Environ. Biosafety Res. (2009)
© ISBR, EDP Sciences, 2009
DOI: 10.1051/ebr/2009007

Available online at:
www.ebr-journal.org

Commentary

Genetic modification through oligonucleotide-mediated mutagenesis. A GMO regulatory challenge?

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In the European Union, the definition of a GMO is technology-based. This means that a novel organism will be regulated under the GMO regulatory framework only if it has been developed with the use of defined techniques. This approach is now challenged with the emergence of new techniques. In this paper, we describe regulatory and safety issues associated with the use of oligonucleotide-mediated mutagenesis to develop novel organisms. We present scientific arguments for not having organisms developed through this technique fall within the scope of the EU regulation on GMOs. We conclude 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.

Keywords: GMO / EU regulation / gene modification / oligonucleotide / new techniques / mutagenesis / risk assessment

INTRODUCTION

The European Directives 2001/18/EC (EC, 2001) and 90/219/EEC (EC, 1990, 1998) provide a general definition of a Genetically Modified Organism (GMO) and a Genetically Modified Micro-organism (GMM) respectively. These Directives include annexes that give additional information regarding the techniques that result in genetic modification, that are not considered to result in genetic modification, or that result in genetic modification but yield organisms that are excluded from the scope of the Directives (see Tab. 1). The European definition of GMO is both technology- and process-oriented. A novel organism will therefore fall under the scope of the GMO Regulation only if it has been developed with the use of certain techniques (such as recombinant nucleic acid techniques). The underlying idea here is that some processes of genetic modification are inherently and potentially associated with risks.

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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. There have been for example 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). Answering this kind of question is of course of utmost importance especially for developers of novel organisms, given the complexity and associated costs of applying the GMO legislation.

In this paper, regulatory and safety issues associated with the use of oligonucleotide-mediated mutagenesis are discussed in the light of the European definition

Table 1. The definition of a GMO according to EU Directives.

Directive 90/219/EEC	**Directive 2001/18/EC**
Article 2	***Article 2***
(a) "micro-organism" shall mean any microbiological entity, cellular or non-cellular, capable of replication or of transferring genetic material, including viruses, viroids, animal and plant cells in culture;	(1) "organism" means any biological entity capable of replication or of transferring genetic material;
(b) "genetically modified micro-organism" (GMM) shall mean a micro-organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.	(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: (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.	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	***Article 3.1***
[...] this Directive shall not apply: – where genetic modification is obtained through the use of the techniques/methods listed in Annex II, Part A.	This Directive shall not apply to organisms obtained through the techniques of genetic modification listed in Annex I B.
Annex I ***Part A***	***Annex I A*** ***Techniques referred to in Article 2(2)*** ***Part 1***
Techniques of genetic modification referred to in Article 2(b)(i) are, *inter alia*: (1) Recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation. (2) Techniques involving the direct introduction into a micro-organism of heritable material prepared outside the micro-organism including micro-injection, macro-injection and micro-encapsulation. (3) Cell fusion or 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.	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 ***Part B***	***Annex I A*** ***Techniques referred to in Article 2(2)*** ***Part 2***
Techniques referred to in Article 2(b)(ii) which are not considered to result in genetic modification, on condition that they do not involve the use of recombinant-nucleic acid molecules or GMMs made by techniques/methods other than techniques/methods excluded by Annex II, Part A: (1) in vitro fertilization; (2) natural processes such as: conjugation, transduction, transformation; (3) polyploidy induction.	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.

Table 1. Continued.

Directive 90/219/EEC	Directive 2001/18/EC
Annex II ***Part A*** Techniques or methods of genetic modification yielding micro-organisms to be excluded from the Directive on the condition that they do not involve the use of recombinant-nucleic acid molecules or GMMs other than those produced by one or more of the techniques/methods listed below: (1) Mutagenesis. (2) Cell fusion (including protoplast fusion) of prokaryotic species that exchange genetic material by known physiological processes. (3) Cell fusion (including protoplast fusion) of cells of any eukaryotic species, including production of hybridomas and plant cell fusions. (4) Self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism which may or may not be followed by reinsertion of all or part of that nucleic acid (or a synthetic equivalent) with or without prior enzymic or mechanical steps, into cells of the same species or into cells of phylogenetically closely related species which can exchange genetic material by natural physiological processes where the resulting micro-organism is unlikely to cause disease to humans, animals or plants. Self-cloning may include the use of recombinant vectors with an extended history of safe use in the particular micro-organisms.	***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.

of a GMO. This paper is largely based on the advice that has been delivered on this matter by the Belgian Biosafety Advisory Council, which advises the Belgian competent authorities on the potential risks associated with the uses of GMOs (BAC, 2007).

WHAT IS OLIGONUCLEOTIDE-MEDIATED MUTAGENESIS?

Oligonucleotide-mediated mutagenesis (OMM) is a technique used to correct or to introduce specific mutations at defined sites of the genome. OMM 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 oligonucleotides induced recombination, oligodeoxynucleotide-directed gene modification, therapeutic nucleic acid repair approach, targeted gene repair (see *e.g.* Andersen et al., 2002; Christensen et al., 2006; Cole-Strauss et al., 1999; de Semir and Aran, 2006; Igoucheva et al., 2006; Zhang et al., 1998).

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).

OMM makes use of different types of oligonucleotides: single-stranded DNA oligonucleotides containing 5′ and/or 3′ modified ends to protect the molecule against cellular nuclease activities (Campbell et al., 1989), chimeric RNA/DNA or DNA/DNA molecules

(Igoucheva et al., 2004a; Parekh-Olmedo et al., 2005), RNA oligonucleotides (Storici, 2008), and triplex-forming oligonucleotides (Simon et al., 2008).

Introduction of the oligonucleotides in the cells can be performed without using any delivery vector system *via* different techniques such as electroporation, lipofection, transfection or particle bombardment (biolistic) (see *e.g.* Radecke et al., 2006). OMM does not involve the introduction or integration of foreign genetic material (prepared outside the target organism) but alters natural chromosomal or episomal sequences. Mutations are introduced *in situ* (*i.e.* site-specific mutations) and can target any nucleotide sequence (regulatory, coding or non-coding), for instance to inactivate a deleterious gene, to induce local modification in expression, by controlling elements which may lead to changes in the level of gene expression or to change an amino-acid in the corresponding protein resulting in a protein with possible new properties. The technique builds on the observation that small specific changes in the amino acid sequence at some critical sites within a protein are in many cases responsible for differences in the performance of the protein and phenotypic character of the corresponding organism.

The observed frequencies of mutagenesis in the treated cells are highly variable (from less than 1% to up to 60%), and appear to depend on the type and design of the oligonucleotide, the cell type and on the target locus. Although the origin of this variability as well as the mechanisms of action at the molecular level are poorly understood, DNA repair enzymes are involved in this process mainly through the activation of the mismatch repair and/or nucleotide excision repair pathway (Andersen et al., 2002; de Semir and Aran, 2006; Engstrom and Kmiec, 2008; Igoucheva et al., 2004b, 2006; Parekh-Olmedo and Kmiec, 2007). The oligonucleotide hybridizes at the targeted location in the genome to create a mismatched base-pair(s) which acts as a triggering signal for the cell's repair enzymes. All reviews clearly indicate that the process being involved is a type of gene repair and not homologous recombination.

POTENTIAL APPLICATIONS OF THE TECHNIQUE IN THE CONTEXT OF THE DEVELOPMENT OF NOVEL ORGANISMS

Microorganisms

OMM has been used successfully in bacteria and yeast mainly as a tool to perform fundamental research on gene expression and regulation aiming at better understanding the possible mechanisms underlying the genetic modification (Andersen et al., 2002; Huen et al., 2006; Li et al., 2003; Liu et al., 2001, 2002). In general, this technique is not expected to have major applications in microorganisms. One of the reasons is that the development of modified microorganisms through the use of recombinant DNA technology is now common practice and offers many advantages in terms of selection.

Mammals

Targeted gene repair directed by chimeric RNA/DNA oligonucleotides has proven successful in animal cells in which the wild-type gene can be restored or knocked out, *e.g.* with globin genes, genes involved in muscular dystrophy, tyrosinase and *c-kit* genes (Alexeev et al., 2002; Rando, 2002; Yin et al., 2005; see also review by Suzuki, 2008). The creation of mouse mutants by modification of embryonic stem cells by ssDNA oligonucleotides has also been reported (Aarts et al., 2006; Murphy et al., 2007). This is an interesting approach, since the organisms created do not contain any marker gene. However, typical efficiencies are lower than 10^{-4}, leaving mutations too rare to be effectively identified, and conditions that can improve the mutagenesis efficiency remain to be found.

The potential of this approach for the directed genetic improvement of livestock animals has also been illustrated through various examples (Laible et al., 2006).

Last but not least, the technique seems to offer opportunities for the future in the field of human gene therapy to correct point mutations, for instance in monogenic inherited diseases and cancer (Christensen et al., 2006; de Semir and Aran, 2006; Kmiec, 2003; Wu et al., 2001). The therapy using oligonucleotides or RNA/DNA chimeras can result in a fraction of cells in which the wild-type gene can be restored. Bertoni et al. (2005) demonstrated repair of the dystrophin gene in muscle cells in a mouse model (in which dystrophin was knocked out due to a point mutation) for a prolonged period of time.

In many cases, however, there has been a disparity in the frequency or reproducibility of gene correction. The efficacy of delivery of the oligonucleotides into the nucleus, the long-term stability or purity of these molecules, the genetic background of the receiving organism, and the nature of target genes are potential factors that may contribute to this variability (Alexeev et al., 2002; De Meyer et al., 2007; Grabowski, 2008; Sørensen et al., 2005; Yin et al., 2005). These observations underscore the need to better understand the underlying mechanisms of gene repair and also to improve animal models.

Plants

Although the usefulness of the technique has been first demonstrated in mammalian cells, preliminary studies at the end of the nineties have shown 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).

Major drawbacks include the low frequency of the gene modification, the difficulty to further select and regenerate plants bearing mutations (in case of absence of a selective marker), as well as the spontaneous occurrence of somatic mutations which obscure the effect of the oligonucleotide-mediated approach (Li et al., 2007; Reiss, 2003; Ruiter et al., 2003). Despite these drawbacks commercial applications of this technique could be expected in the short term (BASF, 2009).

OLIGONUCLEOTIDE-MEDIATED MUTAGENESIS IN THE CONTEXT OF THE EU REGULATION ON GMOs

Regulatory issues

The EU definition of GMOs implies a division of organisms between GMOs and non-GMOs according to the techniques involved. When assessing to what extent oligonucleotide-mediated mutagenesis can be compared to techniques already listed in the Annexes of the Directives, the following observations can be made. The OMM technique:

- does not fall in the category of techniques that are not considered to result in genetic modification (referred to in Annex I A, Part 2 of Directive 2001/18/EC or in Annex 1, Part B of Directive 90/219/EEC). Organisms developed through OMM should therefore be considered as GMOs in the meaning of the EU Directives;
- is not a recombinant nucleic acid technique, does not make use of any vector system, and does not involve insertion of DNA into a genome;
- is not a cell fusion or hybridization technique;
- can involve micro-injection or micro-encapsulation (in liposomes) to deliver the oligonucleotide in the cell, although other techniques such as electroporation or particle bombardment are more commonly used. However we argue that the oligonucleotides should not be considered as "heritable material prepared outside the organism" in the meaning of the annexes of the Directives;
- is a form of mutagenesis. The nucleic acid molecules used in the technique are oligonucleotides which should not be considered as being recombinant nucleic acid molecules.

Considering OMM in relationship with the EU GMO regulatory framework is for the moment particularly relevant in the context of the contained use activities (EC, 1990, 1998), given the fact that most organisms developed through the technique are used in the laboratory for research and development. However, organisms produced through OMM could soon reach the commercial stage, and some patents have already been deposited (Davis et al., 2004; May et al., 2001), which makes it also relevant in the context of environmental releases or marketing applications (EC, 2001).

Another important point to consider is that organisms developed through OMM 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). Detection and traceability are key aspects in the EU regulatory system on GMOs, in particular for GMOs used as Food or Feed (EC, 2003). As a consequence, adequate molecular methods must be available that enable the detection and identification of each GMO individually (the so-called "transformation event"). Traditionally, identification is achieved by mapping a segment of DNA in the GMO corresponding to the junction areas, *i.e.* the regions where the transgenic DNA is inserted in the genome of the host organism. It is thus important to realize that emerging techniques such as OMM that do not involve the introduction into the genome of foreign DNA sequences from other species could pose challenges for unambiguous detection and testing, and ultimately enforcement of the EU regulatory system.

Safety issues

The main advantage of OMM is that in many cases it should theoretically be more precise than other mutational techniques (such as irradiation or chemical treatment) and recombinant DNA technology. OMM acts on specific genes in a very targeted manner and does not use

integrative vectors, thus eliminating the risk of inadvertent insertional effects (such as mutagenesis or transactivation) associated with the introduction of foreign sequences in the host cell genome. In consequence, OMM should lead to fewer unintended effects. The high specificity of the technique has been demonstrated in several studies, and the risk of potential unwanted mutagenesis has been shown to be very low when the oligonucleotide structure and chemistry were properly designed (Agarwal et al., 2003; Beetham et al., 1999; Kochevenko and Willmitzer, 2003; Okuzaki and Toriyama, 2004; Zhu et al., 1999, 2000). Altered genes have also been shown to be heritable and stable, at least in plants (see Sect. 2.3).

Moreover, unintentional changes are possible with all conventional (such as traditional breeding) and biotechnological methods for genetic modification. The development of novel organisms through OMM is not expected to generate more unintentional changes or effects than those faced by organisms generated by irradiation or chemical treatment. The extent to which these changes and potential effects should be assessed differently in GMOs from organisms developed with "traditional" methods underlies part of the controversy surrounding the use of GMOs (Nielsen, 2003).

CONCLUSIONS

As mentioned in the introduction, the definition of the term "genetically modified organism" in the EU is process-based rather than product-based. The focus of the definition is on the alteration of the genetic material *per se*, that is the genotype, without reference to the changes induced in the phenotype.

OMM has potential applications in fundamental research, medicine, agro-food and pest control. The terminology "oligonucleotide-mediated mutagenesis" covers various experimental approaches, but always aims at one objective: the site-specific correction or mutation of a target gene mediated by a chemically synthesized oligonucleotide. Broadly speaking, we consider that the technique does not pose other biosafety questions than those associated with similar techniques already listed in the GMO Directives, and could be considered similar to techniques currently excluded from the scope of the EU GMO regulatory framework.

The following are our main conclusions and recommendations:

- It seems obvious that OMM must be considered as leading to genetic modification in the meaning of the EU Directives. However, it is important to note that the technique does not involve homologous recombination, and is not used for introducing new genes in organisms, but for altering chromosomal or episomal sequences *in situ* in their natural genetic background.
- OMM uses oligonucleotides, which should not be considered as being recombinant nucleic acid molecules.
- OMM should be considered as a form of mutagenesis, a technique which is excluded from the scope of the EU regulation.
- OMM is more specific than recombinant DNA technology and other mutational techniques, such as irradiation or chemical treatment, which makes the risk to generate unintended effects in the genome of the recipient cells very unlikely, provided that the oligonucleotide structure and chemistry are properly designed.
- The reliability, efficacy and reproducibility of OMM show nevertheless a great variability, and further studies are still needed to improve the efficiency of mediating mutations, the effectiveness of their detection, and the knowledge on the mechanisms of action at the molecular level.
- Organisms developed through OMM can in many cases not be distinguished at the molecular level from those developed through "traditional" mutation techniques or from wild-type organisms, thus challenging the enforcement of the EU detection and identification rules for GMOs.

Based on these conclusions, we consider that there are scientific arguments for having OMM excluded from the scope of the EU Directives on GMOs.

This vision is shared by COGEM (the Dutch "Commissie Genetische Modificatie") which also concluded that directed mutagenesis through the use of oligonucleotides was a form of "traditional" mutagenesis and therefore could be excluded from the scope of the GMO regulation (COGEM, 2005, 2006).

The final decision as to whether or not organisms produced by a specific technique should fall under the scope of the EU regulation on GMOs is ultimately a matter of political and legal choices. We plead for a broad reflection at the EU level in order to build a common understanding and to develop further guidance on how new techniques should be considered in the light of the EU regulatory system on GMOs.

Moreover, we think that without similar discussions at the international level, it is likely that the same products of emerging new techniques might be considered GMOs or not depending on the regulatory jurisdiction. For instance, in the United States, modified plants developed through oligonucleotide-mediated mutagenesis have been declared non-GM by APHIS (personal communication, Dr. Lawrence Christy, Phygenics LLC). Such discrepancies should be avoided as they would pose challenges for the international regulation of transboundary movement of GMOs.

Received March 24, 2009; accepted May 25, 2009.

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