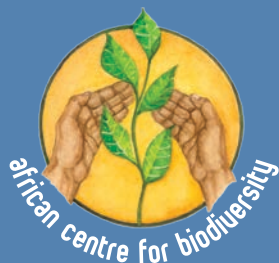


Biosafety Risks of Genome Editing Techniques in Plant Breeding

February 2017



african centre for biodiversity

www.acbio.org.za

Contents

Acronyms	3
Introduction	4
What is genome editing and what distinguishes it from classical mutagenesis and transgenic approaches?	4
CRISPR/Cas9 genome editing	5
CRISPR/Cas9 gene drives	6
Zinc-finger, transcription activator-like effector nucleases (TALENs) and meganucleases	6
Oligonucleotide-directed mutagenesis	7
Risks associated with gene editing techniques	7
Regulating gene-editing techniques as GMOs	8
Conclusions	9
References	10



On 7 April 2015 the African Centre for Biosafety officially changed its name to the African Centre for Biodiversity (ACB). This name change was agreed to by consultation within the ACB, to reflect the expanded scope of our work over the past few years. All ACB publications prior to this date will remain under our old name of African Centre for Biosafety and should continue to be referenced as such.

We remain committed to dismantling inequalities in the food and agriculture systems in Africa and to our belief in peoples' rights to healthy and culturally appropriate food, produced through ecologically sound and sustainable methods, and to define their own food and agriculture systems

©The African Centre for Biodiversity

www.acbio.org.za

PO Box 29170, Melville 2109, Johannesburg, South Africa. Tel: +27 (0)11 486 1156

Copy editor: Liz Sparg

Design and layout: Adam Rumball, Sharkbuoys Designs, Johannesburg

Acknowledgements

We are grateful to Dr Eva Sirinathsinghji for her efforts in researching and writing this paper and to Mariam Mayet of ACB for her contributions.

Acronyms

CBD	Convention of Biological Diversity
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
GM	Genetically modified
GMO	Genetically modified organism
HGT	Horizontal gene transfer
ODM	oligonucleotide-directed mutagenesis
RNA	Ribonucleic acid
RTDS	Rapid Trait Development System
TALENS	Transcription activator-like effector nuclease

Introduction

Recent debate surrounding genetically modified organisms (GMOs) has focused strongly on the development of new techniques for plant breeding. Technical advances for generating novel plant traits have now moved beyond the scope of current regulations for GMOs, raising concern that GMO producers may be able to push such products onto the market without regulatory testing and monitoring, or labelling (where labelling laws are in place). This would remove the requirement to assess any potential effects on food, feed or environmental safety, or the impacts on farmers and society, and would reduce consumer choice for those wishing to avoid such products.

There are various terms and techniques being employed, but, as highlighted by the New Techniques Working Group established by the EU Commission there are several that have obtained consensus in the discussions: genome editing techniques –CRISPR and gene drives; TALENS and oligonucleotide-directed mutagenesis, summarised in this report; as well as cisgenesis; grafting; agro-infiltration and RNA-mediated DNA methylation (covered in ACB's report entitled: 'Biosafety Risks of Novel Plant Breeding Techniques II').

What is genome editing and what distinguishes it from classical mutagenesis and transgenic approaches?

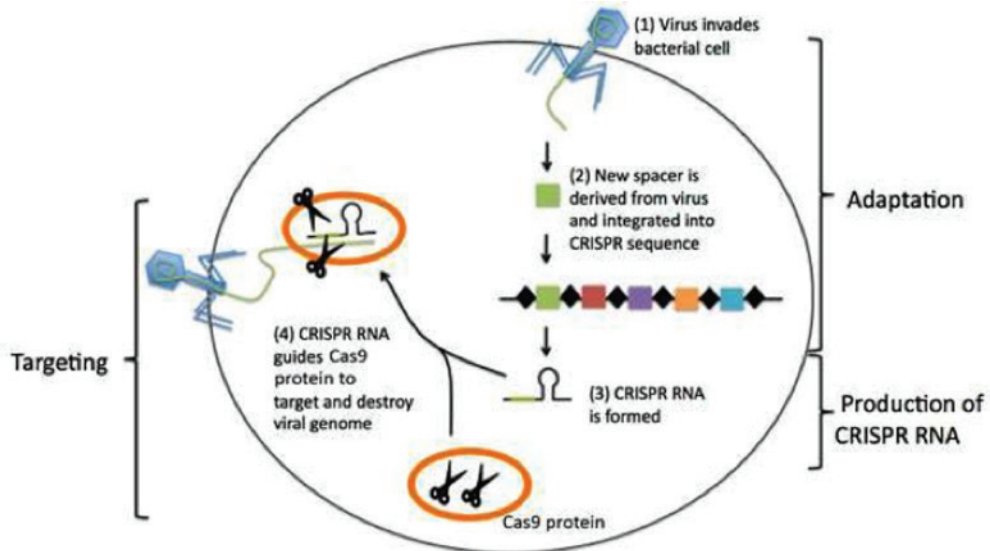
Gene editing refers to the modification of the genome at a specific, targeted location. Taking advantage of natural DNA repair mechanisms of cells and enzymes that act as molecular scissors to cut DNA the genome can be modified through adding, deleting, or altering parts of the DNA sequence. This can be distinguished from classical transgenic approaches, which involve the introduction

and permanent integration of foreign genetic material into unspecified locations within the plant genome. This distinction is crucial with regards to discussion surrounding the regulation of genome edited products.

GMO producers and others supporting these new technologies in food and agriculture argue that, without the permanent introduction of foreign DNA, and just the small alteration in DNA sequences, gene edited plants are more similar to conventional crops, or those produced by mutagenesis techniques such as irradiation, than they are to first generation GM crops. This argument ignores that fact that most genome editing techniques still employ classical genetic engineering tools, such as the use of recombinant DNA (a combination of DNA elements from multiple sources, generated in the laboratory), and also involve transformation of the plant cells (uptake of DNA by a cell). For a detailed summary on the risks associated with the genetic modification process itself, see *GMO Myths and Truths*, (Open Earth Source). When introducing genetic material into a cell, plant cells are grown in the laboratory, as cultured individual cells that will later be regenerated into a full GM plant. Transformation techniques are needed to force the genetic material through the cell membrane of the cultured cells and into the plant genome. The most common is *Agrobacterium*-mediated transformation, which takes advantage of a type of an infectious DNA element from the bacterial *Agrobacterium tumefaciens* species that, in the wild, releases DNA elements to infect plants, causing crown-gall disease. These transformation processes are already well recognised as being highly mutagenic in themselves. Further, there are novel risks introduced by these methods that are expanded upon below.

Classical mutagenesis on the other hand, involves the use of chemicals or ionizing radiation to generate random mutations, a few of which may offer crop advantages, depending on where and what type of mutation is generated, though the technique is hardly natural. There have been an estimated 3 000 varieties of crops generated with mutagenesis techniques, a number dwarfed by the millions generated through conventional

Figure 1. CRISPR mediated immunity – bacterial immunity against viruses (Ho, 2016)



CRISPR regions in the bacterial genome are composed of short DNA repeats (black diamonds) and spacers (colour boxes). When a new virus infects the bacterium, a new spacer derived from the virus is incorporated among the existing spacers. The CRISPR sequence is transcribed and processed to generate short CRISPR RNA molecules. The CRISPR RNA associates with and guides bacterial DNA cutting protein (Cas9 protein) to a matching target sequence in the invading virus. The Cas9 protein cuts up and destroys the invading viral genome (Ho, 2016).

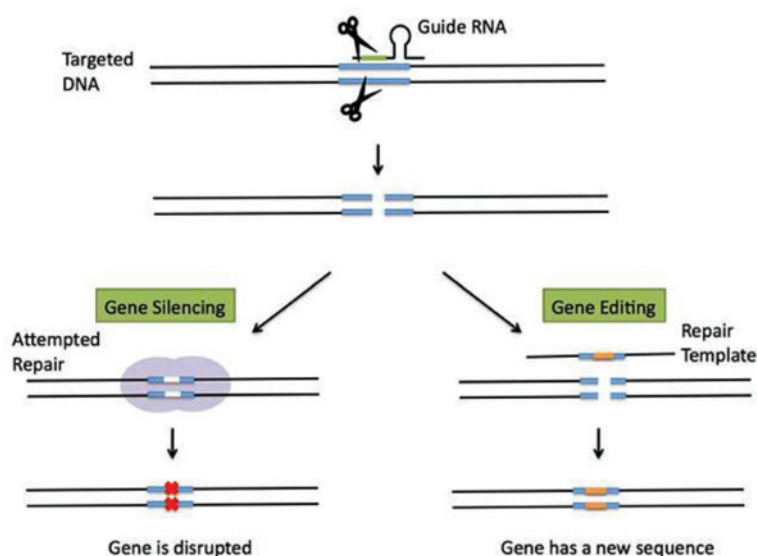
breeding techniques. Further, mutagenesis is unpredictable and it is thought that around 70 % of mutations lead to detrimental and not beneficial effects. It is also worth bearing in mind that the initial motivation for introducing mutagenesis into plant breeding was to find alternative uses to nuclear energy, after the end of World War II (as was the case with the synthetic nitrogen and phosphorous fertilizers that were initially used to make bombs).

CRISPR/Cas9 genome editing

CRISPR (clustered regularly interspaced short palindromic repeat) refers to short, partially repeated DNA found in the genome of bacteria and other microorganisms that protect the organism against viruses (see Figure 1). These repetitive sequences are taken from novel viruses that have infected the cell, acting as a ‘molecular library’ of pathogen sequences that can be employed to identify viruses. This is then copied into RNA molecules (guide RNAs), that are complementary to the virus, which then direct the Cas9 protein, the molecular ‘scissor’ (nuclease) to chop up the viral DNA and thus kill it.

For the purposes of genome editing techniques, theoretically the CRISPR/Cas9 system can be manipulated to target any sequence of interest (see Figure 2). To do that, geneticists first design and synthesise a short RNA molecule that matches not a virus, as would occur naturally, but instead a specific DNA sequence of interest. Then, as in the targeting step of the bacterial system, this guide RNA shuttles the Cas9 protein to the intended DNA target, and can silence a gene or change the sequence of a gene by adding a repair template with a specified change in sequence, so that it is incorporated into the DNA during the repair process. The targeted DNA is now altered to carry the new sequence (see Figure 2). It enables precise changes to be made in the genes of fruit flies, fish, mice, plants and human cells. This flexibility, as well as the efficiency and cost-effectiveness has made CRISPR the genome editing tool of choice for many researchers. CRISPR can also be used to target multiple genetic sequences at once.

Figure 2. CRISPR gene silencing or editing (Ho, 2016)



CRISPR/Cas9 gene drives

Named for the ability to ‘drive’ themselves through populations of organisms over many generations, these genetic elements can spread even if they reduce the fitness cost of the individual organisms. Promoting the inheritance of a particular gene, gene drives act to increase its prevalence in the population. This has now been used to engineer mosquitoes, spreading lethal genes in *Anopheles gambiae* mosquito populations that carry diseases, such as malaria. Gene drives were a largely theoretical idea until the last two years saw publications in yeast, fruit flies and mosquitoes.

Potential horizontal gene transfer (HGT) spread of such gene drive constructs to non-target organisms, through either interbreeding with related species or HGT to non-target species, including humans (Ho, 2016) has raised concern. There are enormous ecological risks to the ability to genetically alter an entire species: threatening biodiversity, likely having knock-on effects for example, on predators, or on plants being pollinated by the targeted organism. The endpoints of such a technique are hard to predict (Latham, 2017). What is the evolutionary trajectory of such a product?

As we have seen in the past with other strategies to kill mosquitoes, there is also potential for the empty niche left behind by

the targeted species to be filled with other disease-carrying mosquito species. Such effects cannot be accurately risk assessed prior to the release of the product. These risks have rightly prompted calls for a global moratorium on CRISPR-based gene drives. World governments at the meeting of the Conference of the Parties under the auspices of the United Nations’ Convention of Biological Diversity in Cancún, Mexico in December 2016, however, rejected proposals for a moratorium proposed by 160 organisations. There is still no international framework that specifically governs the technology’s use, even though its effects can spread across borders.

It is also worth noting that the very latest research on gene drives in mosquitoes has led to the detection of resistance development against it (Callaway, 2017). Clearly, these technologies are still in development, the ramifications of which cannot be entirely predicted.

Zinc-finger, transcription activator-like effector nucleases (TALENs) and meganucleases

These techniques are similar to that of CRISPR/Cas9, the difference being that they lack an RNA molecule as a guide. Instead, the enzyme itself has recognition domains to target particular DNA sequences that are fused to the nuclease, or ‘molecular scissor’. Once it

has found its target, it cuts the DNA, causing a double-strand break that is then repaired by the natural DNA repair mechanisms of the cell.

Since these techniques require engineering enzymes that recognise specific DNA sequences, as opposed to using a guide RNA as with CRISPR, it makes them harder to design and implement.

Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis (ODM), is a form of gene editing that involves the introduction of short DNA sequences (oligonucleotides), synthesised to be complementary to the target sequence to be modified, except for the intended changes that are to be introduced. The oligonucleotide acts as a template for the plant's natural defence mechanism, which detects the mismatch between the template and the plant genomic DNA, and copies the intended changes into the plant DNA. Theoretically, the oligonucleotide is not introduced into the plant DNA. This principle has been used for many years in the creation of targeted mutations in mouse models of disease, for example.

Cibus® have combined the techniques of CRISPR and ODM to increase the efficiency of editing to generate flax (*Linum usitatissimum*) that is tolerant to glyphosate.

Risks associated with gene editing techniques

Gene editing can be used to perform minor changes in DNA sequences, but it can also be used to add or delete whole genes or sequences. However, what is not possible is confining the changes to the intended one alone. During the process, intended products are separated from unintended products.

As seen with conventional transgenesis, the disruption of the genome via genetic engineering can have long-distance effects on the intricate balance of global expression of genes, leading to unexpected, abnormal levels

of constituents in the plant, including new RNA or protein molecules, allergens, nutrients and/or anti-nutrients (Mesnage et al., 2016). The cellular physiology, nutritional profile, crop performance and safety of the product could therefore be compromised. The size of the change does not alter the risk associated with it, since even single-base changes can have adverse effects, while some large alterations may not.

Two of the main causes of unintended products are:

1) off-target activity elsewhere in the genome; and 2) additional changes at the target site of alteration.

1) Off-target activity

This can occur when the gene editing complex, whether it is the guide RNA used in CRISPR, the oligonucleotides involved in ODM, or the recognition domains of the zinc-fingers, meganucleases and TALENS, bind to other regions of the genome that may be similar to the intended target. Off-target changes in DNA sequences elsewhere in the genome could alter gene expression patterns or generate novel RNAs or proteins, all of which could be potentially hazardous (see Agapito-Tenfen, 2016).

Off-target effects could also arise from the transformation process itself, as well as the random integration of nucleotide sequences when DNA is introduced, for example with *Agrobacterium*-mediated transformation or the oligonucleotides introduced with ODM (Antoniou, 2013). Gene editing techniques are not restricted to agricultural applications and are being widely researched for medical applications to correct disease causing mutations. A 2015 study detected off-target effects in edited human embryos with a technique that analyses DNA sequences for protein-coding genes (exome sequencing) on a global level i.e. an unbiased wide-reaching search across the genome (Liang et al., 2015). This is a type of global profiling technique, which is the sort of comprehensive analysis recommended by biosafety experts to be included in GMO risk assessment legislation.

2) Additional changes at the target site

Risks derive from uncertainties about which natural DNA repair mechanisms are employed by the plant. Such mechanisms are incompletely understood. Depending on the mechanism, the repair process can introduce changes at the junctions of the repair process. This depends on several factors we do not understand.

A study analysing four plant species with CRISPR modifications found that at the target sites alone, there were various different deletions and additions of DNA. With regards to ODM, studies have revealed non-target modifications, including the introduction of a single nucleotide change, and, as published by Pioneer Hi-Bred International, six out of 40 maize clones generated had alternative mutations to the single-base mutation they were attempting to generate (Zhu *et al.*, 1999). The above study by Liang *et al.*, 2015, also detected unexpected mutations due to inefficient, and variable repair mechanisms being employed by the human embryonic cells.

Other safety aspects, such as unintended consequences from on-target events and the stability of the modifications also still need to be addressed.

It is clear there are important gaps in scientific knowledge regarding gene editing techniques – both their desired and undesired effects. Most studies on these techniques have been performed on organisms other than plants, so our understanding of species-dependent outcomes is inadequate.

Regulating gene-editing techniques as GMOs

The most relevant regulations in question regarding GMOs are the Cartagena Protocol for Biosafety, a protocol of the Convention of Biological Diversity (CBD), and domestic regulations, such as the EU directive 2001/18/EC.

The argument that genome-edited products are indistinguishable from other products outside of GMO legislation, such as classic mutagenesis can be disputed on various grounds. The argument goes further that it is the products and not techniques that are regulated by the Cartagena Protocol on Biosafety.

However, in most cases, there is an introduction of foreign DNA material, thus rendering the crop a GM crop as defined by the EU directive (2001/18/EC Annex IA), which states that: ‘The following techniques are considered to result in genetic modification: ... techniques involving the direct introduction into an organism of heritable material prepared outside the organism, including micro-injection, macro-injection and micro-encapsulation.’

And even if there is no introduction of foreign DNA, EU biosafety legislation defines GMOs not only by the end product itself, but the process whereby the crops are generated. So, specific techniques are considered as resulting in GMOs, such as the use of DNA plasmid vectors, while mutagenesis – defined as excluding the use of recombinant DNA – is exempt (Directive 90/219/EEC Annex II Part A). Conversely, Canada has taken a “novel traits” approach and regulates new varieties based on the risks posed by its characteristics regardless of the breeding methods used. However, a number of new techniques, including ODM are not defined. Though ODM may or may not include the introduction of foreign DNA, it does involve the use of recombinant molecules, which therefore makes it GM and distinct from mutagenesis.

Genome edited crops in development

In September 2016, **DuPont Pioneer** and **CIMMYT**, a non-profit organisation based in Mexico, announced that they are to develop CRISPR products to address maize lethal necrosis disease that was first seen in Kenya in 2011.

Monsanto reached various licensing agreements with the **Broad Institute** to use CRISPR genome-editing technology in 2016 and early 2017, including a global non-exclusive agreement to use the CRISPR-Cpf1 system for agricultural applications (Seedworld, 2017). The Bayer-Monsanto merger will likely accelerate development of such products, increasing proprietary knowledge would provide them with significant cost savings (ACB, 2017).

The Roslin Institute, UK have developed pigs modified by CRISPR, to resist African swine fever (Reardon, 2016). Whether or not they are destined for the African market is not yet clear.

Dow AgroSciences LLC have used zinc-fingers to engineer glufosinate resistance into Maize (Shukla et al., 2009).

Cibus[®] were awarded their first EU patents for the ODM that they trademarked as the Rapid Trait Development System (RTDS) crop in 2010, which was recently upheld in 2012. They have filed additional patents on RTDS-generated glyphosate-tolerant crops (corn, wheat, rice, barley, soybean, cotton, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tobacco, tomato, alfalfa, poplar, pine, eucalyptus, apple, lettuce, pea, lentil, grape, turf grass and *Brassica sp.*), and sulfonylurea herbicide-tolerant canola/rapeseed (Patents: EP 2203565, WO 2009046334, EP 2600710 A2, EP 1223799 B1).

There have been suggestions to utilise gene drives to re-confer sensitivity of weeds to herbicides in areas where they have gained resistance, threatening the efficacy of herbicide-tolerant GM crops (Esvelt *et al.*, 2014). Such uses are included in gene-drive patents submitted by the Broad Institute (WO 2015105928 A1). This is a clear strategy to prolong marketability of such crops and their associated pesticides.

No definitive definition has yet been made by parties to the CBD or Cartagena Protocol or member states of the EU.

Strict bans on the use of these new technologies should be implemented until safety can be conclusively proven. In addition, efforts must be made to develop international comprehensive rules to regulate and assess the risk of any genome-edited products in the future. 'Novel biotechnical' techniques are covered by the Cartagena Protocol, which acknowledges the need to cover future techniques. As such, methods of risk assessment must keep up with novel associated risks, including the assessment of

off-target effects of the latest technologies with global profiling of genome sequencing, gene expression analyses and RNA profiling to look for alterations in RNA molecules, as well as metabolomic analyses that can be compared to conventional parental lines. Predictive bioinformatics analyses of potential off-target activity is not reliable enough to predict all unanticipated effects.

Well-established methods for detection of such products must also be in place, since many products will lack the distinguishing traits that make conventional GMOs easier to detect, i.e. the presence of transgenic DNA.

Conclusions

Because crops awaiting commercial approval are not publicised, it is difficult to keep track of which crops are reaching the market, though some examples are summarised in Box 1. Of the few that have been publicised so far, it appears that, despite the employment of the latest techniques in molecular biology, the outcome appears to be the same – more herbicide tolerant traits.

This is testament to the fact that altering complex traits required for agriculture is far more complicated than modifying single genes, which is why, after decades of genetic engineering we are still only growing two

main types of GM traits – herbicide tolerance and Bt insecticides. The idea of addressing complex traits, such as abiotic stress (drought, salinity, yield, disease resistance) through such reductionist thinking has thus far failed to come to fruition.

Claims that these technologies are safer than classical transgenesis techniques are unproven and are continuing to promote a chemical industrial agricultural model. It is critical that such thorough risk assessment protocols and regulations are in place for such products before they are released onto the markets.

For further in depth reading, please refer to Agapito-Tenfen (2016).

References

- ACB (African Centre for Biodiversity). 2017. The MONSANTO-BAYER merger: Implications for South Africa's agricultural future and its smallholder farmers. ACB, Johannesburg.
- Agapito-Tenfen SZ. 2016. Biosafety aspects of genome-editing techniques. Biosafety Briefing. Third World Network, African Centre for Biodiversity. https://www.biosafety-info.net/file_dir/110834677458380735d4d3e.pdf.
- Antoniou M. 2012. Sources and mechanisms of health risks from genetically modified crops and foods. Biosafety Briefing, TWN, ENSSER, Tara Foundation. <http://www.ensser.org/fileadmin/files/2.1-Antoniou-paper.pdf>
- Callaway E. 2017. Gene drives thwarted by emergence of resistant organisms. *Nature News* (542): 7639. <http://www.nature.com/news/gene-drives-thwarted-by-emergence-of-resistant-organisms-1.21397>.
- Esvelt KM, Smidler AL, Catteruccia F, Church GM. 2014. Concerning RNA-guided gene drives for the alteration of wild populations. *Elife*. 2014, 3. pii: e03401. doi: 10.7554/eLife.03401.
- Fagan J, Antoniou M, Robinson C. 2014. GMO Myths, and Truths, second edition. Open Earth Source
- Ho MW, 2016. CRISPR too fast for comfort. *Science in Society* 69. http://www.i-sis.org.uk/CRISPR_too_fast_for_comfort.php
- Latham J. 2017. Gene drives: A scientific case for a complete and perpetual ban. *GeneWatch* 2017(30): 8.
- Liang P, Xu Y, Zhang X, Ding C, Huang R, Zhang Z, Lv J, Xie X, Chen Y, Li Y, Sun Y, Bai Y, Songyang Z, Ma W, Zhou C, Huang J. 2015. CRISPR/Cas9-mediated gene editing in human triploid zygotes. *Protein Cell*. 6, 363-72
- Mesnager R, Agapito-Tenfen SZ, Vilperte V, Renney G, Ward M, Séralini G-E, Nodari RO & Antoniou MN. 2016 An integrated multi-omics analysis of the NK603 Roundup-tolerant GM maize reveals metabolism disturbances caused by the transformation process. *Scientific Reports* 6, Article number: 37855 (2016) doi:10.1038/srep37855.
- Reardon S. 2016. Welcome to CRISPR zoo. *Nature* (531): 160–163.
- Seedworld. 2017a. Monsanto reaches new licensing agreement on crispr technology. [Online] Available: <http://seedworld.com/monsanto-reaches-new-licensing-agreement-on-crispr-technology/>. Accessed: 15th February 2017.
- Shukla VK et al., 2009. Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. *Nature* 2009(459): 437–41
- Zhu T, Peterson DJ, Tagliani L, St Clair G, Baszczyński CL, Bowen B. 1999. (Targeted manipulation of maize genes in vivo using chimeric RNA/DNA oligonucleotides. *Proc Natl Acad Sci USA* 96(8768):73.



PO Box 29170, Melville 2109, South Africa
www.acbio.org.za