Trans Atlantic Consumer Dialogue

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Resolution on consumer concerns about new genetic engineering techniques

Introduction

This resolution builds on TACD's February 2000 Resolution: Consumer Concerns about Biotechnology and Genetically Modified Organisms (GMOs), by considering the implications of new genetic engineering techniques for TACD's existing recommendations in this area.

TACD considers that new genetic engineering techniques will create genetically modified organisms (GMOs) that require risk assessments and labelling, consistent with the aforementioned TACD February 2000 Resolution, and more recent resolutions regarding international trade of products of modern biotechnology[1]. Risks to human health, animal welfare and the environment must be assessed before products derived from these new techniques are placed on the market or released into the environment. Products must also be labelled in accordance with consumers' rights to know and choose what they are buying, including what they eat.

Recommendations

TACD urges the EU and US governments to:

- Regulate products of new genetic engineering techniques as genetically modified organisms (GMOs);
- Strengthen regulatory systems to include mandatory pre-market human health evaluation that will screen all foods produced using new genetic engineering techniques for potential hazards;
- Develop strong systems of pre-market environmental safety evaluation and post-market monitoring;
- Fully consider the welfare of animals altered using new genetic engineering techniques prior to approval;
- Adopt mandatory labelling rules for all food produced using new genetic engineering techniques;
- Adopt and enforce strict rules for corporate liability and mandatory insurance for companies that want to release organisms altered using new genetic engineering techniques into the environment;
- Establish and maintain systems to ensure that identity-preserved supplies of non-genetically-engineered ingredients remain available.

[1] Resolution on the proposed chapter on Sanitary and Phytosanitary Measures in the Transatlantic Trade and Investment Partnership (TTIP) Agreement. TACD Doc. No. Food 37/16. 21 January 2016. http://tacd.org/wp-content/uploads/2015/01/TACD-Resolution-TTIP-SPS_-GREEN_rev0216.pdf ; Resolution on the proposed chapter on Sanitary and Phytosanitary Measures in the Transatlantic Trade and Investment Partnership (TTIP) Agreement (Update).TACD Doc. No. Food 38/16. 5 July 2016. http://tacd.org/wp-content/uploads/2015/01/TACD-Resolution-TTIP-SPS_-UPDATE_July2016.pdf

Background

New genetic engineering techniques

Currently, genetically modified organisms (GMOs) are mainly plants grown as commodity crops, for use in human food, animal feed, clothing (cotton) and biofuels, although a genetically engineered salmon has been approved in both the US and Canada.

Genetic engineering involves altering the genetic material of organisms using artificial laboratory techniques. Genes, which are made up of the chemical known as DNA, act as instructions to make molecules called proteins or to produce non-coding RNAs. Changing the genes of a plant or animal can change its properties or traits e.g. how it responds to disease, pesticide products or lack of water.

There are currently two main methods that are used to genetically modify plants that are available commercially: altered DNA is inserted using a bacterium that has the ability to infect plants and insert DNA into a plant's genome; or minute gold, tungsten, or silver particles are coated with the new DNA and fired into plants' cells. More recently, many new methods have been developed to change the DNA of plants and animals, so they have new traits (see Appendix). Collectively many of these new genetic engineering techniques are referred to as "gene editing," Gene editing will not be used only for crops but also for trees, farm animals, fish and insects with a wide range of new properties.

New genetic engineering techniques still produce GMOs

Although the new laboratory methods used are different, all new genetic engineering techniques still produce genetically modified organisms (GMOs). These organisms have genetic material that has been altered in a laboratory to give them new properties or traits. The definition of "modern biotechnology" by the Codex Alimentarius Commission is instructive for understanding how to regulate the GMOs produced by the new techniques.

The Codex Alimentarius Commission is the food standards organisation of the United Nations, jointly run by the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO) of the United Nations. Its main goal is to protect the health of consumers and promote fair practices in international food trade. Codex Alimentarius standards, guidelines and codes of practice are recognised by the World Trade Organisation (WTO) as presumptively authoritative standards and guidelines for the resolution of disputes concerning food safety and consumer protection. Codex principles state that a pre-market risk assessment should be undertaken for all food derived from modern biotechnology.[2] The Codex Principles use the following definition of modern biotechnology:

"Modern Biotechnology" means the application of:

i) In vitro nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or

ii) Fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombinant barriers and that are not techniques used in traditional breeding and selection.

The term "*in vitro* nucleic acid techniques" means any technique that alters the genetic material of an organism (which consists of nucleic acids, e.g. DNA and RNA) in the laboratory. *In vitro* (meaning "in glass" in Latin) refers to the technique of performing a given procedure in a controlled environment

[2] Codex Alimentarius (2011) Principles for the Risk Analysis of Foods Derived from Modern Biotechnology.

outside of a living organism. The new genetic engineering techniques, e.g. RNAi or gene/genome editing technologies, invariably involve "in vitro nucleic acid techniques," and so must be considered to be products of "modern biotechnology." Given the Codex standards and guidelines are referenced by WTO, this means that countries could require safety assessments and labelling of food derived from these new genetic engineering techniques and such assessments would not automatically be considered as non-tariff trade barriers, e.g., such safety assessments would be considered trade legal.

There are currently 188 Codex Members, including the United States and the European Union (EU), which have agreed these standards.

EU law and GMOs

In the EU, GMOs produced using new genetic engineering techniques fall within the definition of GMOs used in current legislation and therefore should continue to be regulated under these laws. The basic laws governing GMOs in the EU are Directive 2001/18, Regulation 1829/2003 and Regulation 1830/2003. Whether an organism is regulated as a GMO or not is determined by Directive 2001/18, which defines a "*genetically modified organism*" on the basis of the process by which it has been created. According to the law, it is 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*" (Article 2.2).

This definition is legally and scientifically sound because it is the process of genetic engineering that invariably leads to both intended and unintended outcomes, including also unpredictable changes to the DNA and its functioning, which may compromise the final product's health and environmental safety.

The EU Directive lists a number of processes as resulting in GMOs that fall under the EU definition. However, this list is explicitly open-ended ('inter alia') so that the Directive can be applied to technical developments in genetic engineering. One example of a GM process is the insertion of genetic material (e.g. stretches of nucleic acid such as RNA or DNA) prepared outside the organism ('in vitro') into a host organism, which causes an alteration of the organism's own genetic make-up (Annex IA, Part 1).

Importantly, it is only the characteristics of the process, not the characteristics of the resulting organism, which determine whether or not an organism is a GMO. In terms of determining whether an altered organism is a GMO, it is irrelevant whether the intended genetic alteration could, in theory, also arise from mutations that are induced by chemicals or radiation, or that occur spontaneously. It is also irrelevant whether the inserted genetic material originates from a crossable species, or whether such genetic material is present in the final product.

The Directive mentions two processes of genetic modification whose products are exempt from the scope of the law. These are mutagenesis and cell fusion between crossable organisms. However, these processes are only exempt "*on the condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms*" (Annex 1B). This means that organisms whose genetic material has been altered using RNA or DNA sequences prepared outside the cell, or using GMOs, cannot be exempt from the law.

The exemptions are presented as a closed list. They encompass "*certain techniques of genetic modification which have conventionally been used in a number of applications and have a long safety record*" (Recital 17). None of the new techniques can claim to have such a "*long safety record".* The list is part of the Directive, which specifies that the precautionary principle must be taken into account when interpreting it.

The EU Directive also applies to organisms that are derived from GMOs. This includes organisms produced through grafting on a GM rootstock, reverse breeding and some types of RNA directed DNA Methylation (RdDM). The functioning of these organisms could be impeded by compounds and metabolites of the GMO, giving rise to safety implications.

GMOs produced with new techniques continue to require risk assessments

New genetic engineering techniques will produce GMOs with properties which may pose risks to human health and the environment.

For example:

- New GM crops produced using new genetic engineering techniques might produce toxins, allergens or altered nutrients that could harm human health;
- Meat, milk or eggs from GM farm animals produced using new genetic engineering techniques, might also produce toxins, allergens or altered nutrients that could harm health;
- New GM plants intended to produce industrial chemicals or pharmaceuticals might inadvertently end up in the food chain.

Genetically modifying farm animals also raises concerns about animal welfare, including:

- A high failure rate for genetically engineered mammals often leads to many aborted, dead or deformed embryos;
- Genetically engineered animals may suffer as a result of the new genetically engineered trait e.g. if they are too heavy to walk easily, or over-produce milk;
- Herds or flocks of genetically engineered animals or birds may be more vulnerable to some diseases if they are all genetically similar;
- Genetic engineering technologies could be even more harmful as they may strive to push animals even further beyond their physiological limits.

These concerns also apply to GM animals produced using new genetic engineering techniques. It is highly troubling that such technologies will mainly be used to further intensify the livestock sector and will entrench the use of intensive animal farming systems with inherently poor welfare. New genetic engineering techniques have the potential to further damage the health and welfare of farm animals.

The long-term consequences of releasing GMOs into the environment are difficult to predict and the precautionary principle should be applied. For example:

- Genetically modified fish or insects may disperse long distances, including through human transport of their eggs. Ecosystems may be altered by releases of GM fish and insects in ways that are not easy to predict.
- Genetically modified trees have long life cycles and their pollen, seeds and vegetative material spreads over long distances;
- Genetically modified plants can spread through pollen or seeds and spread into new areas or cross with wild relatives.

In the EU, the open release of GMOs into the environment is regulated and all new products require risk assessments which consider the risks to the environment, animal welfare and human health.[3] Similar risk assessments should be required for GMOs produced using new genetic engineering techniques, under the same legislation.

[3] EU GMO legislation. http://ec.europa.eu/food/plant/gmo/legislation/index_en.htm

The World Trade Organisation's Agreement on Sanitary and Phytosanitary measures (SPS Agreement) defines international standards, guidelines and recommendations for food safety as the standards, guidelines and recommendations established by the Codex Alimentarius Commission.[4] This means that Codex has far reaching implications for resolving trade disputes. Although countries are not obliged to cite such standards, if a country's regulations—such as those requiring safety assessments and labelling of food derived from these new genetic engineering techniques—are consistent with Codex, then such assessments would not automatically be considered as non-tariff trade barrier, provided other requirements were met, such as making assessments in a timely way[5].

Thus, a country that requires safety assessments of food produced using these new genetic engineering techniques could ban the import of such foods if they haven't already been explicitly approved in the country of import. Thus, since there are no specific laws requiring GMOs to go through a food safety assessment in the United States and GMO plants in the US only go through a "Generally Recognised as Safe" process run by the companies themselves, food products produced using modern biotechnology, including gene edited products, may be banned from import into countries that do require such food safety assessments.

GMOs produced with new techniques must be labelled for consumers

Consumers have a fundamental right to know and choose what they are buying, including what they eat. Many consumers have concerns about the safety and nutritional content of GMOs, the welfare of GM farm animals, or about the environmental impacts of releasing GMOs into the environment.

Currently, there is a legal requirement for foods containing GMOs to be labelled in the EU.[6] Foods containing GMOs produced using new genetic engineering techniques should also be labelled, under the same legislation that is used for existing GMOs. In the United States, state GMO labelling legislation has been passed in Vermont, Connecticut, Alaska and Maine and federal legislation has since been passed which will pre-empt such state laws and, although controversial, may require some form of GM labelling for some of the products of GMOs.

The choice of whether to eat or not to eat GM foods should remain with consumers, but this right to choose can be undermined by the mixing of GM products with conventional non-GM products, as a result of commercial production or field trials. Therefore, it is fundamental to ensure traceability through the segregation of GM products – including those produced using new genetic engineering techniques - from traditional products. If the manufacturer does not have full knowledge of the genetic status of the ingredients, the consumer's right to be informed and right to choose will not be guaranteed.[7]

The right to choose also requires non-GM supplies of crops and animals to be maintained and GM products to be labelled as such.

[4] The WTO Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement). https://www.wto.org/english/tratop_e/sps_e/spsagr_e.htm
[5] US vs. EU Biotech Products Case: WTO Dispute Backgrounder. IATP. 14 September 2005. http://www.iatp.org/documents/us-vs-eu-biotech-products-case-wto-dispute-backgrounder
[6] Regulation (EC) 1830/2003. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=URISERV:l21170
[7] See the "Right to Choose" section of TACD Resolution on Consumer Concerns about Biotechnology and Genetically Modified Organisms (GMOs) of February 2000

Appendix : New genetic engineering techniques[1,2,3,4,5,6,7,8,9,10,11,12,13,14]

This Appendix provides background information on new genetic engineering techniques, including the gene editing techniques:

1. Clustered regularly interspaced short palindromic repeats system (CRISPR/Cas);
2. Zinc Finger Nucleases (ZFN) types -1, -2and -3;
3. Transcription activator-like effector nucleases (TALENs);
4. Meganucleases (MN); and
5. Oligonucleotide Directed Mutagenesis (ODM);

And other new genetic engineering techniques:

6. Cisgenesis and intragenesis;
7. RNA-dependent DNA methylation (RdDM);
8. Grafting: of non-GMO graft (scion) on GMO rootstock (and vice versa);
9. Reverse breeding (RB)
10. Agro-infiltration: Agro-infiltration 'sensu stricto' & Agro-infection.

Gene editing using site directed nucleases (SDNs)

Gene editing (or genome editing) using site directed nucleases (SDNs) is a type of genetic engineering in which DNA in the genome of an organism is inserted, deleted or replaced using engineered enzymes called nucleases, or "molecular scissors", which can cut DNA, creating double strand breaks (DSBs). Targeted DSBs are achieved using SDNs (also known as sequence-specific nucleases, SSNs) - enzymes that recognize and cleave the target locus (position on the genome) with high specificity. Gene editing then uses the cell's own DNA repair pathways to create a variety of targeted DNA sequence modifications, ranging from DNA deletions to the insertion of large arrays of transgenes (genetic material that has been transferred from one organism to another).

After breaks are introduced into the chromosome using the molecular scissors, two possible self-repair mechanisms are used by cells repair them. One is non-homologous end-joining (NHEJ), the other is homologous recombination (HR):

- Using NEHJ, broken chromosome ends can be joined together or also be joined to other DNA molecules that are introduced into the cell simultaneously with the SDN/SSN. The capture of heterologous DNA sequences (i.e. DNA sequences from a different organism) can be used to achieve a targeted gene knock-in (targeted insertion). If two breaks are introduced into the chromosome simultaneously, targeted gene deletions or other rearrangements can result.

- In HR, a repair template is used as a source of DNA sequence information that is copied to the broken chromosome to restore its integrity. HR can be harnessed to achieve targeted DNA sequence modifications by introducing into the cell both an SDN/SSN and a DNA repair template with sequence similarity to the break site (this process is referred to as gene targeting). Sequence variation that is carried by the repair template is copied by HR into the chromosome, thereby achieving targeted DNA sequence modification. The user specifies the type of sequence variation in the repair templates, allowing many different possibilities for changes to the genome. HR has the potential to insert multiple (stacked) transgenes at the same site and to create traits such as herbicide tolerance in plants.

Most published examples of gene editing currently use NEHJ repair, as it is easier to implement. NHEJ is prone to errors and can cause unintended effects as small deletions or (more rarely) insertions can be introduced at the junction of the newly rejoined chromosome. If the sequence modification causes a frameshift mutation or alters key amino acid residues in the target gene product, a knockout (loss of function) mutation can be created.

There are three categories of gene editing techniques using SDNs/SSNs : SDN-1, SDN-2 and SDN-3:

- SDN-1: small site-directed random DNA changes, which may be small deletions, substitutions or insertions of nucleotides. In this case the cell will 'repair' the break in a random fashion, using the NHEJ repair mechanism.
- SDN-2: small site-directed intended DNA changes, such as 'point mutations' (one nucleotide change). Here the repair mechanism is HR, following instructions provided by a DNA 'template' that has been added (a stretch of DNA that has the same sequence as the target area but with one or two small additional alterations or a short insertion).
- SDN-3: large site-directed insertions of genes or regulatory sequences. In the genetic engineering process a DNA template will be added as in SDN-2, but the template will also contain an additional long DNA sequence (e.g. one or more genes) for integration.

Gene editing methods use four major classes of SSNs/SDNs: Clustered regularly interspaced short palindromic repeats system (CRISPR/Cas); Zinc Finger Nucleases (ZFN) types -1, -2and -3; Transcription activator-like effector nucleases (TALENs); and Meganucleases (MN). Each of these methods may also have unintended ("off target") effects and may be used to create a wide variety of traits in plants and animals that may have implications for human health and the environment.

New unintended effects that derive from the use of such nucleases are mainly related to uncertainties regarding target specificity (i.e. whether the nuclease cuts only the intended target site or also other sites in the DNA) and double-stranded break repair (i.e. whether the repair mechanism works as intended or introduces errors). In addition, because gene editing allows the introduction of new traits into plants or animals, the effects of these traits and their impact on health and the environment need to be considered. When gene editing is used in animals, animal welfare concerns may also arise, particularly as gene edited animals require cloning, which has a high failure rate in mammals.

1. Clustered regularly interspaced short palindromic repeats system (CRISPR/Cas)

The CRISPR/Cas system is part of the immune system of some single-celled organisms, which confers resistance to foreign genetic elements by cutting them out of the organism's DNA. CRISPR/Cas9-based technologies utilize two components, CRISPR-associated protein 9 (Cas9) and a single guide RNA (sgRNA), to perform genome-editing. The sgRNA typically is designed to contain a 20-nucleotide DNA sequence complementary to a target sequence directly in front of a protospacer adjacent motif (PAM). The Cas9 can then interact with the sgRNA and target DNA creating a double strand break in the target DNA 3 to4 base pairs (chemical letters) upstream of the PAM site. The PAM is an essential targeting component (not found in bacterial genome) which distinguishes bacterial self from non-self DNA, thereby preventing the CRISPR locus from being targeted and destroyed by nuclease. The process is shown schematically in Figure 1.

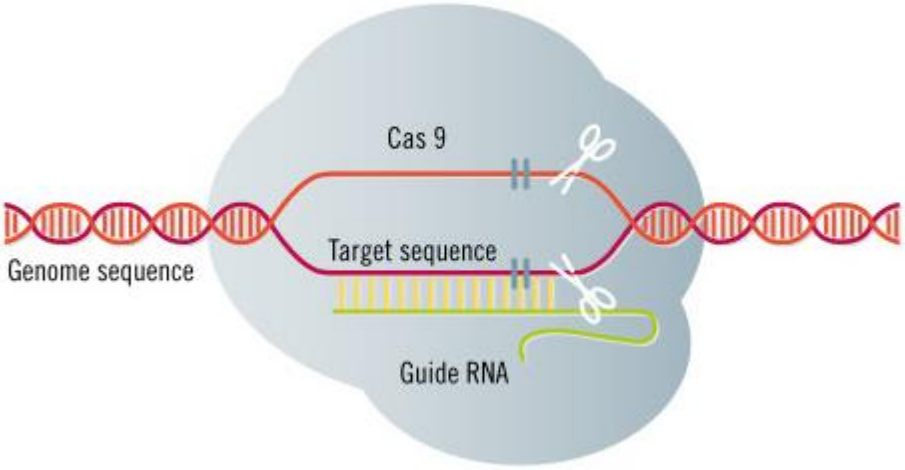

Figure 1: The CRISPR/cas9 genome editing process. Source: TestBiotech

CRISPRs have been now been used experimentally in a wide variety of species, including a variety of plant species, zebrafish, mice, chickens and mosquitoes. CRISPR/Cas9 is becoming the gene editing method of choice due to its versatility and ease of use. Designing and producing the synthetic sgRNA long is much simpler than the production of custom ZFNs and TALENs enzymes described below. By delivering the Cas9 nuclease and appropriate guide RNAs into a cell, the cell's genome can be cut at a desired location, allowing existing DNA to be removed and/or new DNA to be added.

However, RNA-guided endonucleases have demonstrated off-target effects, which can cause collateral damage in the genome. Further, CRISPR/Cas may be used to develop a wide variety of genetically engineered traits in many different organisms, some of which might have adverse effects on human health and the environment. Proposals to use CRISPR/Cas to create a “gene drive” mechanism , potentially allowing a genetically engineered trait to spread throughout the entire population of a species (e.g. plants or insects), have led to debate about the need for strict regulation, to avoid potential adverse effects to entire ecosystems. To date, attempts to use CRISPR in mammals such as pigs, goats and cattle have been less successful than TALENs (described below), resulting in lower recovery of genetically modified cloned animals.

2. Zinc Finger Nucleases (ZFN) types -1, -2 and -3

ZFNs are proteins that are custom-designed and utilised to cut DNA at a specific location. Zinc fingers are small protein domains that bind to several compounds, ranging from nucleic acids to proteins and other small molecules. The “zinc finger” (ZF) component can recognise a specific short stretch of DNA (9 to 12 bases) and the nuclease (N) component will cut the DNA at that site. It requires two ZFNs – each to dock diagonally across the double stranded DNA – to cut through both strands. This DNA cut will then trigger one of the cell’s two DNA repair mechanisms to stick the loose ends together again, with a number of possible outcomes. The three categories of ZFN-1, -2 and -3 refer to the three types of SDNs described above.

When ZFNs are used in plants, the gene for the specifically designed ZFNs will commonly be introduced into the plant through genetic engineering with standard GM transformation, making it a GMO at this stage. Once the ZFN proteins have been expressed and done their work, plant lines will be selected that do not carry the transgene. Alternatively, plant virus expression systems have been developed where the ZFN gene is intended to stay within the viral expression system and not be integrated into the plant’s own DNA. The loss, change or insertion of a single nucleotide (point mutation) can be

sufficient to change traits in a plant, such as: herbicide tolerance, male or female sterility, flower colour, delayed fruit ripening.

ZFNs have also been used in animals, including pigs.

Unintended changes and risks:

- Off-target effects: ZFN technology is known for its non-specific binding to non-target DNA and hence to result in a significant level of off-target mutations in the genome. These mutations can a) if in the coding sequence, result in changes of function of proteins, or b) if in regulatory sequences, result in changes of the expression of genes, such as increased presence of plant toxins, or absence of proteins important for nutrition, plant defence or disease resistance.
- Template DNA (ZFN-2 and 3) may integrate randomly into the genome, similar to transgenic insertions, either as a whole or in parts, disrupting genes and regulatory sequences or potentially resulting in altered proteins. This may lead to a decrease in performance, heightened disease susceptibility, accumulation of toxins and residues, increase in allergens.
- Tissue culture and also transformation and transfection processes are used in the production of ZFN genetically modified plants. Such processes are known to lead to additional mutations.

3. Transcription activator-like effector nucleases (TALENs)

Transcription activator-like effector nucleases (TALENs) are restriction enzymes that can be engineered to cut specific sequences of DNA. TALENs are similar to ZFNs in that they have a DNA-binding domain derived from TALE proteins fused to a *Fok1* cleavage domain, to cut the DNA. TALE proteins are transcription factors from the plant bacterial pathogen *Xanthomonas*. In a similar process to the use of ZFNs, TALENs can be used to edit genomes by inducing double-strand breaks in the DNA, which cells respond to with repair mechanisms. The off-target activity of an active nuclease may lead to unwanted double-strand breaks and may consequently lead to unintended chromosomal rearrangements. TALENS have been used experimentally to produce genetically modified pigs, goats and cattle, as well as plants. Gene-edited animals require cloning which has been criticised for its high failure rate in mammals, resulting in spontaneous abortions, deformed foetuses and early death of cloned offspring.

4. Meganucleases (MN)

Meganucleases are naturally occurring restriction enzymes that can be used to modify the genome of any species. As with other gene editing techniques, off-target effects occur as a nuclease will still have some likelihood of acting even if the sequence does not match perfectly. As the number of naturally occurring meganucleases is limited, attempts have been made to modifying the specificity of existing meganucleases by introducing a small number of variations to the amino acid sequence, or by associating or fusing protein domains from different enzymes. Research has also been undertaken into combining their use with TALENs.

Other new genetic engineering techniques are described below. ODM is also a gene editing technique, although it does not use the “molecular scissors” (SSNs/SDNs) described above. The other techniques listed are not regarded as gene editing techniques.

5. Oligonucleotide Directed Mutagenesis (ODM)

In ODM an oligonucleotide, which is a short stretch of single-stranded genetic material, is synthetically produced. It is designed to be almost identical to the DNA sequence of the target gene, except for 1 to 4 nucleotides. This will create a sequence mismatch when the oligonucleotide binds to the target

gene, inducing a site-specific DNA change (mutation) once the cell's own DNA repair mechanism is triggered, so that it copies the mismatched sequence rather than its own original sequence. The aim is to create small and predesigned changes within very specific sites in genes, to either change the function of the gene product or to stop its production.

ODM is a genetic engineering technology that can give rise to the same or similar direct and indirect impacts as current GMOs, both due to the intended traits (e.g. herbicide tolerance) and the processes and methods used.

Unintended changes and risks:

- Off-target effects: The oligonucleotide can bind to other DNA sites to which it is sufficiently similar and where it will cause unintended mutations. These in turn can result in changes or loss of function of proteins, or changes in the expression of genes, leading to problems such as increased presence of plant toxins.
- The oligonucleotide can also integrate into the plant DNA, in a manner similar to transgenic insertions, disrupting genes and regulatory sequences or potentially resulting in altered proteins.
- The utilisation of tissue culture and GM type transformation or transfection methods are known to lead to genome-wide unintended mutations.
- Near target site mutations have been observed in ODM derived GM organisms.
- Depending on the oligonucleotides used, there is a risk that the oligonucleotides may interfere with a cell's regulation of gene expression, by triggering the RNAi pathway , which can lead to gene silencing that lasts for many generations. This may be more the case for oligonucleotides that contain RNA nucleotides.

6. Cisgenesis and intragenesis

Cisgenesis and Intragenesis are basically the same as transgenesis, but instead of sourcing the DNA from totally different species or inventing a new synthetic DNA sequence, the DNA inserted will be sourced from the same or closely related species, those with which the plant would, in theory at least, be able to interbreed/cross. In 'Cisgenesis' an exact copy of an entire gene sequence as found in the donor organism is used. In 'Intragenesis' the inserted gene sequence is a composite, made up of sequences and elements from different genes of one or more closely related species.

Unintended changes and risks:

Whether or not the gene sequences come from closely related species, the process of genetic engineering is still the same, involving the same risks and unpredictability that occurs with transgenesis. There will be:

- random integration of the transferred gene, capable of disrupting another gene or interfering with the regulation of neighbouring genes (positional effects).
- insertion-site mutations and genome-wide mutations resulting from the transformation processes, including the effects of tissue culture. These can include deletions, rearrangements and multiplications of DNA sequences.
- potential for gene silencing of the introduced gene or the plant's own genes if promoter sequences share high similarity (homology).
- re cisgenesis: The fact that the inserted gene comes from a related species is no guarantee that there are no unintended or unpredictable effects, as neither this particular gene nor its product would have been present before in this genetic context or position. Hence it may express in a different way from the way it did in the plant from which it is taken and/or interact

(e.g. interfere) with wider gene regulation or metabolic pathways. This can give rise to altered behaviour and performance, higher susceptibility to disease, increased fitness and /or invasiveness, altered composition of signalling molecules, nutrients, toxins and allergens.

- re intragenesis: the genetic sequences assembled in such a gene will never have existed in this composition and in this regulatory context before. Their behaviour and interactions cannot be predicted simply by knowing the DNA sequence or by knowing that these sequences are derived from related organisms.

7. RNA-directed DNA methylation (RdDM)

RNA-directed DNA methylation (RdDM) is a process where RNA molecules direct the cell to add methyl groups (-CH3 groups) to certain nucleotides along a specific stretch of DNA in order to silence a gene. The methylation of the promoter region of a gene will stop the expression of that gene. Whilst such gene silencing is not a permanent alteration, it will be inherited for many generations. It eventually fades, though the triggers for the reversal of the methylation are not known or understood. Any small double-stranded RNA that has a matching sequence to that of a stretch of the DNA will initiate the methylation of these DNA sequences, and thus silence the associated gene. There are a number of ways to get specific sequences of double-stranded RNA into a cell, for example:

- genetically engineering/modifying the plant with a gene that will produce such an RNA (with an 'inverted' (reversed) sequence). To have transient gene silencing, i.e. for a few generations only, the inserted gene can be removed (de-selected) by back-crossing in the breeding process.
- infection of plants with genetically engineered plant viruses (containing the targeted promoter sequence), which will result in the silencing of the targeted gene through methylation. ('Virus Induced Gene Silencing' (VIGS) – RdDM)
- spraying of a plant with dsRNA (double stranded RNA).

One aim of RdDM is to obtain a new trait for a number of generations of seed, and to do so without changing the actual DNA sequence, i.e. the sequence of nucleotides, within the organism. Instead a process of RdDM can be utilised within the cell to silence a specific gene, so there will be no gene product from that gene. This in turn can give rise to desired traits such as delayed fruit ripening, different coloured flowers, enhanced content of specific nutrients, male sterility.

Unintended changes and risks:

- off target effects: silencing of other genes, leading to altered traits, with potential negative impacts such as production and accumulation of toxins and allergens, lowered nutrient content, disease susceptibility.
- the silencing of the target gene may not only stop the manufacture of the gene product (i.e. the protein), but depending on the possible involvement of this protein in other pathways, may cause other unpredicted effects (often referred to as pleiotropic effects). Consequences may include anything that is linked to those pathways, e.g. growth factors, defence and signalling mechanisms, accumulation of compounds, etc.
- specific to dsRNA: Depending on the methodology used, the presence of dsRNA molecules in the food chain and the environment may negatively impact organisms ingesting them, as they can be passed down the food chain, and may be amplified and lead to the switching off of vital genes, which could have wide ecological and health consequences.

8. Grafting: of non-GMO graft (scion) on GMO rootstock (and vice versa)

Grafting (e.g. of fruit trees, grapevines, tomatoes) is a way to combine the strength or desired traits of two organisms into one, without having to cross-breed them, e.g. by choosing a rootstock for disease resistance and the graft or scion for fruit flavour. Though in combination a chimera (a single organism composed of genetically distinct cells), the graft and rootstock in themselves will largely keep their own genetic identities with regard to the basic sequence of their DNA. To obtain the GM chimeric plant, by definition, requires genetic engineering. The aim of using a GM rootstock is to create grafts that would benefit from the GM characteristics without being defined as GM or sharing the GM DNA, though, as a whole, the plants are GM. Thus, strictly speaking, the tissue of the graft would not have been genetically engineered, but the rootstock has. Yet many of the molecules produced by the GM rootstock, whether proteins, certain types of RNA (eg: dsRNA), hormones, signalling or defence molecules, can spread throughout the whole of the chimeric plant.

Unintended changes and risks:

- Impact of the GM rootstock on the environment: genetic engineering processes, such as transformation and tissue culture, are known to induce genome wide mutations, as well as insertion site mutations. These can lead to altered and unexpected traits, potentially with negative impacts on soil and environment. Positional effects of inserted genes, such as affecting gene expression of neighbouring genes, may equally lead to negative impacts.

- Compounds and metabolites produced by the GM rootstock will be present in the graft and its products (e.g. in fruit) and may alter the composition of the fruit/product, which in turn may change the nutrient, allergen or toxin composition.

- If RNAi (RNA interference) methodology has been used in the GM rootstock, the gene silencing present in the DNA of the rootstocks could transfer to the DNA of the graft via the movement of small RNA molecules from the rootstock into the graft. This may silence genes in the graft and alter its traits.

9. Reverse breeding (RB)

RB is a GM technology intended to reconstitute genetically uniform and pure (homozygous) parental lines from an existing hybrid whose parental lines are no longer available or no longer exist. A major hurdle in this is, that every time gametes (reproductive cells) are being produced, the chromosomes previously acquired from the parental lines swap information in a stage of genetic recombination , thus mixing the DNA. To avoid this, the selected hybrid seed is genetically engineered to suppress genetic recombination (using RNAi). With the help of tissue culture, individual resulting gametes are used to reconstitute plants with two sets of the same chromosomes (called 'double haploid'). At a later stage the GM gene is deselected and parental lines chosen that – in combination – will give rise to the envisaged hybrid.

Unintended changes and risks:
As the same genetic engineering processes are being used, both to insert genes and to reconstitute plants through tissue culture, the same risks and unpredictable outcomes are possible as with other GM. There usually will be:

- insertion site and genome wide mutations (e.g. deletions, rearrangements, multiplications) resulting from the transformation processes, including tissue culture, with unpredictable consequences, which could lead to altered performance and disease susceptibility, accumulation of toxins, increased production of allergens, changes in nutritional composition.
- The vast majority of these mutations would remain present in the reconstituted parental lines even if the GM gene itself is deselected and with it the mutations most closely associated with the insertion site itself.

- The GM gene silencing method of RNAi may lead to non-target gene silencing of other genes, effects that will be maintained for many generations of seed.
- Functional components or full sequences of the genetically engineered gene may have integrated themselves elsewhere in addition to the primary insertion. They may thus not be removed in the de-selection process, leaving them potentially still able to initiate gene silencing in the target region or in off-target areas.

10. Agro-infiltration: Agro-infiltration 'sensu stricto' & Agro-infection

This method involves two distinct technologies. It is not intended to result in specific GM genes being stably inserted and integrated into a plant genome, but rather for such genes to be present within the plant cell transiently, for at maximum just one generation.

To this end, genes either coding for specific proteins or for RNAs to interfere with the plant's own genes (e.g. via RNAi) are engineered into the plasmid of the bacteria *Agrobacterium tumefaciens*. A solution of such Agrobacteria or their plasmids is then used to treat specific tissues of living plants (e.g. leaves) so as to have the plasmids with the GM genes delivered to the cells in that tissue, where these genes will be expressed in the specific RNA.

The aims may be to: test potential transgenes; study the function of the plant's own genes (e.g. through gene silencing via RNAi); express and produce high value proteins in plants (e.g. pharmaceuticals); produce plants, seeds, hybrids with altered traits through RdDM (RNA dependent DNA methylation); or as a delivery system for other GM-based NBT tools, such as site directed nucleases. There are two distinct technologies:

- Agro-infiltration 'sensu stricto' (i.e. in the strictest sense): The intention is to keep the gene expression and effect localised, thus the genetic construct prepared and used is non-replicating.
- Agro-infection: The intention is to spread the specific GM gene throughout the whole plant into almost all the tissues, but without integrating the gene into the plant's DNA. For this purpose, in addition to the chosen gene, the gene construct contains a viral vector sequence in order to replicate the construct in all infected cells. The gene for the RNA is meant to be expressed from the vector location, i.e. not from a location on the plant's DNA.

Unintended changes and risks:

- Though applied locally, the gene construct can spread throughout the plant, due to the agrobacteria and/or the viral vector sequences used. Although meant to be transient, the genetic material may become integrated into the plant's DNA, including reproductive tissue, thus unintentionally giving rise to GMOs and to GM progeny.
- Integration may happen at random places within the genome and may also involve any of the genetic sequences introduced, including vector DNA. Disruption of genes due to positional effects or due to sequences present in the gene construct could give rise to negative impacts on plant performance, environment and biodiversity, or on its safety as food.
- Accidental release of genetically engineered Agrobacteria into the environment could occur (either due to the spread of and contamination from infiltrated plant material that has been discarded or removed, or simply through spillage, e.g. from the lab, greenhouse or test plots). This in turn could give rise to adverse effects if the gene constructs get transferred to other plants or to microorganisms.

References

[1] Steinbrecher R (2015) Genetic Engineering in Plants and the "New Breeding Techniques (NBTs)": Inherent risks and the need to regulate. http://www.econexus.info/publication/genetic-engineering-plants-and-new-breeding-techniques

[2] Eckerstorfer M, Miklau M, Gaugitsch H (2014) New Plant Breeding Techniques and Risks Associated with their Application. Environment Agency Austria. Umweltbundesamt. REPORT REP-0477 Vienna 2014. http://www.umweltbundesamt.at/fileadmin/site/publikationen/REP0477.pdf

[3] Voytas DF, Gao C. (2014). Precision Genome Engineering and Agriculture: Opportunities and Regulatory Challenges. *PLOS Biol*, **12**(6), e1001877. http://doi.org/10.1371/journal.pbio.1001877

[4] Esvelt, K. M., Smidler, A. L., Catteruccia, F., & Church, G. M. (2014). Concerning RNA-guided gene drives for the alteration of wild populations. *eLife*, e03401. http://doi.org/10.7554/eLife.03401

[5] Khatodia S, Bhatotia K, Passricha N, Khurana SMP, Tuteja N (2016). The CRISPR/Cas Genome-Editing Tool: Application in Improvement of Crops. *Frontiers in Plant Science*, **7**, 506. http://doi.org/10.3389/fpls.2016.00506

[6] Schaeffer, S. M., & Nakata, P. A. (2016). The expanding footprint of CRISPR/Cas9 in the plant sciences. *Plant Cell Reports*, **35**(7), 1451–1468. http://doi.org/10.1007/s00299-016-1987-x

[7]Agapito-Tenfen SZ, Wikmark O-G (2015) Current status of emerging technologies for plant breeding: Biosafety and knowledge gaps of site directed nucleases and oligonucleotide-directed mutagenesis. Biosafety report 02/2015. GenØk - Centre for Biosafety, Tromsø, Norway. January 2015. http://genok.no/wp-content/uploads/2015/06/250615_Emerging_technologies_final.pdf

[8] Hartley S, Gillund F, van Hove L, Wickson F (2016) Essential Features of Responsible Governance of Agricultural Biotechnology. *PLoS Biology*, **14**(5), e1002453. http://doi.org/10.1371/journal.pbio.1002453

[9] Tan W, Carlson DF, Lancto CA, Garbe JR, Webster DA, Hackett PB, Fahrenkrug SC (2013) Efficient nonmeiotic allele introgression in livestock using custom endonucleases. *Proceedings of the National Academy of Sciences*, 201310478. http://doi.org/10.1073/pnas.1310478110

[10] Hammond A, Galizi R, Kyrou K, Simoni A, Siniscalchi C, Katsanos D, Gribble M, Baker D, Marois E, Russell S, Burt A, Windbichler N, Crisanti A, Nolan T (2015). A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nature Biotechnology*, advance online publication. http://doi.org/10.1038/nbt.3439

[11] Lezaun J, Porter N. (2015) Containment and competition: transgenic animals in the One Health agenda. *Social Science & Medicine*, **129**, 96–105. http://doi.org/10.1016/j.socscimed.2014.06.024

[12] Heinemann JA, Agapito-Tenfen SZ, Carman JA (2013) A comparative evaluation of the regulation of GM crops or products containing dsRNA and suggested improvements to risk assessments. *Environment International*, **55**, 43–55. http://doi.org/10.1016/j.envint.2013.02.010

[13] Lillico SG, Proudfoot C, Carlson DF, Stverakova D, Neil C, Blain C, WA Ritchie, Tan S, Mileham A, Mclaren D, Fahrenkrug SC, Whitelaw CBA (2013). Live pigs produced from genome edited zygotes. *Scientific Reports*, 3. http://doi.org/10.1038/srep02847

[14] Then C (2016) Synthetic gene technologies applied in plants and animals used for food production. TestBiotech. http://www.testbiotech.org/sites/default/files/Gene_editing_plants_and_animals_0.pdf