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Biosafety aspects of genome-editing techniques

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The new techniques of genome-editing

Recent scientific and technical developments in modern biotechnology have intensified the debate about the regulation of organisms resulting from new techniques. More specifically, the debate is addressing whether or not organisms resulting from new techniques fall within the scope of legislation regulating genetically modified organisms (GMOs). This debate is taking place at national (e.g., Brazil, Germany, Sweden, USA, etc.), regional (e.g., European Union) and international (i.e., Convention on Biological Diversity and its Protocols) levels.

In general terms, GMO regulations set mandatory approval and risk assessment requirements, sometimes also taking into account socioeconomic and ethical considerations. They were originally established in response to the modern biotechnological techniques emerging in the 1970s and have evolved over time and jurisdiction to better capture the scope of coverage. The question now is whether variations of certain techniques are creating potential products for release into the environment that might not be subject to current GMO regulations and/or if these regulations require revision and adaptation (Heinemann 2015).

New biotechnological techniques can be described as a range of techniques that create organisms with novel traits or alter the expression of an already existing trait. Up until now, these techniques have mainly been used on yeast and bacteria, but most environmentally released products will be plants. Although the terms used to define these new techniques vary among regulators and scientists, the New Techniques Working

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The AFRICAN CENTRE FOR BIODIVERSITY is committed to dismantling inequalities in the food and agriculture systems in Africa and to its belief in people's rights to healthy and culturally appropriate food, produced through ecologically sound and sustainable methods, and to define their own food and agriculture systems.

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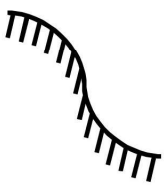
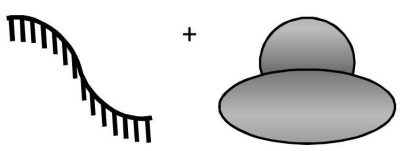
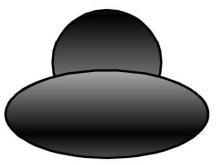
Table 1. List of current genome-editing techniques

Genome-editing category	Genome-editing techniques
Site-directed nucleases	Zinc-finger nuclease (ZFN)
	Transcription activator-like effector nucleases (TALENs)
	Meganucleases or homing endonucleases
	Clustered regularly interspaced short palindromic repeats (CRISPR). The first CRISPR associated protein discovered, and still most used, is Cas9 nuclease. This is the reason for the designation CRISPR/Cas9.
Oligonucleotide directed mutagenesis or oligonucleotide genetic engineering	Oligonucleotide directed mutagenesis or oligonucleotide genetic engineering

Figure 1. Comparative explanatory table of genome-editing techniques and their main characteristics

Genome-editing techniques		
Oligonucleotide genetic engineering (OGE)	**CRISPR**	**Zinc-fingers, TALENs and meganucleases**
Made of nucleotides (DNA or RNA) 	Made of a nuclease (enzyme that works like a 'molecular scissor') bound to an oligonucleotide (RNA) 	Made of a nuclease that has a nucleotide-binding domain. 
Synthetic oligonucleotides homologous to the target DNA, but containing mismatches, are introduced into plant cells. The mismatches in base pairing between the oligonucleotide and target DNA are modified by the native repair mechanism of the cell, resulting in permanent modification in the targeted DNA.	The nuclease Cas9 or its variants bind to an RNA molecule forming a complex. RNA enables interaction with DNA molecules that have a matching sequence. The complex functions like a sentinel in the cell and searches the sequences in the bound RNA. When the sites are found, it allows the protein complex to cut and break DNA at that site. The cell native DNA repair mechanism will join the broken DNA ends by replacement of one or a few nucleotides or introduction of short deletions. When an oligonucleotide template is also provided, repair and insertions can be achieved as intended in the oligonucleotide template sequence.	Functions similarly to CRISPR/Cas9. The difference is the lack of an RNA molecule. The nuclease itself has enzymatic domains that recognize nucleotides in a specific manner and order, thus requiring a specific DNA sequence to bind. Also working like a sentinel in the cell, once sites are found it also produces double-stranded DNA breaks at host genomic sequences. The cell native repair system will then 'repair' the break. Also works with an oligonucleotide template.

Group established by the European Commission in 2007 has identified several new techniques that have obtained consensus in the discussions (EC 2012).

Among the new techniques, genome-editing (Table 1, Figure 1), specifically CRISPR (clustered regularly interspaced short palindromic repeats), is gaining special attention due to its various possibilities and relatively easy manipulation. The CRISPR technique has rapidly emerged as a leading tool for investigating gene function and for creating genetic variation using site-directed genomic alterations (Travis 2015).

Genome-editing, classical genetic engineering and mutagenesis

Genome-editing techniques differ from those of classical genetic engineering because of their ability to (1) modify target genes *in vivo*, and not only *in vitro* followed by re-introduction; (2) increase the efficiency of introducing the intended modification at an intended place; and (3) increase the range of organisms in which the first two possibilities can be achieved.

These techniques have multiple and novel steps in the process of producing a GMO, such as the naked delivery of nucleases (e.g., Cas9 and zinc-fingers), and in some cases may be used without requiring any DNA sequence integration. However, up until now, the majority of the applications in plants still rely on classical genetic engineering tools (e.g., recombinant DNA, particle bombardment, *Agrobacterium*-mediated transformation). Thus, in many instances new DNA sequences could still be introduced.

Genome-editing techniques utilize externally supplied nucleotides and/or enzymes in addition to endogenous nucleotides, nucleic acids and enzymes, to modify DNA in a targeted manner. Such techniques exploit the sequence-specific interaction of the nucleotide (i.e., oligonucleotide genetic engineering (OGE) and CRISPR) or nucleotide-binding domains of enzymes (i.e., zinc-fingers, transcription activator-like effector nucleases (TALENs), meganucleases) with DNA sequences in the cells.

Genome-editing techniques are said to be specific because the tools make use of the biochemical property of hybridization (ability of single-stranded nucleic acid molecules to 'base-pair' with one another) or protein-nucleic acid recognition which, like hybridization, involves an optimal formation of a large number of weak bonds between the nuclease and the target nucleic acid. The number and strength of the bonds, in both cases, is a function of the order of nucleotides in the target molecule (Heinemann 2015).

Some define genome-editing techniques as a form of 'mutagenesis' (i.e., oligonucleotide mutagenesis, site-directed mutagenesis). In fact, any change to a DNA sequence can be described as a mutation and any process by which it is delivered can be called 'mutagenesis'. However, it is necessary to understand the characteristics each of these techniques possesses that are relevant to the GMO regulations. Heinemann (2015) conducted a review of the OGE technology and described how the scientific literature before 2001 had frequently referred to OGE as a 'genetic engineering' process and not 'mutagenesis'. Apart from the inconsistencies and multiple meanings of such terms, choosing to call these techniques 'mutagenesis' does not make them less like the mutagenesis caused by a transgene insertion (within the scope of GMO regulations) or more like mutations caused by a chemical or radiation mutagen (outside the scope of GMO regulations) (Heinemann 2015).

Why genome-editing techniques should be regulated: Risk aspects

Genome-editing techniques raise the possibility of targeting, *in vivo*, a specific gene or sequence in the genome of virtually any species. Targeted gene modification is the deletion, insertion or alteration of nucleotide order in an existing molecule of DNA or RNA. It is, however, also possible

to insert or delete entire new genes or large sequences. Insertion of genes requires the supply of a DNA template along with the nuclease. However, confining the change to the intended template only is not possible. Thus, after the procedure, intended products are separated from unintended products.

The safety of such genome-editing techniques relies on two premises:

1. Changes only in the intended places
2. Only the changes intended

Premise 1 is relevant to the issue of off-target activity of genome-editing techniques. Off-target activity occurs when the oligonucleotide or the nuclease complex bonds tightly to a particular sequence of nucleotides separate from that which was intended. The more off-target activity, the more likely unintended and potentially adverse effects might arise. This is because the characteristics of the organism may be altered due to changes in genes that might occur.

Unintended changes in DNA sequences can also lead to changes in gene expression that are tolerated by the GMO but still create hazards (to animals and humans or the environment). If there are unintended gene product(s) (e.g., RNA, proteins) which are different from those originally intended, these genotypic and/or phenotypic changes, including changes in native/endogenous gene expression and regulation at the transcriptional, translational and post-translational levels (for example, toxic products of endogenous upregulated genes), might directly impact the nutritional and safety value of that product (AHTEG 2010).

Therefore, when evaluating genome-edited organisms, one has to take into consideration what other sites could be affected. In maize, for example, which has one of the largest genomes of crops, only 30% of the genes have been predicted by bioinformatics to be targeted by specific CRISPR guide RNAs (gRNAs) (Bortesi and Fischer 2015). The lack of specific gRNAs for so many maize genes probably reflects the genome complexity (duplication events) and genomic sequence context. It is therefore anticipated that wheat and barley, with genomes that are similarly as large and complex as the maize genome, may present similar challenges for genome-editing techniques (Xie et al. 2014). Therefore, a significant number of off-target modifications should be expected.

While bioinformatics tools can help identify off-target activity, they are inferences or judgments made based on predictions from what is already known and this is not exhaustive for any organism or every genome, much less large plant genomes. In addition, unanticipated off-target modifications can be difficult to detect and they are not possible to reliably predict using bioinformatics techniques because very short matches in the oligonucleotide or weak binding of the nuclease might cause an off-target effect. Therefore, potential adverse effects cannot be ruled out only through genome sequencing and post-sequencing analysis. Unintended changes should also be identified in the genetically modified (GM) product through a semi-targeted qualitative profiling of small RNA molecules using next generation sequencing or other *-omics* techniques in a comparative assessment between the GM and conventional parent (Heinemann et al. 2011). In addition, to specifically test for adverse effects on humans and the environment, more dedicated testing would be needed; depending on the outcome of the experiments, further testing may be required.

The current scientific knowledge about the safety of genome-editing techniques is based on a relatively small number of studies on tested genome-edited organisms and off-target sites. General conclusions would be premature at this point. A comprehensive review of off-target activity of OGE, CRISPR, zinc-fingers, TALENs and meganucleases, and the corresponding knowledge gaps are provided in Agapito-Tenfen and Wikmark (2015).

Much of what is being written about genome-editing suggests that these techniques are precise and thus can only generate 'small' mutations at target sites. What this generally means is sometimes a confusing mix of concepts from 'precision of targeting' to 'efficiency' of the intended reaction at the target site.

Even as off-target activities become better understood, in the context of genetic screens, it is still critical to require that multiple perturbations targeting a gene show consistency in order to conclude that the observed phenotype is due to on-target activity (Doench 2016). In other words, genome-editing techniques can be called 'targeted' but not yet 'precise'. 'Targeted' and 'precise' have different meanings: while 'target' suggests the idea of modifying a specific gene, 'precision' is the idea of modifying *only* that gene. Figure 2 provides a graphical representation of how a technique can be accurate and precise in terms of its target and off-target activity.

Size matters?

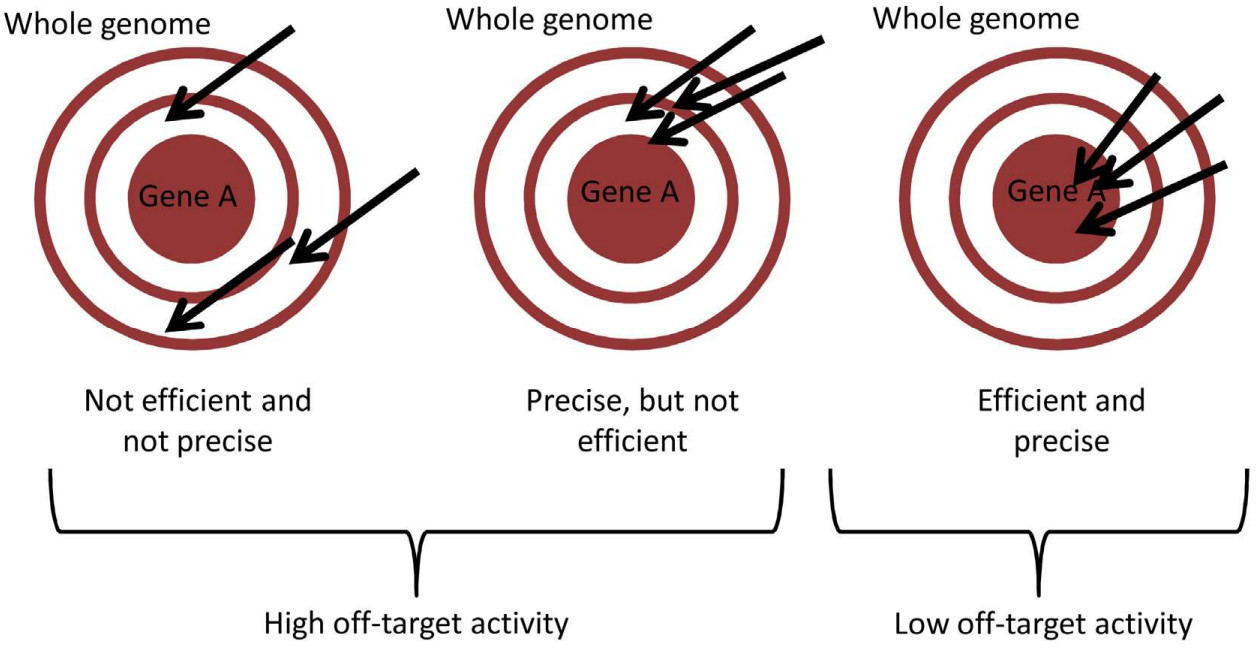
The size of the mutation (as in number of altered bases) has limited value for predicting unintended or adverse effects. Single nucleotide changes can be important, while some large changes (depending on what and where they are) may not be. That is why case-by-case assessment remains so crucial.

Precision is a double-edged sword

High efficiency of change at the target is due to the interaction between the tools and the target and, therefore, any 'off-target' site, even if there are fewer of them, will also be efficiently processed. Thus, the probability of two specific changes (the intended and one or more of the small number of off-target sites) in a single organism is greater than the probability when using 'random' mutagenesis techniques. If any off-target change causes an adverse effect on the environment or human health, then it will more likely occur along with the intended change in the products of these genome-editing techniques (Heinemann 2015).

Premise 2 is relevant to the resulting type of DNA modification. Double strand breaks can be both detrimental to organisms (e.g., leading to carcinogenesis or apoptosis) or beneficial to organisms (e.g., generation of a fitness-improving new trait). The repair of a double strand break depends on several factors that are not yet fully understood, particularly in plants. Knowledge gaps in the basic functioning of such repair mechanisms

Figure 2. Graphical representation of off-target activity of genome-editing techniques



show that most assays are error-prone trials with large variation.

In a recent study, Woo et al. (2015) tested four plant species for CRISPR gene modification. The analysis of target sites alone showed a variation from -29 nt (or a deletion of 29 nucleotides) up to +33 nt (or the addition of 33 nucleotides). But even single nucleotide changes can have a variety of effects on endogenous gene expression and might not cause a phenotypic change and thus can be difficult to detect and identify.

Harmonized detection methods for the identification of off-target mutations at a pre-market risk assessment step have not yet been developed. Accurate detection methods are crucial since many of these products might not be distinguishable from already existing products. Studies on the inheritance and stability of such mutations over generations are also still lacking in the scientific literature.

In the context of future agricultural products, it is relevant to highlight that the large majority of the studies are still based on experience using mammalian cells or microorganisms and not plants. Thus, they are not adequate for future environmental risk assessments because the likelihood of different outcomes is species-dependent and there have been only a few comparative studies looking at the basis of such diversity (Bortesi et al. 2016).

It is clear that the scientific knowledge about these new techniques is evolving, and as new information becomes available, knowledge gaps will no doubt diminish. However, it is also clear that proper regulation and mandatory risk assessment should be in place before genome-edited products are allowed on the market. It is crucial that regulators ask for experimental evidence to address potential adverse effects of genome-editing techniques in order to avoid a vacuum in the risk assessment of such organisms.

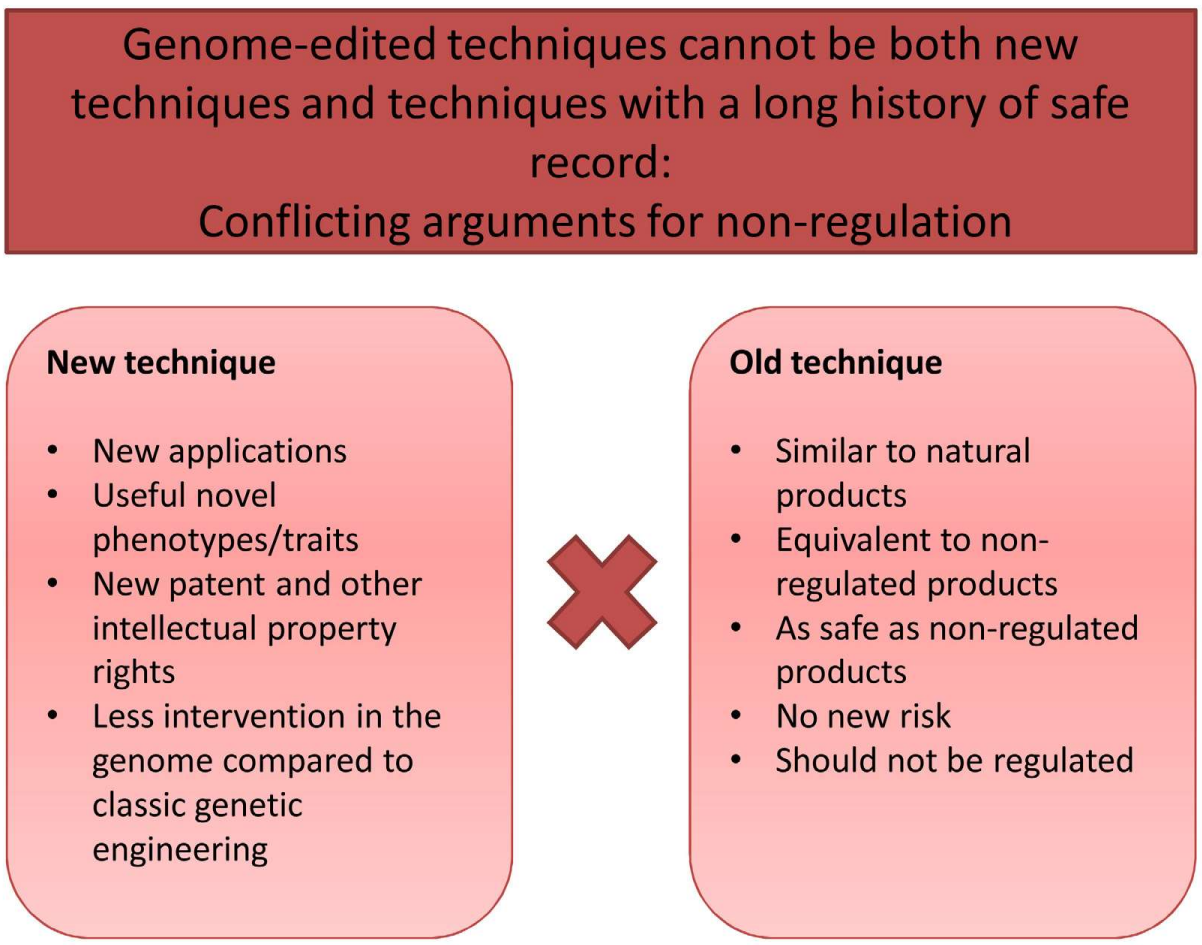
Regulation of genome-editing techniques

It is important to consider the origins and history of GMO regulations when addressing novel and controversial issues. The most relevant regulations in question are the Cartagena Protocol on Biosafety, a protocol of the Convention on Biological Diversity (CBD), and domestic GMO regulations such as EU Directive 2001/18/EC.

The similarity between genome-edited organisms and organisms that originate from classic mutagenesis techniques or even those found in nature has been used as a justification for excluding these products from the scope of GMO regulations. The argument goes that these products are indistinguishable from the products created by processes already excluded from legislation and, therefore, they have an equal potential to (not) cause harm and neither should they be regulated. It is important, however, to make clear that the reasoning based upon distinguishability of products and not techniques is not relevant to the Cartagena Protocol or the EU Directive because it is relevant neither to the definition of a GMO nor to the description of a process by which it was made (Heinemann 2015). Moreover, distinguishability is a function of existing technology. As the technology changes, so might the ability to distinguish. Regulations do not define their scope by the similarity or otherwise of products, but by the differences between techniques (scientifically, ethically and through history of use). In addition, a genome-edited technique cannot be both a new technique and a technique with a long history of safe use (Figure 3) (Heinemann 2015).

In October 2010, the Parties to the CBD first turned their attention to a new and emerging topic, synthetic biology. In their discussions, genome-editing was highlighted as a supporting technology to synthetic biology applications. Follow-up decisions established an Open-Ended Online Forum and an Ad Hoc Technical Expert Group (AHTEG) on Synthetic Biology to provide information

Figure 3. The contradictory arguments for non-regulation of organisms and products derived from new techniques



regarding the potential positive and negative impacts, as well as possible gaps and overlaps with provisions in the Convention and its Protocols. Discussions are still ongoing and will continue as several processes under both the CBD and the Cartagena Protocol are mandated to take up the issue of synthetic biology.

Although no definitive decision about genome-editing regulation has yet been made by Parties to the CBD or the Cartagena Protocol or member states of the EU, some countries that are Parties to the Protocol are close to reaching a conclusion about genome-editing techniques, based on the interpretation of their domestic laws. For example, the Office of the Gene Technology Regulator in Australia has opened a call for submissions for technical review of the 2001 Gene Techniques regulations and it is expected that genome-editing techniques will be discussed. The National Technical Biosafety Commission in Brazil is currently revising a new standard on new techniques and this should come into force any time soon.

In February 2015, the German Federal Office of Consumer Protection and Food Safety (BVL) issued a decision allowing the release of oilseed rape produced by US company Cibus without it being subject to regulation required for GMOs. This particular oilseed rape is produced by OGE[1] and had earlier been deregulated in the US. But later that year, a letter sent by the European Commission to the relevant authorities of EU member states clearly confirms that the BVL decision on the release of the OGE oilseed rape could not be implemented due to the legal uncertainty on the matter, and that member states should 'await, as much as possible, the outcome of the Commission legal interpretation before authorizing a deliberate release of

1 https://www.testbiotech.org/en/node/1282

organisms obtained with new plant breeding techniques' since 'the deliberate release of products which are subject to the rules of the EU GMO legislation without appropriate prior authorization, is illegal'.[2]

In November 2015, the Swedish Board of Agriculture concluded that *Arabidopsis* plants that have been produced by CRISPR would be exempted from the GMO legislation.[3] However, in that same letter, the Board stated that their decision was only valid for that specific case, and that their interpretation may be subject to change if there were a future common interpretation of the definitions and exemptions at the EU level. The Board further urged caution during cultivation, as the plant's legal status might change rapidly.

In April 2016, the US Department of Agriculture told Pennsylvania State University that their CRISPR-derived mushroom was not subject to its regulations (Waltz 2016). But the US is not a Party to the Cartagena Protocol and therefore, its domestic legislation does not necessarily meet the standards of the Protocol. In addition, the US does not apply the Precautionary Principle to the issue of GMOs.

Clearly, as the techniques are developing rapidly, regulations are attempting to play 'catch-up' and there is as yet no international legal clarity. It is therefore important for the Precautionary Principle to be applied, and to hasten the work already begun at international level, under the CBD and its Protocols, to ensure that oversight of these new techniques is robust and comprehensive.

2 European Commission. Letter to Competent Authorities. 5 June 2015. Obtained through Freedom of Information request by Corporate Europe Observatory. http://corporateeurope.org/sites/default/files/attachments/18._2015.06.15_lettre_autorites_competentes_redacted.pdf

3 https://www.upsc.se/documents/Information_on_interpretation_on_CRISPR_Cas9_mutated_plants_Final.pdf

Applications in food and agriculture and status of approvals

Trait improvement through classic breeding in crops can indeed be accelerated by genome-editing. Cibus' herbicide-tolerant OGE oilseed rape has reached commercial phase in the US (*Nature* Editorial 2015), but it is on hold for field trials in Germany, pending a court case. A maize variety developed using OGE is resistant to imidazolinone herbicides, but its commercial status is unknown (Zhu et al. 2000). There have been a few scientific developments in crops – such as bacterial leaf blight resistance in rice, powdery mildew resistance in bread wheat, soybean with enhanced fatty acid profile, potatoes with reduced acrylamide levels, maize with both reduced phytate content and herbicide tolerance, etc. – which have been produced through site-directed nucleases such as zinc-fingers and TALENs but their commercial status is also unknown (Wolt et al. 2016; Schaart et al. 2016). CRISPR has been used in commercial crops in attempts to increase yield, improve drought tolerance, and increase growth in limited-nutrient conditions to breed crops with improved nutritional properties and to combat plant pathogens, but these are not yet on the market (Barrangou and Doudna 2016).

CRISPR-modified animals are also in the agricultural pipeline. Roslin Institute in Edinburgh is creating genome-edited pigs to be immune to African swine fever (Reardon 2016). African swine fever virus (ASFV) is a haemorrhagic virus that causes the disease to sweep through pig populations in sub-Saharan Africa and Eastern Europe. Whether or not the genome-edited pigs will be available to small-scale farmers in Africa is yet unknown.

Another application of CRISPR technology is 'gene drives'. Gene drives can be understood as a genetic engineering approach to promote the inheritance of a particular gene to increase its prevalence in a population. The term is also used for specific genetic elements (i.e., a piece of DNA) that can implement the technique

(Pennisi 2014). It has been tested in fruit-flies and in *Anopheles gambiae*, the mosquito species that transmits malaria in sub-Saharan Africa (Ledford and Callaway 2015). Gene-drive approaches can be used to spread particular genetic alterations through wild populations over many generations. Because they can alter the traits of entire species, they represent a potentially high risk to biodiversity. Many civil society organizations are calling for a global moratorium on CRISPR-based gene drives.

Because applications for commercial approvals are not disclosed in many countries, it is hard to keep track of what is reaching the market in which country. In addition, the Biosafety Clearing House[4] set up by the CBD Secretariat, which should ideally list all field trials and approvals by Parties to the Cartagena Protocol, is unfortunately not always updated by Parties. The field is constantly changing and most of the monitoring activities are conducted by the scientific community or non-governmental organizations (NGOs), but the information remains scattered.

New techniques, old power relations

Genome-editing techniques are likely to have similar commercial and socioeconomic implications as classical GMOs. Results of genome-editing are bound to be protected by intellectual property rights, which will therefore have market-power and purchasing-power implications for seed and biotech companies as suppliers, on the one hand, and farmers, on the other.

A good example is the current patent battle over CRISPR technology. Seed giant DuPont Pioneer has entered into a strategic alliance with genome engineering pioneer Caribou Biosciences, a company co-founded by patent claimer Jennifer Doudna from the University of California, Berkeley. In October, the companies signed an agreement for sharing intellectual property rights for CRISPR applications in major staple crops like corn and soybean (Grushkin 2016). On the other end is Broad Institute of Massachusetts Institute of Technology (MIT)'s patent fight against Berkeley. Recently, Monsanto announced a global licensing agreement with Broad Institute for agricultural applications of CRISPR genome-editing techniques.[5]

Companies and institutions are clearly positioning themselves for the next big battles ahead, in terms of claiming intellectual property over these new techniques.

Recommendations: What should be the next steps?

Exemption of plants produced by genome-editing techniques from GMO regulations, as called for by industry and some other quarters, would mean that there is no specific requirement to assess any potential effects on food, feed or environmental safety caused by the use of these techniques. It would also mean exemption from GMO labelling requirements, restricting the choices available to consumers who wish to avoid food derived from such new techniques. To prevent this scenario, current GMO regulations need to be interpreted in their intended sense, to encompass all modern biotechnological processes that directly modify genomes (Achterberg 2015).

It is clear that the drafters of the Cartagena Protocol recognized that any definition of 'modern biotechnology' should cover new techniques not yet envisaged at the time the Protocol was adopted but which may emerge in the future. This is because the technology is developing all the time, and the legal instrument had to be drafted so as to not exclude new technological processes not yet identified but which may give rise to novel combinations of genetic material through the use of modern biotechnology.

Therefore the definition of 'modern biotechnology' in the Protocol reflects the

4 https://bch.cbd.int

5 http://news.monsanto.com/press-release/corporate/monsanto-announces-global-licensing-agreement-broad-institute-key-genome-edi

need to cover future techniques,[6] and a close reading of the various definitions in both the CBD and the Protocol shows that such techniques are indeed covered by these international legal instruments. Hence, the progression of work in these fora on synthetic biology, including genome-editing techniques, is currently underway. Efforts need to be made to ensure that genome-editing techniques are explicitly and comprehensively regulated so as to minimize adverse effects on health, the environment and society. This means that national biosafety legislation should also be applied to these new techniques.

Further, current methodologies for risk assessment should be adapted to consider the potential new risks and new challenges posed by these techniques. One identified potential risk is the off-target activity of genome-editing techniques, which presents the challenge of reliable detection – more specifically, how to look for small nucleotide changes across the genome without prior knowledge of the type and range of such changes. It would be critical to assess whether and how these unintended changes cause environmental harm or hurt human health. Genome-editing's ability to edit small bits of DNA sequences may generate small changes in DNA sequences, and it also makes it more difficult for regulators and farmers to identify a modified organism once it has been released. Lack of detection of genome-edited crops would raise concerns over labelling and consumer rights, as well as risk monitoring issues. Further research is therefore needed to develop reliable identification and detection techniques for genome-edited organisms.

6 An Explanatory Guide to the Cartagena Protocol on Biosafety. 2003. IUCN Environmental Policy and Law Paper No. 46. International Union for the Conservation of Nature and Natural Resources: Gland, Switzerland and Cambridge, UK. https://bch.cbd.int/database/attachment/?id=10858

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