is clearly not the case. However, it can be argued that when the ZFN protein is expressed from a vector or mRNA that is present transiently in plant cells (i.e. not inserted into the plant's genome) the criteria for Annex 1B are not met. COGEM has argued that whilst the question as to whether an oligonucleotide is a recombinant nucleic acid molecule cannot be answered unequivocally, an oligonucleotide used for site-directed mutagenesis should not be considered a recombinant nucleic acid. ACRE considers that describing some mutagens (that are not inserted into a host's genome) as recombinant nucleic acid molecules and not others will lead to inconsistency in which organisms are regulated, without any credible scientific basis to this differentiation. ACRE notes that the use of nucleic acids as mutagens that do not insert into the host DNA would not

have been envisaged at the time when Annex 1B was drafted. However, it would have been reasonable to refer to 'recombinant nucleic acid molecules' in this annex to prevent the exclusion of transgenic organisms, which have been mutagenised through the insertion of DNA into their genomes.

It should be noted that significant research effort is now being made in the development of other designer nucleases based on transcriptional activator-like effectors (TALEs) (Doyle et al. 2012; Mussolino and Cathomen, 2012: Reyon et al. 2012). These TALENs nucleases may provide improved accuracy of targeting. Other recent modifications include one that involves an *in planta* gene targeting method that does not rely on efficient transformation and regeneration techniques (Fauser et al. 2012).

In many cases, organisms developed through these technologies could not be distinguished at the molecular level from those developed through "traditional" mutation techniques (using chemicals or ionizing radiations) or from wild-type organisms (when the introduced change results in the restoration of the wild-type sequence).

It should also be noted that the technologies discussed here will also interact with those such as artificial chromosome techniques (Gaeta et al., 2012) and other methods for the 'stacking' of transgenes.

10. Conclusions

ACRE's views on whether the new techniques under discussion would be captured by the EU's GMO legislation are based on a scientific interpretation of the terms in the legislation (refer to Table 1 for a summary of our conclusions). As these terms are open to interpretation, Ministers should also seek legal advice. This flexibility in the EU's definition of a GMO presents a problem for regulators and will affect innovation in plant breeding and crop science in the EU. Therefore, ACRE advises that a more transparent, scientifically robust interpretation be adopted if the EU continues to employ the current definition. ACRE intends to explore how this might be achieved in a way that is as close to the current definition as possible.

Ultimately, however, regulators should consider a different system that is neutral with respect to the technology used. In particular, there is no evidence or rationale that would suggest that the current EU system is more effective in protecting human health or the environment as compared to systems that have adopted a product-based approach. Indeed, there is a contrary argument because organisms with the same phenotype (and sometimes genotype) as a GMO can be produced using conventional techniques (e.g. herbicide tolerant plants) but these are not risk assessed. It is arguable that there should be a comparative assessment of the risks and benefits of novel organisms whether produced by GM on non-GM technologies on a product-by-product basis.

As summarised by Morris and Spillane (2008) it is clear that, as both fundamental and biotechnological research with plants advances, the EU's process-based regulatory framework for GM crops will find it increasingly difficult to consider the possibility of similar or equivalent risks posed by other, *sensu stricto*, non-GM-based approaches that can elicit similar effects, varieties and products.

These discussions about the status of new techniques have also highlighted the fact that with the advance of biotechnology, it is becoming increasingly difficult to distinguish between GM and other plant biotechnological techniques.

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