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Why genome edited organisms are not excluded from the Cartagena Protocol on Biosafety

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New and emerging genome editing techniques are being promoted as faster methods to genetically engineer changes in genetic information and expression at targeted regions of the genome. Such techniques include clustered regularly interspaced short palindromic repeats (CRISPR), meganucleases (MNs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and oligonucleotide directed mutagenesis (ODM). The development of such techniques has prompted intense debates about (1) whether they are regulated under current biosafety legislation, (2) whether they are safe, and (3) whether they should undergo the same risk assessment and risk management procedures as living modified organisms (LMOs).

One argument promoted by those seeking to exclude genome edited organisms from regulation, including at the national level, is that organisms engineered with genome editing techniques do not fall within the definition of an LMO under the Convention on Biological Diversity's (CBD) Cartagena Protocol on Biosafety.

This paper sets out to show that currently deployed genome editing technologies and applications, including all techniques involving CRISPR-based systems, clearly fall within the Protocol's definition of an LMO, whether they involve inserting, deleting or editing sequences of genomes.

The definition of "living modified organism" under the Cartagena Protocol

Article 3 of the Cartagena Protocol provides three definitions that are interrelated and have to be read together: "living modified

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organism", "living organism" and "modern biotechnology".

"Living modified organism" means "any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology".

A living modified organism is thus defined in the Protocol to include only those living organisms that
– contain novel combinations of genetic material; and
– have been produced using the techniques of modern biotechnology (paragraph 208, *An Explanatory Guide to the Cartagena Protocol on Biosafety*: Mackenzie et al., 2003).

"Living organism" means "any biological entity capable of transferring or replicating genetic material, including sterile organisms, viruses and viroids".

While the Cartagena Protocol does not define "genetic material", the CBD does: "any material of plant, animal, microbial or other origin containing functional units of heredity". Functional units of heredity are understood to be nucleic acids containing genetic information. In the context of the Cartagena Protocol, genetic material can be understood to refer to nucleic acids that contain functional units of heredity (paragraph 201, *Explanatory Guide*).

A "novel combination of genetic material" can be regarded as a combination that was not previously known to exist at the time it was first produced. Linked to the CBD definition of genetic material, this can then be understood to refer to a novel combination of nucleic acid containing functional units of heredity (paragraph 209, *Explanatory Guide*). It is important to note that the novel combination relates solely to a combination of genetic material, even if this does not result in an observational change (paragraph 210, *Explanatory Guide*).

The novelty of a combination could arise through a novel form of a functional unit of heredity, e.g., resulting from a change that modifies the overall sequence of nucleotides within the unit, whether by altering, inserting or deleting one or more nucleotides. Novelty could also arise from a novel arrangement of functional units of heredity, e.g., introduction of genetic material from different species, or rearrangement of genetic material of the same species. A novel combination could arise from a single change in a nucleotide sequence or from much larger changes (paragraphs 211-212, *Explanatory Guide*).

According to the Cartagena Protocol, the novel combination of genetic material must be "obtained through the use of modern biotechnology". This is a fundamental criterion for the definition of an LMO. Whether or not an organism is an LMO under the Protocol depends on whether "modern biotechnology" is used to create a novel combination of genetic material.

"Modern biotechnology" is defined in the Cartagena Protocol as:
"The application of:
a. In vitro nucleic acid techniques, including recombinant DNA and direct injection of nucleic acid into cells or organelles, or
b. Fusion of cells beyond the taxonomic family,
that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection".

This therefore includes, but is not limited to, in vitro nucleic acid techniques applied to the insertion, deletion and alteration of genetic material (paragraph 215, *Explanatory Guide*). The two qualifications are that natural physiological reproductive or recombination barriers must be overcome, and that they are not techniques used in traditional breeding and selection.

In summary, in order for a living organism to be defined as an LMO under the Cartagena Protocol, it has to fulfil three criteria:
1. the organism contains novel combinations of genetic material;
2. it has been produced using the techniques of modern biotechnology (including the application of in vitro nucleic acid techniques, or fusion of cells beyond the taxonomic family); and
3. the modern biotechnology techniques used have overcome natural physiological reproductive or recombination barriers and are not techniques used in traditional breeding and selection.

Criterion 1: the organism contains novel combinations of genetic material

For CRISPR-based systems, the technique involves the use of guide RNA (gRNA) sequences that are synthetically produced to target a DNA (or RNA) sequence of interest for 'editing'. The gRNA then directs the Cas9 DNA-cutting enzyme, or endonuclease, towards the sequence of interest, where it then cuts the DNA. Once the DNA is cut, the cell's DNA repair machinery is activated, and the DNA is repaired. If the repair process results in the insertion or deletion of small pieces of genetic material ('indels'), the outcome is called a site-directed nuclease (SDN) 1 application. SDN-1 applications are used to imprecisely destroy genes. Alternatively, if the intention is to 'edit' a gene, additional DNA is also introduced that provides the template for the desired alteration that can then be copied into the target organism's gene. This is termed SDN-2. CRISPR-based systems are also being used to insert transgenic DNA into a target site, by introducing a DNA template alongside the CRISPR machinery. This is termed SDN-3.

Similarly, TALENs, MNs and ZFNs can be designed to perform SDN-1, -2, and -3 applications of 'indels', edits, or insertions of transgenic DNA sequences. However, they differ from CRISPR-based systems in that they do not deploy gRNAs to target specific DNA sequences, but are instead protein-based enzymes that have DNA recognition sites to bind and cleave particular DNA sequences of interest.

ODM, on the other hand, involves the introduction of short stretches of DNA, called oligonucleotides, or DNA/RNA oligonucleotide hybrids. These oligonucleotides are identical to the target sequence to be modified, except for the desired alteration to be introduced. These DNA templates bind to the target sequence, after which the cell's natural repair machinery recognises the single base mismatch between its own DNA and that of the repair template.

The entire purpose of all genome editing techniques is to introduce previously non-existent genetic changes in order to generate new traits in living organisms regardless of whether DNA has been inserted or not. Whether deployed to generate 'indels', 'edits' or 'insertions', all aim to generate novel traits by generating novel combinations of DNA, regardless of whether transgenic DNA is indeed inserted.

*It is thus clear that **all** genome editing techniques produce a novel combination of genetic material, and thus fulfil criterion 1.*

Criterion 2: the use of modern biotechnology (including the application of in vitro nucleic acid techniques, or fusion of cells beyond the taxonomic family)

Currently, the vast majority of genome editing techniques involve the application of nucleic acid techniques, involving the use of either RNA or DNA nucleic acids at a certain stage of the process.

CRISPR-based techniques (including double-nickases and base 'editors') can be performed in a number of ways, all of which involve the introduction of either DNA or RNA nucleic acids. The classic approach is to introduce DNA plasmids that encode for the CRISPR machinery. Either the plasmid is permanently introduced into the target organism's genome, and can later be removed by backcrossing, or it can be transiently expressed. More recent DNA-free techniques have been developed that involve either introducing messenger RNA molecules that encode for the CRISPR machinery, or the direct introduction of ribonucleoproteins consisting of the Cas9 protein along with the guide RNA molecule (Metje-Sprink et al., 2019).

As with CRISPR-based techniques, the classic TALEN, ZFN and MN methods involve the introduction of DNA plasmids that express each of TALENs, ZFNs or MNs respectively, either introduced permanently into the target organism's genome (and later removed if desired), or transiently. Messenger RNA encoding for TALEN machinery in plant cells has also been demonstrated (Stoddard et al., 2016).

Limited exceptions have been demonstrated in the laboratory using TALENs and MNs (Luo et al., 2015) where direct delivery of the respective protein forms of the nucleases has been performed in plants to generate a

genome edited organism. While this would arguably fall outside of fulfilling criterion 2, such methods are currently much less efficient than delivery of DNA plasmids and unlikely to be widely used.

ODM techniques are performed either by directly introducing the oligonucleotides, which can be in the form of DNA, or DNA/RNA hybrids, into the target organism's genome.

CRISPR-based, ZFN and ODM techniques all involve the use of nucleic acids (either DNA, or RNA, or DNA/RNA hybrids) to perform genome editing functions, and thus fulfil criterion 2. Most TALENs and MNs techniques, with some limited exceptions, involve the use of nucleic acids (either DNA, or RNA hybrids) to perform genome editing functions, and those thus fulfil criterion 2.

Furthermore, even if the novel combination of genetic material obtained through modern biotechnology is subsequently transferred into another organism through traditional breeding or selection techniques, the resulting organism is also an LMO under the Protocol (paragraph 214, *Explanatory Guide*). Such a definition would therefore include genome edited organisms that introduce a DNA cassette encoding for nucleases that is later removed by conventional breeding (as often performed with CRISPR, TALENs, ZFNs and MNs), which would fulfil criterion 2, as the earlier step would have already involved the application of nucleic acid techniques to generate a novel combination of genetic material.

Ensuring the process and not just the outcome is regulated under criterion 2

It is vital to highlight that criterion 2 ensures that the process, not the outcome, is regulated and assessed, in order to allow for detection of unintended effects, some of which undermine the theoretical distinction between applications of ODM, SDN-1, -2 and -3. Unintended insertions of genetic material have been detected, at both on- and off-target sites (Li et al., 2015; Liang et al., 2017; Ono et al., 2019; Norris et al., 2020; Skryabin et al., 2020), an effect associated with all nucleases that introduce double-stranded DNA breaks (including CRISPR, TALENs, ZFNs and MNs). As such, while the intention of ODM, SDN-1 or -2 does not involve permanent insertion of DNA, current evidence suggests this may often be a theoretical rather than practical outcome.

Other documented unintended effects include off-target modifications elsewhere in the genome, e.g., mutations, complex large-scale rearrangements, translocations, insertions and deletions and novel protein production (Wolt et al., 2016; Mou et al., 2017; Shin et al., 2017; Zhu et al., 2017; Kosicki et al., 2018; Tuladhar et al., 2019). Even single nucleotide alterations can have impacts on the function of a gene, whether at the targeted or any off-target location.

Further, the process of genome editing usually involves identical supporting techniques to the older transgenesis techniques, including transformation of cells grown in culture. Such processes are associated with unintended effects such as deletions and rearrangements (see, for example, Kim et al., 2003; Latham et al., 2006; Makarevitch et al., 2003; Rang et al., 2005; Windels et al., 2003).

Criterion 3: modern biotechnology techniques used have overcome natural physiological reproductive or recombination barriers and are not techniques used in traditional breeding and selection

It is clear that genome editing techniques are not techniques used in traditional breeding and selection, as genome edited organisms are generated without any breeding taking place, but through the use of modern biotechnology techniques to introduce genetic changes. It is also evident that they bypass natural reproductive or recombination barriers, with genome editing allowing for modifications that would not otherwise naturally arise (see review by Kawall, 2019). As detailed below, the described techniques can be used to perform one or multiple modifications that overcome natural reproductive or recombination barriers.

One of the major advantages afforded to LMO developers is the ability to use genome editing to perform simultaneous or successive alterations of genetic material, an application termed multiplexing. Many plant traits are dependent on a multitude of genes. CRISPR, TALENs, ZFNs and MNs techniques now offer a means to target many genes at once, or multiple copies of the same gene (as well as paralogues), something that has not yet been achieved with conventional breeding, chemical mutagenesis or transgenic techniques to date (e.g., see Kawall, 2019; Ran et al., 2018; Wang et al., 2018; Li et al., 2018; Qi et al., 2016; Cai et al., 2018; Bao et al., 2015; Suzuki et al., 2013; Hauschild et al., 2011). The alteration of all copies of a gene is highly relevant to plants, with many species having multiple copies of a gene as a result of polyploidy (having more than two copies of each chromosome) or genetic redundancy (having multiple copies of a gene). Neither conventional breeding nor naturally occurring mutations are able to alter all copies of a genetic sequence. While demonstrations of multiplexing of different genes or multiple copies of genes currently appear lacking with ODM, in theory such alterations are possible (Jansing et al., 2019).

Genome editing further allows for the generation of mutations in regions of the genome that are ordinarily protected by naturally existing DNA repair mechanisms. Genetic variation occurs naturally in organisms as a result of numerous mechanisms. Mutations may arise spontaneously as a result of external environmental factors, e.g., UV sunlight or exposure to mutagenic substances, or internally as a result of mistakes during DNA replication or by mutagenic metabolic by-products, e.g., reactive oxygen species. Mutations arising from such factors result in the recruitment of DNA repair mechanisms, with preferential protection of particular regions of DNA, such as those containing genes. DNA repair mechanisms thus function to prevent the excessive accumulation of mutations in DNA regions where the sequences need to be conserved to maintain their key functions, e.g., gene-encoding sequences. Genome editing techniques thus override endogenous repair mechanisms with the ability to alter conserved genetic sequences. Further, evidence suggests that mutations resulting from genome editing techniques are not repaired with the same processes as those that have occurred naturally, with high error rates in repairs of CRISPR-induced mutations (Brinkman et al., 2018). CRISPR/Cas systems have been used to modify conserved sequences, for example when applied to gene drive technologies (Kyrou et al., 2018). TALENs, as well as ZFNs, have also been demonstrated to target conserved sequences (Suzuki et al., 2013; Bilichak et al., 2020).

Natural genetic variation is also generated during meiosis, the production of gametes such as sperm and egg cells. Meiosis, a fundamental biological process, is responsible for generating genetic variation in sexually reproducing organisms by recombining the genetic content received from both parents. The exchange of genetic material between chromosomes during this process occurs in a non-random manner, in defined regions of the chromosomes, sometimes termed recombination 'hotspots'. In contrast, genome editing allows for altering DNA in regions that are so-called 'coldspots', overriding these natural limitations. CRISPR/Cas has been used to overcome linkage drag effects where desirable genes are linked to undesirable genes in low recombigenic regions (Roldan et al., 2017; Soyk et al., 2017). Further, CRISPR is being explored for manipulation of meiotic recombination events in order to increase crop genetic diversity, though this appears to be in early stages of research and development (Taagen et al., 2020).

*In summary, **all** the genome editing techniques allow for bypassing of natural reproductive and recombination barriers via a multitude of mechanisms, as well as not being techniques used in traditional breeding and selection, and thus fulfil criterion 3.*

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