Should Novel Organisms Developed Using Oligonucleotide-mediated Mutagenesis be Excluded from the EU Regulation on GMOs?

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In the European Union, genetically modified organisms (GMO) and genetically modified microorganisms (GMM) are defined respectively according to Directives 2001/18/EC[1] on deliberate release of GMO and 2009/41/EC[2] on the contained use of GMM. The definition of a GMO is both technology- and process-oriented. A novel organism will fall under the scope of the GMO Regulation only if it has been developed with the use of certain techniques. The EU Directives therefore 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.

The underlying idea here is that some processes of genetic modification are potentially associated with risks. This approach is now challenged with the emergence of new techniques for which it is not always clear whether the resulting organisms shall be subject to the prevailing European GMO legislation or not. In a recent paper published in Environmental Biosafety Research[3], we discussed in detail regulatory and safety issues associated with the use of oligonucleotide-mediated mutagenesis and provided scientific arguments for not having organisms developed through this technique fall within the scope of the EU regulation of GMOs.

Oligonucleotide-mediated mutagenesis

Oligonucleotide-mediated mutagenesis (OMM) is a technique used to correct or to introduce specific mutations at defined sites of an episomal or chromosomal target gene. OMM is also referenced in the literature under other names, e.g., targeted nucleotide exchange, chimeraplasty, oligonucleotide-mediated gene repair, or targeted gene repair. OMM is mediated through the introduction of a chemically synthesized oligonucleotide (single-stranded DNA oligonucleotide, chimeric RNA/DNA or DNA/DNA, RNA oligonucleotide) with homology to the target gene, except for the nucleotide(s) to be changed. The mechanisms of action at the molecular level are poorly understood, but DNA repair enzymes are involved, and the process involves primarily the activation of the mismatch repair and/or nucleotide excision repair pathway. 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. The gene modification is induced directly and exclusively via the effect of the oligonucleotide itself, indicating that the process is a type of gene repair and not homologous recombination.

Potential applications of the technique

OMM has potential applications in fundamental research, medicine, agro-food and pest control. 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.

In bacteria and yeast, OMM has been used successfully mainly as a tool to perform fundamental research on gene expression and regulation, aiming at better understanding of the possible mechanisms underlying the genetic modification. In general, this technique is not expected to have major applications in microorganisms.

OMM has been successful in restoring or knocking out wild-type genes in animal cells, creating mouse mutants by modification of embryonic stem cells, and in directing genetic improvement of livestock animals. The technique seems to offer the potential to correct point mutations in human gene therapy, for instance in monogenic inherited diseases and cancer. 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.

Oligonucleotide-mediated mutagenesis is also applicable to plants. 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. Commercial applications of this technique in plants could even be expected in the short term. BASF and Cibus recently announced that they had reached a significant research milestone for developing CLEARFIELD Production System plants in Brassica winter oilseed rape and spring canola using Cibus' patented Rapid Trait Development System (RTDSTM) to enhance the tolerance levels of spring canola plants to CLEARFIELD herbicides[4].

Regulatory issues in the context of the EU legislation on GMOs

The EU definition of GMO implies a division of organisms between GMOs and non-GMOs according to the techniques involved. When considering OMM in the context of the GMO definition and techniques already listed in the Directives, the following conclusions can be drawn:

- OMM must be considered as 'leading to genetic modification' in the meaning of the EU Directives.
- All reviews clearly indicate that the process is a type of gene repair and not homologous recombination.
- The technique does not involve the introduction or integration of new genetic material in organisms, but alters chromosomal or episomal sequences in situ in their natural genetic background. OMM should therefore not be considered as a recombinant nucleic acid technique in the meaning of the EU Directives. We are also of the opinion that the nucleic acid molecules used in the technique (oligonucleotides) should not be considered recombinant nucleic acid molecules.
- OMM does not make use of any vector system. Delivery of the oligonucleotide in the cell can involve micro-injection or micro-encapsulation (in liposomes), although other techniques such as electroporation or particle bombardment are more commonly used.
- OMM can be considered as a form of mutagenesis, a technique that is excluded from the scope of the EU regulation.

Another important point to consider is that organisms developed through OMM in many cases 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). Detection and traceability are key aspects in the EU regulatory system on GMOs, in particular for GMOs used as Food or Feed. As a consequence, adequate molecular methods must be available that enable the detection and identification of each GMO individually (the so-called "transformation event"). It is important to realize that 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 reliability, efficacy, and reproducibility of OMM show a great variability, and further studies are 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.

Nevertheless, 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 unlikely when the oligonucleotide structure and chemistry were properly

designed. Altered genes are also stably maintained during mitosis and transmitted in a Mendelian fashion to subsequent generations.

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[5].

Conclusions

The terminology "oligonucleotide-mediated mutagenesis" covers various experimental approaches, but always has 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 biosafety questions other than those associated with similar techniques already listed in the GMO Directives, and could be considered similar to mutagenesis, a technique currently excluded from the scope of the EU GMO regulatory framework. This vision is shared by the COGEM (the Dutch GMO biosafety advisory committee)[6].

OMM is just one amongst several techniques (including Zinc Finger Nuclease technology, cisgenesis, reverse breeding, agroinoculation, grafting on GM rootstock, RNAi, synthetic biology) that are currently challenging the process-based approach followed in the EU to define a GMO. There have been for example scientific papers arguing for the exemption of cisgenic plants from the scope of the EU Directives[7].

In this context, a Working Group has been established recently by the European Commission to evaluate these techniques and to develop further guidance on how they should be considered in the context of the existing legislative framework and to make its findings available to the relevant competent authorities for further follow-up. Indeed, 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.

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 USDA APHIS. Such discrepancies should be avoided, as they would pose challenges for the international regulation of transboundary movement of GMOs.

Last but not least, it is important to realize that the outcome of these discussions is of utmost importance for developers of novel organisms and in turn may have ramifications for plant breeders, agro-industry development and biomedicine in the European Union. In the absence of legal clarity, the commercial applications of new techniques may be restrained, owing to the complexity and associated high costs of applying GMO legislation in the EU.

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