RAGES

RISK ASSESSMENT OF GENETICALLY ENGINEERED ORGANISMS IN THE EU AND SWITZERLAND

New genetic engineering technologies

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Summary

Genetically modified organisms (GMOs), predominantly plants, have been commercially grown in some countries, notably the Americas, since the mid-1990s. Current GMOs have been developed using ‘first generation’ genetic engineering technologies. More recently, new applications of GMOs and new modes of creating novel traits have been developed alongside new genetic engineering technologies. Grafting, cisgenesis and intragenesis, reverse breeding and RNA-directed DNA methylation (RdDM) either utilise GMOs created using first generation techniques as an intermediary stage or can, in the case of agro-infiltration, unintentionally give rise to GMOs. Most, if not all, of the principal concerns regarding first generation GMOs apply to these new types of GMOs and new genetic engineering techniques. Some novel types of GMOs (e.g. RNA interference (RNAi)-based GM plants) present additional challenges for risk assessment, as do new genetic engineering techniques, such as genome editing.

RNAi-based GM crops

For RNAi-based GM crops, major uncertainties and knowledge gaps exist, resulting in open questions remain on how to assess the risks of RNAi-based GM crops to both the environment and food and feed. Despite the lack of guidance from the European Food Safety Authority (EFSA) on the risk assessment of RNAi-based GM crops, two RNAi-based GM crops have been approved for food and feed use in the EU and one has received a positive opinion, also for food and feed use, from EFSA. This is not acceptable, and it is strongly recommended that the issue of risk assessment guidance for GMOs developed through new techniques, particularly those developed by genome editing, precedes any consideration of applications to cultivate or market.

Genome-edited GMOs

New techniques of creating genetically modified organisms (GMOs) have been developed in the past decade. In particular, the so-called ‘genome editing’ technologies have been much discussed. These include oligonucleotide-directed mutagenesis (ODM), zinc-finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), meganucleases and CRISPR (clustered regularly interspaced palindromic repeats) techniques with CRISPR becoming the predominant genome editing technology. Genome editing tools can also be applied to produce cisgenic and intragenic organisms, applied to synthetic genomics and to induce RdDM.

Genome editing techniques can give rise to a broader spectrum of new genetic combinations and novel traits compared to the classical traits introduced by first generation GMOs (predominantly herbicide, insecticide resistance and combinations thereof). However, genome editing is limited in its applications when it comes to editing of polygenic traits. Whilst several sites in the genome can be targeted at once, these are edited outside the context of their genetic and epigenetic regulation. Many traits required by consumers and/or farmers (e.g. drought tolerance in plants) are controlled by ‘complex traits’. Modern conventional breeding techniques such as genomic selection and marker assisted selection are, in general, more suited to breeding complex traits. One principal reason is that, with conventional breeding the whole genome is encompassed so that genetic and epigenetic regulation of genes remains intact. Conventional breeding has had, and will undoubtedly continue to have, success in breeding varieties with traits such as enhanced drought and/or flood tolerance.

EU regulation covers genome editing

The technologies involve the direct modification of genomes. That means that changes in the genome are achieved by directly introducing either genetic material or material that enacts a change to genetic material into the cell, with the material produced, or at least handled in the laboratory by humans. This concept of direct modification of genomic material is important as it underlies the concept and definition of both a genetically modified organism GMO in the EU and a living modified organism in the United Nations Cartagena Protocol on Biosafety.

Broadly, these new genetic engineering techniques can be grouped into three groups:

1) those giving rise to novel types of GMOs (synthetic genomics, RNAi-based crops, cisgenesis and intragenesis);

2) infrequent applications of GMOs in plants (grafting; agro-infiltration; reverse breeding) and

3) new techniques of producing GMOs (RdDM and genome editing techniques: ZFN, ODM, CRISPR, TALEN, meganucleases).

Unintended effects

As with plants developed through first generation genetic engineering technologies, both intended and unintended changes can be important in terms of plant protein production and metabolism. Thus, it is possible, even likely that, like first generation techniques of genetic engineering, genome editing techniques can give rise to plants displaying unexpected and unpredictable effects with implications for food, feed and environmental safety. Although genome editing techniques are often described as ‘precise', in reality there is potential for unforeseen genomic interactions, genomic irregularities and unintended biochemical alterations. These can produce unexpected effects in the resultant GMO.

Unintended effects associated specifically with genome editing fall into two main categories:

- off-target effects where the nuclease unintentionally alters DNA at a site in addition to the target site;

  unintended on-target effects, where the intended change generates further alterations, e.g. to genomic regulation.

Farm animals

Currently, there are no commercial GM farm animals, and the only GM animal approved for food use is limited to a GM salmon in Canada and the U.S. The production of GM animals is thought to be limited by difficulties with first generation genetic modification techniques for animals. In contrast, CRISPR is reported to have high efficiencies in animals, meaning that there may be applications to market genome-edited farm animals as food. But besides risk-related issues, ethical and welfare concerns of genome-edited animals are pressing and largely similar to those that have been raised for genetic engineering and/or cloning.

Gene Drives

Gene drives are genetic elements that do not follow the Mendelian pattern of inheritance as they increase the probability that a specific genetic condition is being transmitted to the next generation above the normal 50% for sexual reproduction. With gene drives, contrary to most other applications of genetic engineering, the GMOs are not intended to be contained within the laboratory or restricted to a single generation of hybrid plants. They are intended to genetically engineer wild (uncultivated) populations of animals and plants. In this backdrop, new layers of risk-related issues emerge including a lack of spatio-temporal control and disruptive processes that can affect whole species and/or associated ecosystems. Gene drives, no matter if supposed to replace or suppress a population, can give rise to genetically engineered populations that persist in the environment with little or no opportunity for recall. If persistence of genetically engineered organisms goes along with lack of spatio-temporal control, it becomes difficult or largely impossible to predict either the short-term or the long-term ecological impact. There is a broad range of further negative or adverse impacts that require consideration, such as spontaneous transboundary movements, introgression into organic production systems in agriculture, socio-ecological and ethical considerations. As a consequence, there are many serious and valid concerns regarding uncontrolled spread of organisms with gene drive systems. It is not clear how the approval of local communities could be sought as at present (as required under the Conventional for Biological Diversity) as there is no mechanism for societal consultation on GMOs in the EU. Application of the precautionary principle, as enshrined in EU law would preclude the release of GMOs as part of a gene drive system.

Risk assessment for organisms developed through genome editing techniques

Just like first generation techniques, new genetic engineering techniques can produce unexpected and unpredictable effects in the resultant GMOs, even if any inserted genes (whether intentionally or unintentionally inserted) are subsequently removed prior to commercialisation. Therefore, it is important that any applications for cultivation (including field trials) and marketing of GMOs produced by these techniques undergo full environmental and health risk assessment. The current risk assessment guidance in the EU would need to be expanded in order to assess the additional unintended effects that genome editing can cause. The molecular characterisation element of the risk assessment will need to be expanded to include analysis for unintended changes at the genomic level, including off-target effects, unintended on-target effects and effects on genomic regulation.

There are several techniques that can be used to detect and assess any unintended effects generated by the genome editing process. These could also be used to improve the risk assessment of GMOs created by first generation techniques. These are collectively summarized as ‘omics’-approaches and include analysis of the RNA profile (transcriptomics), the protein profile (proteomics) and the metabolite profile (metabolomics). Metabolic profiling characterizes the current status of all molecules involved in the metabolism using methods combining chromatography and spectrometry.

The risk assessment will need to consider a broader range of traits conferred by the genetic engineering process, for some of which there may be a lack of experience. It will need to consider direct and indirect implications for agricultural practices and ecological impacts caused by any changes in animal diets. Genome-edited GM plants should also be analyzed with regard to the composition of their microbiome as the microorganisms colonizing the surfaces and inner tissues of plants play an important role for functional traits of the plant such as crop yield and nutrient quality.

Detectability of GMOs developed with new techniques

As with current GMOs, labelling of GMOs created by genome editing is necessary to enable consumer choice and to protect agricultural systems that exclude GMOs, e.g. organic agriculture. GMOs developed by genome editing are detectable, provided prior information is available regarding the intended genomic changes. It is evident that advances in detection technologies are needed, not only for genome-edited organisms, but for other new genetic engineering techniques such as RdDM. Therefore, there needs to be political will to develop suitable detection technologies. Regulatory requirements of traceability and labelling would be likely to spur research into developing new detection technologies.

1 Introduction

Genetically modified organisms (GMOs), predominantly plants, have been commercially grown in some countries, notably the Americas, since the mid-1990s (International Service for the Acquisition of Agri-biotech Applications, 2018). Concerns regarding the risks of GMOs to the environment, animal and human health have led to them requiring a risk assessment prior to cultivation and marketing in the EU (European Commission, 2001) and many other regions and countries around the world.

Current GMOs have been developed using what are termed here as ‘first generation’ genetic engineering technologies. More recently, new applications of GMOs and new modes of creating novel traits (e.g. RNA interference (RNAi)-based GM crops) have been developed alongside new genetic engineering technologies, e.g. so-called ‘genome editing’ or ‘gene editing’ technologies such as clustered regularly interspaced short palindromic repeat (CRISPR) technologies (Scientific Advice Mechanism, 2017; Lusser et al., 2012). Table 1 gives groupings of these new types and applications of GMOs, together with new techniques of producing GMOs.

New techniques of creating GMOs have especially been developed within the past decade. In particular, the so-called ‘genome editing’ technologies have been much discussed (Yin et al., 2017; Scientific Advice Mechanism, 2017). These include oligonucleotide-directed mutagenesis (ODM), zinc-finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), meganucleases and CRISPR techniques (Table 1, Sander and Joung, 2014) with CRISPR becoming the predominant genome editing technology. Many of these genome editing technologies can also be applied to produce cisgenic and intragenic organisms, applied to synthetic genomics and to induce RNA-directed DNA methylation (RdDM). Additionally, there are new applications of genetic engineering (Table 1), including RNA-interference (RNAi)-based genetically modified (GM) crops (where the genetic engineering results in either gene silencing in the resultant organism or insecticidal properties) and gene drives (where the inserted gene(s) are designed to persist and transgress through the natural population).

All the technologies in Table 1 involve the direct modification of genetic material. That means that changes in the genome are achieved directly, without mating, by introducing either genetic material or material that enacts a change to genetic material into the cell, with the material produced, or at least handled in the laboratory by humans, i.e. *in vitro* techniques. This concept of direct modification of genetic material is underlies the concept and definition of a GMO in both the EU (European Commission, 2001) and a living modified organism in the Cartagena Protocol on Biosafety (United Nations Convention on Biological Diversity, 2003).

Broadly, these new genetic engineering techniques can be grouped into three groups (Table 1):

1) those giving rise to novel types of GMOs (synthetic genomics, RNAi-based crops, cisgenesis and intragenesis);

2) applications of genetic engineering in plants that are rarely used in commercial applications (grafting; agro-infiltration; reverse breeding) and

3) new techniques of producing GMOs (RdDM and genome editing techniques: ZFN, ODM, CRISPR, TALEN, meganucleases).

Table 1 Grouping of novel types of GMOs, new applications of GMOs and new techniques of producing GMOs.		
Novel types of GMOs	**Commercially infrequent applications of GMOs**	**New techniques of producing GMOs**
Synthetic genomics	Grafting	RNA-dependent DNA methylation (RdDM)
RNAi-based crops	Agro-infiltration	Genome editing techniques: ZFN, ODM, CRISPR, TALEN, meganucleases.
Cisgenesis and intragenesis	Reverse breeding	

This chapter outlines the current concerns with GMOs, and describes the risks to the environment, humans and animals associated with each of the groups in Table 1. Finally, it examines considerations for the risk assessment of GMOs developed using genome editing techniques, including the detection of GMOs created by genome editing.

2. Principal concerns regarding genetic modification of living organisms

Briefly, the fundamental concern regarding GMOs and the direct modification of genetic material is that it can unintentionally interfere with the gene expression of an organism or interfere with complex biochemical pathways within an organism. For example, genetic modification can give rise to unintended or altered proteins or altered secondary metabolites, particularly in plants whose secondary chemistry is complex (Aharoni and Galili, 2011). Hence, the biological and biochemical characteristics of the organism might be changed in a way that impacts human and animal health and/or the environment. In addition, the novel trait conferred by the genetic engineering, e.g. herbicide tolerance in plants is also of concern as this can have consequences for agricultural systems, the environment and often for food and animal feed safety. Further, in the EU, a system of traceabilty and labelling is necessary to allow for segregation of GM foods from non-GM foods to enable consumer choice and monitoring of any adverse effects in the human population post-marketing of GMOs.

Most, if not all, of the principal concerns regarding current GMOs, created using first generation methods, apply to these new types of GMOs and new genetic engineering techniques, as described in the following sections. Additionally, these new genetic engineering techniques can give rise to a broader spectrum of new genetic combinations (Kawall, 2019) and novel traits compared to the classical traits introduced by first generation GMOs (predominantly herbicide, insecticide resistance and combinations thereof in plants) (Agapito-Tenfen et al., 2019; Eckerstorfer et al., 2019).

3. Novel types of GMOs

Novel types of GMOs include RNAi-based GM crops, synthetic genomics, cisgenesis and intragenesis.

3.1 RNAi-based GM crops

RNAi is a mechanism of gene silencing that occurs naturally in the cells of fungi, plants and animals, but RNAi-based GM crops artificially induce this gene silencing through the production of novel RNAi in the GMO. Although the effects of gene silencing have been observed for a considerable time, the RNAi mechanism has only been elucidated in the past 15 years (Couzin, 2002; Roberts et al., 2015). Naturally occurring RNAi is a gene-silencing mechanism and can also confer resistance to the invading nucleic acids. Initially it was found that RNAi is exogenously triggered as an immune response by the infiltration of foreign RNA (in most cases double stranded RNA (dsRNA)) into an organism's cell (for example by an invading viral pathogen) (Mello and Conte Jr, 2004). Shortly after, endogenous genomic sources for RNAi (centromeric regions, transposons and other repetitive sequences) were also uncovered (Lippman and Martienssen, 2004).

In normal gene expression, genes (encoded by DNA) are transcribed into an intermediate product, messenger RNA (mRNA), which is subsequently translated into a protein. RNAi acts at the RNA level by, for example, cleaving an invasive viral RNA or mRNA, preventing it from being translated into a protein. There are two kinds of small RNA molecules that act in the RNAi pathway: miRNAs (micro RNAs) and siRNAs (small interfering RNAs). miRNAs are derived from short stemloop RNA molecules and typically silence genes by repression of translation. siRNAs are derived by longer regions of dsRNAs and typically work by cleaving the mRNA before translation (Wilson and Doudna, 2013). The structure and length of siRNAs are very similar to miRNAs. In both the exogenously and endogenously induced RNAi pathway, dsRNA is incorporated into the nucleus and is enzymatically cleaved into smaller pieces of RNA (Carthew and Sontheimer, 2009; Pačes et al., 2017). These small RNA molecules are directed to mRNA molecules or invasive RNAs that have a complementary sequence. This binding event can either lead to the cutting of the mRNA or prevent the mRNA from getting translated into the corresponding protein, both of which can lead to an overall reduction in the level of the protein (see Pačes et al., 2017).

Commercially-orientated (i.e. those intended for uncontained cultivation, food or feed) RNAi-based GM organisms are, so far, restricted to plants. In RNAi-based GM crops, a single functional gene or, more typically, a suite of novel genes is inserted into the organism (usually a plant) using first generation genetic engineering techniques. These novel genes induce the production of dsRNA. The dsRNA is then processed into small interfering RNAs, which then mediate the interference with mRNA in a sequence-specific manner. The processing of dsRNA into siRNAs can be directed to occur either within the GM organism itself, or within an organism that ingests the GM organism. For more details, see Pačes et al. (2017).

In the study of molecular biology, RNAi mechanisms are widely used as a research tool under contained-use conditions to explore cellular regulation of the expression of protein-coding genes in plants and animals. In contrast, commercially-orientated (i.e. those intended for uncontained cultivation, food or feed) RNAi-based GM plants have two main applications: those intended to change composition, e.g. the compositional changes (but not the herbicide tolerant trait) in MON 87705 resulting in low-linolenic, high-oleic soybean, known as Vistive Gold (see the RAGES

report, “Nutritionally Enhanced GM crops”) and those intended to act as a plant pesticide, e.g. the insecticidal element DvSnf7 in MON 87411 maize (United Nations Biosafety Clearing House, 2015).

Genetic modification for RNAi is not intended to produce a novel protein as most commercial GMOs do (e.g. production of the Bt protein in GM Bt crops and production of the EPSPS protein in GM Roundup Ready crops). However, the production of a novel protein is not the only risk that is considered in the risk assessment of GMOs. The main concerns regarding RNAi-based GM crops relate to the lack of specificity of siRNAs and the potential adverse impacts this may cause on the GM plant, ecosystems and human health. Whilst all GM crops are subject to concerns regarding safety for the environment and inclusion as food for humans and animals, there are specific concerns regarding the GM plants that contain RNAi constructs, as described below.

Unintended effects in RNAi-based GM crops

One major concern regarding unintended effects within RNAi-based GM plants is the lack of specificity of siRNAs. siRNAs may be generated that are sufficiently complementary to mRNAs in the GM plant other than the mRNA intended to be silenced. This could enact gene silencing of an unintended gene (an off-target effect) within the GM plant. During cleavage of dsRNA, many siRNAs are produced. Each siRNA has the potential to recognize putative off-target sites due to a lack of specificity (Ramon et al., 2014).

Off-target effects could result in unexpected effects in the resulting GM plant, e.g. by altering a biochemical pathway (e.g. starch or oil production). Off-target effects would need to be assessed robustly as plant chemistry is complex. Altering one biochemical pathway has a high probability of altering another pathway (Hines and Zahn, 2012). This is discussed further in the RAGES report, “Nutritionally Enhanced GM crops”. Additionally, further research is needed on how transgenic siRNA can affect the expression levels of other non-coding RNAs and other RNAs such as mRNAs within RNAi-based GM plants. This could lead to changes in protein and enzymatic content, putatively altering the nutritional value of the GM plant. Alterations of the RNA level in GM plants in comparison to unmodified plants can be identified by sequencing of the transcriptomes (EFSA, 2019a).

Ecological concerns of RNAi-based GM crops

One of the principal ecological concern regarding RNAi-based GM crops are unintended adverse effects on non-target organisms in the environment. That is, the dsRNA produced by the GM plant may affect organisms in the environment other than the intended pest, for example beneficial insects. Whilst research to date has primarily considered this concern in relation to RNAi-based GM crops intended to control a specific pest (Zhang et al., 2017a), it could equally apply to RNAi-based GM crops with altered composition.

Environmental risk assessment is hindered by large knowledge gaps in the genomics and understanding of RNAi in both invertebrates and vertebrates (Christiaens et al., 2018; Ramon et al., 2014). For example, without full information of the genome sequence for a potential non-target organism, it is difficult to assess whether a specified novel dsRNA could be absorbed from the gut of the non-target organism, processed into siRNAs, or what genes in the non-target organism may be affected by the siRNA produced (Roberts et al., 2015).

Genomic datasets for most model (i.e. well-studied) organisms are complete and publicly available but, for the majority of invertebrate organisms, genomic datasets are incomplete with huge gaps in the genome sequences, as shown in the systematic literature review by Christiaens et al. (2018). Beside invertebrates, vertebrates, such as birds or rodents, may also be adversely affected by RNAi-based GM crops – or at least the possibility of adverse effects cannot be dismissed. Like invertebrates, the genomic datasets of most vertebrate organisms are also still incomplete, although some, e.g. for extant birds (Zhang 2016; Bird 10,000 Genomes Project 2016) are under construction.

These factors make assessment of the ecological safety of RNAi-based crops difficult, if not impossible at present (Heinemann et al., 2013; Lundgren and Duan, 2013). Unlike conventional pesticides, different species can have different sensitivities to dsRNA, even within the same order (e.g. butterflies) (Roberts et al., 2015; Terenius et al., 2011; Christiaens et al., 2018) so it's difficult to select focal species upon which to perform ecotoxicity testing (as with conventional pesticides). Also, there are only a few publications reporting expression data of the RNAi constructs in RNAi-based GM crops which is necessary to evaluate the impact on non-target organisms (Christiaens et al., 2018). Similarly, there is limited knowledge on the fate of dsRNA from GM plants in the environment, although persistence of dsRNA is expected to be limited (Christiaens et al., 2018).

Unintended transmission of dsRNA via ingestion

Concerns for human and animal health exist but are poorly constrained as there is a considerable lack of knowledge regarding the unintended transmission of dsRNA and siRNAs following ingestion. Processing of dsRNA into siRNAs following ingestion is the key mechanism for the pesticide action of RNAi-based GM crops. The concern is whether dsRNA, or the siRNAs derived from them, might also be active when the GM plant is ingested as a food. If so, the question is whether this might affect gene expression within the consumer.

It is not yet clear to what extent dsRNA from an RNA-based GM plant could survive in the human or animal gut in order to be absorbed into the body and possibly processed into siRNAs. If dsRNAs are absorbed and processed into siRNAs, there is concern that they could affect gene expression in the consumer, if closely matching mRNA sequences are present (Nawaz et al. 2019; Chan and Snow, 2017; Pačes et al., 2017; Roberts et al., 2015; Ramon et al., 2014). In the literature review published by the European Food Safety Authority (EFSA) concerning risk assessment of RNAi crops on food and feed, it was indicated that plant miRNAs are more stable than anticipated due to structural properties influencing their stability and turnover (EFSA, 2019a):

"*However, when assessing the stability of plant ncRNAs outside the plant, compelling evidence exists that plant miRNAs are highly stable under different conditions including food storage, processing, cooking, or simulated digestion. Moreover, they seem to survive after long incubation in serum, or are detected in the gastric content of mice, suggesting that plant miRNAs are more resistant to degradation than synthetic or animal miRNAs.*"

One critical question is whether naturally occurring micro RNAs (miRNAs) from diet can be taken up by animals and humans from their diet and, if so, what effect it might have on gene expression.

miRNA and siRNA are similar families of small RNAs, both derived from dsRNA (Nawaz et al., 2019; Carthew and Sontheimer, 2009). Hence, if miRNAs can be taken up from the diet by animals and humans, so too might siRNAs from RNAi-based GM crops. Plant dsRNAs need to cross many barriers to find their targets in the host cells in order to perform their mode of action. Whether non-coding RNAs can really cause systemic effects is hotly debated (EFSA, 2019a).

In the past few years, there have been several publications and much discussion on this topic (for reviews, see Nawaz et al., 2019; Zhao et al., 2018), partially summarized in the following list:

- Zhang et al (2012) reported, for the first time, that miRNA produced by plants can enter the bloodstream of mammals (including humans) at the stage of consumption. Initially, the findings of (Zhang et al, 2012) were called into question (see, for example, United States Environmental Protection Agency 2014; EFSA, 2014). However, more recent publications also indicate that plant miRNA can enter the bloodstream, organs, milk and urine of mammals after ingestion (Yang et al., 2015; Liang et al., 2015; Hirschi et al., 2015, Lukaski & Zielenkiewicz, 2014). In contrast, some publications have demonstrated only limited or inconsistent uptake of plant miRNA (e.g. Baier et al., 2014; Witwer et al., 2013). Further research is also needed to investigate if plant miRNA can cross specialized barriers as the blood-brain barrier or placental barrier (EFSA 2019a).
- Publications suggest that small RNAs taken up from the intestine can affect gene regulation in humans and animals. For example, it was found that miRNA transferred via milk shows biological activity (Baier et al., 2014) and small RNAs produced by plants were able to affect the immune system in humans and animals (Zhou et al., 2015; Cavalieri et al., 2016).
- Several studies suggest that uptake of miRNA from the mammalian gut and its detection are dependent on technical and physiological factors, which could explain the contradictory results obtained by different researchers. For example, uptake of plant miRNAs through the digestion tract is thought to be selective as only relatively few of the thousands of plant miRNAs are readily detectable in human and animal plasma (Liang et al., 2015, Zhang et al., 2012); Liang et al. (2015) describe how techniques of RNA extraction might affect detection and suggest standardised protocols for the detection and quantification of miRNA in plasma; Yang et al. (2015), as well as Wang et al. (2012), show that the health status of the recipient can affect the uptake of miRNAs; Baier et al. (2014) speculate that packaging of milk miRNAs in vesicles might protect against degradation in the gut, enhancing uptake and Yang et al. (2015) show that dosage and duration of exposure are important.

As yet, there is little consensus whether plant miRNA can be absorbed into the body of animals and humans (EFSA 2019a, Pačes et al. 2017, Witwer and Zhang, 2017), yet it remains a distinct possibility (Nawaz et al., 2019; Zhao et al., 2018). Pačes et al. (2017) summarise the discussion as follows: *"It is apparent that four years after the original report (Zhang et al., 2012(...)), the field remains split. The essential questions concerning the existence of the proposed mechanism emerged already in 2012. Further research is necessary to clarify the basis of the aforementioned contradictory observations."*

There are at least two ways in which the dsRNA expressed in GMO plants, and its subsequent processing into siRNA, can impact mammalian health via ingestion:

(1) Uptake from the gut into the bloodstream in the same way as other plant miRNAs as described (see, for example, Yang et al., 2015; Liang et al., 2015; Hirschi et al, 2015; Beatty et al., 2014). If the bioactive molecules produced in the plants interfere with mammalian gene regulation, adverse effects could result (see for example Then and Panskus, 2018). Based on current knowledge, this interference scenario cannot be excluded. The need for further investigation is supported by the outcome of a meeting of a USA governmental scientific advisory panel in 2014 which maintained that, in particular, risks from RNAi-based GM crops to immune-compromised individuals should be tested (US Environment Protection Agency, 2014):

"*The stability of dsRNA should be tested in individuals that manifest specific diseases (e.g., Crohn's, colitis, irritable bowel syndrome, etc.), the immune compromised, elderly, as well as children. These individuals may have compromised digestion or increased sensitivity to dsRNA exposure.*"

(2) It is well known that endogenous miRNA plays a key role in gene regulation in the gut microbiome, as well as in the communication between the mammalian host and its gut microbiome (see, for example, Williams et al., 2017). It is plausible that the dsRNA produced in GM maize MON 87411 can interact with the gut microbiome directly without direct uptake from the gut. For example, the Snf7 gene which targeted by the dsRNA as produced in maize MON 87441, is involved in essential biological processes in insects as well as in yeast (Then & Panskus, 2018). Thus, there is a plausible hypothesis on how the additional dsRNA might affect the gut microbiome community and further research is needed to understand the impact of exogenous dsRNA in mammalian host microbiota composition and to identify microbial targets and their effect on physiological conditions (EFSA 2019a). Another point to consider is whether special diets (e.g. vegetarians or vegans) might lead to an increased uptake of dsRNA due to an increased exposure (EFSA, 2019a)

In summary, it is clear that interference with gene regulation following the absorption and processing of dsRNAs to siRNA within humans and animals following ingestion of RNAi-based GM crops is both feasible and plausible. As Nawaz et al. (2019) conclude:

"Based on the currently available evidence, off-target effects from the ingestion of novel siRNAs present in foods derived from either GM crops or foliar insecticidal or anti-viral spray application, cannot be ignored and thus should form an integral part of the risk assessment of these products."

Risk assessment of RNAi-based crops

The risk assessment of RNAi-based GM crops is hindered by large and significant knowledge gaps regarding RNAi mechanisms and pathways for adverse effects within the environment and on human and animal health. More research is needed in this area before any meaningful risk assessment for food and feed can take place (EFSA, 2019a, Pačes et al., 2017; Roberts et al., 2015).

In order to begin identifying these gaps and issues unique to the risk assessment of RNAi-based crops, EFSA convened an international scientific workshop in June 2014 (EFSA, 2014; Ramon et al., 2014). Following the workshop, two literature reviews were commissioned by EFSA, one on baseline data to inform the risk assessment of RNAi-based GM plants in general (Pačes et al., 2017) and one (Christiaens et al., 2018) to inform the environmental risk assessment. Recently, a third review about food and feed risk assessment of RNAi-based GM plants was published by EFSA (EFSA, 2019a). From Pačes et al. (2017) and other published literature, EFSA considers that, currently, bioinformatics searches for off-target effects of siRNAs can usefully (in terms of risk assessment) be conducted for plants but give insufficiently reliable predictions for animals or humans (EFSA, 2017). Thus, EFSA has only issued a protocol for off-target bioinformatic searches in plants, not for animals or humans who might ingest the plant (EFSA, 2017). This means that it may be possible to look for off-target effects within the GM plant that might give rise to unexpected effects. However, for the possibility and potential effects of uptake of dsRNA from GM plants by humans and animals from RNAi-based GM plants, the risk cannot be assessed at the present time (EFSA, 2017; Christiaens et al., 2018). This affects both the environmental risk assessment (for non-target organisms) and also food and feed safety risk assessment (for consumers).

In 2014 an EFSA workshop (ESFA, 2014) identified the following issues as relevant for risk assessment of health effects:

> *"Throughout the different discussion topics, the following issues were identified as knowledge gaps, where more research could be warranted:*
>
> *- The RNAi and metabolic profiling in RNAi-based plants could be further explored and corroborated to support risk assessment. In this context, 'omics' techniques should be further investigated as supporting tools.*
>
> *- The use of bioinformatics to predict potential off-target effects in consumers should be further explored.*
>
> *- Possible changes in microbiota, residing in human or animal guts, following consumption of food and feed products derived from RNAi-based plants could be a research topic."*

None of these research topics have yet been progressed to the point where they can assist the food and feed risk assessment of RNAi-based GM crops (see, for example, the discussion on 'omics' techniques in Risk assessment related to the genome editing process). The EFSA literature review on food and feed risk assessment of RNAi-based crops highlighted, once again, that there are still considerable knowledge gaps and that more research is needed. In particular, it is controversial and a matter of debate whether or not plant miRNAs found in body fluids of mammals can be traced back from the uptake in the gut or whether this might be contaminations (EFSA, 2019a).

Despite the controversy surrounding the possible uptake of miRNAs into humans, two RNAi-based GM crops were authorised in 2015 for food and feed use in the EU (MON 87705 and 305423 soybeans) and EFSA gave a positive opinion for the food and feed safety of maize MON 87411 (EFSA, 2018a), which recently lead to the approval of the maize by the European Commission for import and usage for food and feed in the EU (European Commission, 2019). In EFSA's risk assessment of maize MON 87411, the risks of RNAi-based crops to human and animal health were mostly overlooked (EFSA, 2018a). It appears that a rigorous risk assessment is needed, but a proper solution how to achieve that is still pending.

As the BSE crisis showed, the risk of bioactive compounds being transmitted to humans via the food and feed chain poses a high risk for farm animals and humans (see Pačes et al., 2017). There are clearly knowledge gaps in how RNAi-based GM crops could have environmental effects (via negative effects on non-target wild animals) and affect health in human consumer (via the uptake of dsRNA in food). Therefore, uncertainties and knowledge gaps in any risk assessment are not acceptable and the precautionary principle should be invoked. This means that RNAi-based GM crops would neither be cultivated, nor approved for food and feed use (see also Then and Panskus, 2018). Currently, no applications have yet been made for commercial cultivation of RNAi-based GM crops in the EU, yet open questions remain on how to assess the risks of RNAi-based GM crops that are being used for food and feed production.

3.2 Synthetic Genomics

Synthetic genomics is part of the larger field of synthetic biology. It involves the synthesis of stretches of DNA molecules which are then transferred into an organism which has been pared down to its essential components (a 'chassis') (Secretariat of the Convention on Biological Diversity, 2015). Currently, this has been applied to bacteria (Fredens et al., 2019; Gibson et al.,

2010) and yeast, a single-cell eukaryote (Bao et al., 2018; Garst et al., 2017; Kannan and Gibson, 2017). Although at an early stage of research, synthetic genomics has the ultimate aim of creating GMOs with substantially altered or completely artificial metabolic pathways, or even artificial organisms for which there is no reference conventional counterpart (Scientific Advice Mechanism, 2017). Synthetic genomics is facilitated by genome editing techniques, particularly CRISPR (Scientific Advice Mechanism, 2017) and organisms developed by synthetic genomics, could potentially be used in gene drive systems (see Gene Drives).

Currently, organisms developed through synthetic genomics are intended to be used under conditions of contained use. That is, used within secure facilities that prevent any releases into the environment. However, in the future, the deliberate environmental release of such organisms may be considered. For example, the intention to produce microorganisms designed for bioremediation and biosensors, agricultural crops tolerant to abiotic stress and pests, and to reintroduce extinct alleles, or even work towards the 'de-extinction' of species (Redford et al., 2019; Secretariat of the Convention on Biological Diversity, 2015) would, if successful, entail the release of organisms developed through synthetic genomics.

The environmental release of GMOs developed through synthetic genomics can negatively impact biodiversity in a similar fashion to other GMOs. These include: negative (e.g. toxic) effects on non-target organisms such as soil microorganisms, beneficial insects, other animals and plants; disruption of ecosystems caused by the survival and persistence of GM organisms and the transfer of their genetic material to wild populations, including native microorganisms (Secretariat of the Convention on Biological Diversity, 2015). However, the key aspect in terms of potential environmental impacts is the traits expressed by GM organisms developed through synthetic genomics, which can be extremely far removed from those normally present in the various ecological systems. For example, if GM algae, created by synthetic genomics to produce oil by breaking down sugarcane, were to escape from contained use, it could break down sugarcane in the local environment and could disrupt ecosystems and habitats (Secretariat of the Convention on Biological Diversity, 2015; Snow, 2012). These problems would be compounded if the synthetic organism persisted, multiplied, or passed on genetic elements to other organisms in the environment, either via horizontal or vertical gene transfer (Secretariat of the Convention on Biological Diversity, 2015).

EFSA has received a mandate to develop an opinion on GMOs developed through synthetic biology and their implications for risk assessment methodologies, including synthetic genomics (EFSA, 2018b). Working groups set up by EFSA will consider the adequacy of existing EFSA guidance on molecular characterisation and environmental risk assessment for synthetic biology GMOs (EFSA 2018b), reporting for synthetic biology GM microbes and plants at the end of 2020, following public consultation (EFSA 2018b). Consideration of the adequacy of existing EFSA environmental risk assessment guidance for synthetic biology GM animals, as well as considerations of the adequacy of existing EFSA food and feed guidance for synthetic biology GM microbes, plants and animals are to be considered at an undefined, later stage.

In summary, organisms created by synthetic genomics are currently intended for use under 'contained use' conditions, as regulated by the appropriate GMO regulations. Nevertheless, there is potential for escaped GM organisms developed through synthetic genomics to disrupt ecosystems, especially if they persist in the environment. Thus, extra scrutiny is required to ensure organisms created by synthetic genomics do not accidentally escape into the environment. Although there are currently no applications for environmental releases of organisms created by synthetic genomics, guidelines are being developed by EFSA for synthetic biology organisms, including those

developed by synthetic genomics. Given the potential for ecosystem disruption, the precautionary principle should be employed and no applications for environmental release should be considered.

3.3 Cisgenesis and Intragenesis

The majority of current commercial GM crops, developed through first generation techniques, are transgenic (or intergenic) in that they contain genes from non-sexually compatible species. Cisgenesis and intragenesis differ from transgenesis only in their source material. With cisgenesis and intragenesis, the functional gene(s), at least, are from closely related species. Cisgenesis is sometimes confused with intragenesis. In cisgenesis, intact genes, together with associated promoter/terminator from one species are inserted into the genome of the same or a closely related (i.e. sexually compatible) species. By contrast, in intragenesis the functional gene(s) may be partial and the promoter/terminator may not be associated with the functional gene(s) in the native plant, although all components are derived from the same or a closely related species (EFSA, 2012a). Cisgenesis and intragenesis involve the direct modification of genetic material and, to date, has used recombinant nucleic acids (i.e. first generation techniques). Cisgenesis and intragenesis can also be performed by using SDN-3 type genome editing techniques (see Genome editing techniques).

Concerns regarding unpredictable and unexpected effects in first generation GMOs arise, not only from the source of the inserted genetic material, but also from the act of inserting that genetic material. It is the act of insertion that can cause irregularities in the genome (e.g. deletion or rearrangement of the DNA flanking the inserted genetic material). It was already shown that the transformation process using *Agrobacterium tumefaciens* results not only in large genomic rearrangements in the vicinity of the integration site (Wilson et al., 2006, Jupe et al., 2019), but also in epigenetic alterations (Jupe et al., 2019). Such alterations are of concern because they can disrupt normal function of the genome, causing an alteration of gene expression. In turn, this could produce novel or altered proteins, which may be of consequence to food safety.

Within the context of considering whether existing guidance documents for the risk assessment were applicable to cisgenic and intragenic plants, EFSA has compared characteristics with the potential to cause adverse effects of plants developed through cisgenesis and intragenesis with those developed from conventional breeding and transgenesis (EFSA, 2012a). In this comparison, EFSA (EFSA, 2012a) considered that new combinations of genetic elements may be present in intragenic GM plants which could present novel traits with novel hazards. This renders hazards of intragenic GM plants akin to those of transgenic GM plants. For cisgenic GM plants, however, EFSA considered the hazards similar to conventionally bred plants.

EFSA (2012a) considered that "*the potential for 'random' changes to the genome caused by the insertion event is, however, not limited to transgenesis, cisgenesis and intragenesis*" but that similar changes could take place during conventional breeding. For example, EFSA (2012a) considered that deletions, insertions and rearrangements and the creation of new open reading frames can be created at random during all the techniques, including conventional breeding. However, with conventional breeding, these changes are of evolutionary consequence, and may form part of the desired outcome. With GMOs, these are not the intended change, but the unintended effects that may affect the environmental and food/feed safety of the GM crops. For example, EFSA (2012a) assert that, in conventional breeding, there is "*random movement of numerous mobile genetic elements such as transposons and retrotransposons*". However, evidence that these movements are random is scant (Bennetzen and Wang, 2014; Shapiro, 2010). On the contrary, the importance of transposon

movement for evolution and any associated deletions/rearrangements are highly active areas of enquiry in research, especially for plants (Lisch, 2013; Fedoroff, 2012).

The potential for unintended changes in secondary metabolites levels and composition may be reduced for plants developed through cisgenesis compared to intragenesis and transgenesis as the genes are endogenous, but the potential for unintended changes in general, including those with a potential adverse effect on health or environment is not necessarily reduced. Thus, cisgenesis and intragenesis could still alter plant biochemical pathways in similar ways to transgenesis, potentially giving rise to unexpected effects. Unintended changes to either genetic material and/or plant metabolism in the resulting cisgenic or intragenic plant could be important in terms of plant's impact on the environment and human and animal health.

3.4 Infrequent commercial applications of GMOs

Some applications of genetic engineering are widely used in research, but not generally used in the production of GMOs intended for commercial use (i.e. uncontained cultivation and food/feed) and thus are summarized as 'infrequent commercial applications' of GMOs. Nevertheless, they have received attention over whether they carry the same risks as GMOs produced using first generation genetic engineering techniques (Scientific Advice Mechanism, 2017; Lusser and Davies, 2013; Lusser et al., 2012). They include grafting, agro-infiltration and reverse breeding.

Trans-grafting onto GM rootstocks

Grafting is a traditional method used in horticulture. However, grafting onto GM rootstock is the grafting of a non-GM scion (plant cutting) onto a genetically modified rootstock, and commonly termed 'trans-grafting'. Although the fruits of the plant do not contain the inserted DNA sequence, sugar, metabolites and small RNAs (including siRNA and miRNA) derived from the GM rootstock can be exchanged between the graft and the scion (Scientific Advice Mechanism, 2017; Lusser et al., 2012). This means that any unexpected metabolites from the GM rootstock, or alteration of gene expression via RNAi involving small RNAs in the GM rootstock, could affect the scion, including the fruits (Scientific Advice Mechanism, 2017; Lusser et al., 2012).

Agro-infiltration

Agro-infiltration is a technique where plant tissues, typically leaves, are infiltrated in a liquid suspension of *Agrobacterium sp.* bacteria which carry a gene of interest (Lusser et al., 2012). However, flowers can also be immersed in the bacterial solution to produce a GM offspring containing the gene (floral dip) (Usher et al., 2017).

With leaf infiltration, although the gene of interest is expected to only be locally expressed at a high level, without being integrated into the plant genome, the movement of *Agrobacterium* throughout the plant and therefore the integration of genes carried by the *Agrobacterium*, cannot be excluded (Scientific Advice Mechanism, 2017). The technique is mostly used in a research context (Lusser et al., 2012; Scientific Advice Mechanism, 2017). However, if such plants were to be released to the environment (e.g. cultivated outdoors) or placed on the market, there would be concerns that the *Agrobacterium* may have genetically modified the plant, which means the concerns regarding existing GMOs would apply.

Reverse Breeding

The aim of reverse breeding is to produce homozygous parental lines of a selected heterozygous plant. The genes involved in the meiotic recombination process are silenced through first generation genetic engineering preventing meiotic crossing over. This results in non-recombined haploid lines with doubled chromosomes through the double-haploid technique. The transgenes inserted prior to chromosome doubling to prevent meiotic recombination are subsequently removed through backcrossing.

4. New genetic engineering techniques

4.1 RNA-directed DNA methylation (RdDM)

RdDM is a technique that has, so far, been applied primarily to plants, although its application in animals is also possible. In essence, the effect of RdDM methylation is to render genes inactive without altering the DNA sequence of the genome. This is achieved by the enzymatic attachment of a small chemical group (in this case a methyl group) to certain nucleotides of the DNA sequence, which is then inherited in subsequent cell division to daughter cells. As the DNA sequence is not directly changed by RdDM this clearly falls under the scope of epigenetics. Epigenetics is the study of heritable patterns that influence how genes are expressed without altering the underlying DNA sequence. The epigenome is the collection of all epigenetic patterns such as DNA methylation, histone modifications or histone variants in regard to their distribution along the genome (Mazzio and Soliman, 2012).

Understanding of the role of the epigenome has increased in recent years, from its role in human cancers (Laird-Offringa and Sanchez-Cespedes, 2018), to its importance in defining population characteristics of plants (e.g. Taudt et al., 2016). The epigenome plays a vital role in regulating gene expression and many projects (e.g. the Roadmap Epigenomics Project) are aiming to unravel the epigenetic code of human cells and other organisms using next-generation sequencing techniques (Romanoski et al., 2015). Importantly, epigenetic changes can be heritable for several generations, and the triggers to reverse methylation are not yet fully known or understood (Crisp et al., 2016). The heritability of epigenetic modifications means that any changes produced through genetic engineering of the epigenome can also be present not only in the modified organism, but also in its offspring. This means that many, if not all, of the risks of GMOs where the DNA is altered, also apply to GMOs created by RdDM. For example, if the RdDM trait may persist in offspring arising from outcrossing with wild relatives.

Usually, RdDM generates an RNAi-based GM plant as an intermediate product to induce the DNA methylation (see RNAi-based GM crops). The inserted genes can be removed by backcrossing after the DNA methylation has been enacted. Recently, gene silencing has been performed using purified small non-coding RNA (sRNA) applied as a spray on plant leaves, without introducing recombinant DNA into the organism at all (Scientific Advice Mechanism, 2017). Genome editing can also be used to change the epigenome (see Other genome editing applications using CRISPR). In whatever way the change to the epigenome is enacted, RdDM causes a heritable change to genetic material, and alters gene expression.

There are currently no commercial applications of RdDM modified organisms. The technique is mainly (currently at least) used as a research tool to investigate gene function (Lusser and Davies,

2013). Whilst EFSA have not considered an application for cultivation or food/feed of a RdDM GMO, it has considered whether such changes would be considered an alteration of genetic material (EFSA, 2015) and suggested it could be restricted to situations where the nucleotide (i.e. DNA) sequence was modified. This would have the effect of excluding GMOs developed through RdDM from requiring a risk assessment for cultivation or entering the food chain. However, the scientific knowledge of the epigenome, in particular the role of the epigenome in regulating gene expression and the heritability of the genetic alteration strongly indicates that a risk assessment for such GMOs would be necessary. Concerns regarding this type of genetic engineering include: off-target effects, unintended interruption of metabolic pathways through the silencing of a key gene (either unintentionally or as a consequence of an intended silencing) and the unintended effects of dsRNA (see RNAi-based GM crops) (Eckerstorfer et al., 2014).

4.2 Genome editing techniques

Genome editing techniques are substantially different to first generation genetic engineering technologies. They can result in minor or major changes to genomic material, predominantly DNA. Genome editing is applicable to plants, animals and microorganisms (Sander and Joung, 2014). Genome editing is also applicable to humans, e.g. for therapeutic use, although outside the scope of this chapter and EU GMO legislation (European Commission, 2001). These new engineering techniques generally use site-directed nucleases (SDNs), sometimes called ‘molecular’ or ‘enzymatic’ scissors, which cleave DNA at specific sites and trigger the organism’s own repair mechanisms. Alternatively, oligonucleotides can be used to enact a change to DNA, as with ODM.

Although a broader range of traits can be produced through genome editing than first generation genetic engineering, genome editing is limited in its applications because several traits of interest (e.g. drought tolerance in plants) have a complex genotype (‘complex traits’). That is, the trait is very often controlled by several genes operating at once in a coordinated manner. Epigenetic regulation of genes is also an important factor in plant response to stress (Banerjee & Roychoudhury, 2017). So far, genome editing can target several sites in the genome at once (for example by knocking out several copies of a single gene or targeting a limited number of different genes, called multiplexing) (Zetsche et al., 2017; Raitskin and Patron, 2016; Wang et al., 2016). However, like first generation genetic engineering techniques, genome editing operates outside of the organism’s existing regulatory network that controls gene expression (i.e. controlling when, where and to what extent genes are turned on and off) and repair mechanism (Brinkman et al., 2018). Modern conventional breeding techniques such as genomic selection and marker assisted selection are more suited to the breeding of complex traits. One principal reason is that the whole genome is encompassed with conventional breeding, so that genetic and epigenetic regulation of genes remains intact. This is in contrast to genome-engineering where genes are edited in isolation, without regard to their regulatory control. Conventional breeding has had, and will undoubtedly continue to have, successes in breeding. This particularly applies to plant varieties, with complex, polygenic traits such as enhanced flood or drought tolerance, virus resistance, increased nutrient efficiency and increased yield (Maxmen, 2019; Li et al., 2018; Bevan et al., 2017; Crossa et al., 2017; Dar, et al., 2013; Fujita et al., 2013) and also desirable traits in animals.

Oligonucleotide-directed mutagenesis (ODM)

ODM is a genome editing technique that does not use SDNs. Instead, short DNA (or DNA-RNA) fragments (oligonucleotides) are introduced into cells where they trigger the cell to modify its own

DNA to match the introduced DNA fragments (Sauer et al., 2016; Lusser et al., 2012). This technique can change, insert or delete one or a few base pairs of DNA. The term 'mutagenesis' is a misnomer as the technique bears little resemblance to traditional techniques of mutagenesis, which use externally applied stresses originating from chemical or x-ray sources to induce mutations, either in the whole organism (usually a plant) or cell (Jung et al., 2017; Scientific Advice Mechanism, 2017). However, it's worth noting that plants developed using traditional techniques of mutagenesis are classified as GMOs by the EU but are granted exemption from the legislation according Directive 2001/18 (European Commission, 2001, European Court of Justice, 2018).

According to EFSA (EFSA, 2015), ODM is considered as a type of mutagenesis as the end product cannot be distinguished from naturally occurring mutations or induced by mutagenesis. However, ODM is an *in vitro* technique that uses genetic material that is generated in a laboratory outside from the respective target organism or cell and is used to change its genome. As discussed in the Introduction, *in vitro* techniques result in a genetically engineered organism and therefore logically require regulation.

Whilst ODM may involve changes to only small number of DNA bases, there is the possibility of off-target effects (in common with other genome editing technologies and discussed in further detail under Off-target effects below). There are no published data concerning the frequency of off-target or unintended effects (Eckerstorfer et al., 2019; Scientific Advice Mechanism, 2017), but this does not mean that they do not occur. Any risk assessment would have to assess for off-target effects from the introduced oligonucleotide, and any subsequent degradation products of the oligonucleotide. In addition, a risk assessment would also have to assess whether the oligonucleotide has integrated into the genome and if so, what the consequences are. This possibility of oligonucleotide integration cannot be excluded (Scientific Advice Mechanism, 2017).

There is currently one commercial GMO that has been generated using ODM, Cibus' herbicide tolerant oilseed rape (canola), cultivated in USA and Canada (Cibus, 2014), although it may, in future, produce commercial GMOs in conjunction with other genome editing techniques, such as CRISPR/Cas (Eckerstorfer et al., 2019; Sauer et al., 2016). Overall, there are only few studies published that utilise or further develop the ODM technique (Eckerstorfer et al., 2019).

CRISPR/Cas, ZFN, TALEN and Meganucleases

CRISPR/Cas, ZFN, TALEN and meganucleases all use SDNs to break DNA at specific sites. Depending on whether a repair template is used or not, these methods can induce random (non-specific) changes to one or more base pairs via non-homologous recombination (termed SDN-1) or specific changes via homologous recombination (termed SDN-2) changes to nucleotide sequences. These changes can be substitutions, deletions or insertions of one or more base pairs. More extensive changes involve, for example multiplexing, which targets several genes at once, or repeated applications (Zetsche et al., 2017; Raitskin and Patron, 2016; Wang et al., 2016). Changes involving the insertion of whole genes (including gene-stacking) are also possible, involving donor DNA (termed SDN-3) (Eckerstorfer et al., 2019; Lusser et al., 2012; Sander and Joung, 2014).

ZFN, TALEN and meganucleases are protein-based systems that use engineered proteins to both target the site and cleave it, i.e. act as DNA nuclease. These techniques have been largely eclipsed by the CRISPR/Cas system in recent years. CRISPR/Cas uses RNA (in its natural bacterial system encoded on CRISPR sequences) to guide the protein nuclease (Cas) to the DNA site to be cleaved (Eckerstorfer et al., 2019; Joung and Sander, 2013; Sander and Joung, 2014). There are now at least two main CRISPR/Cas classes in use, which are further subdivided in different types depending on

their bacterial origin. The most commonly used CRISPR/Cas type is CRISPR/Cas9 but other subtypes, e.g. CRISPR/Cpf1, have been characterized (Zetsche et al., 2017) and more may yet be developed. The focus here is on CRISPR/Cas systems, of which only brief details are given here but are discussed in-depth elsewhere (Chen et al., 2019; Wang et al., 2016).

Delivery of the nuclease complex

Typically, genetic elements encoding the CRISPR/Cas nuclease complex are inserted into the organism using first generation genetic engineering techniques at random sites of the genome (Gaj et al., 2013; Weeks, 2017). Once CRISPR/Cas has achieved the intended change, the inserted genes can be removed by segregation using conventional breeding. However, the act of inserting genetic material can give rise to genomic irregularities (as described in Cisgenesis and Intragenesis), including large genomic rearrangements and epigenetic alterations in the vicinity of the integration site (Jupe et al., 2019; Wilson et al., 2006). Multiple copies of the CRISPR/Cas complex may also be inserted. For example, in experiments delivering the DNA encoding CRISPR/Cas9 in soybeans, the intended DNA segment was detected at the target location, but also at other multiple, apparently random, locations (Li et al., 2015). In addition, the removal of the inserted genetic elements may be incomplete and may not be feasible for crops that produce asexually (Woo et al., 2015; Yin et al., 2017).

Genome editing can be performed by introducing a plasmid encoding the CRISPR/Cas complex into plant cells without integration into the genome. The aim of this delivery technique is to avoid any genomic irregularities created by the insertion of CRISPR genetic elements. Dupont Pioneer's genome-edited waxy maize is an example of this approach (Dupont Pioneer, 2015). However, there is potential for the introduced plasmid DNA (or fragments thereof) to unintentionally recombine into the organism's DNA (Dupont Pioneer, 2015; Liang et al., 2017; Malnoy et al., 2016) (see below in Unintended effects of genome editing).

A strategy has been developed to apply CRISPR/Cas genome editing without the introduction of DNA to plant cells, sometimes called 'DNA-free CRISPR/Cas' (Jung et al., 2017). In these cases, the CRISPR/Cas complex can be processed outside the cell and directly inserted into the cell as a pre-assembled ribonucleoprotein (RNP) incorporating a single guide RNA (sgRNA) with Cas9 (Jung et al., 2017; Weeks, 2017; Woo et al., 2015). Examples of CRISPR/Cas genome editing without introducing DNA into the organism include apple, grape, maize and wheat (Jung et al., 2017; Liang et al., 2017; Malnoy et al., 2016).

Other genome editing applications using CRISPR

Various types of CRISPR/Cas complexes are being developed to utilise different strategies of genome editing. These include changing DNA bases without entirely cutting through DNA and targeting changes at RNA and the epigenome.

One field of interest in genome editing research is the development and improvement of so called 'dead' Cas9 (dCas9) approaches. The enzymatic 'cutting' function of dCas9 is prevented, which allows the targeting of dCas9 to specific DNA loci without cleavage (Qi et al., 2013). This is used in various applications: dead Cas9 can, for example, be coupled to a variety of other enzymes that can introduce biochemical changes to the target DNA sequence or associated proteins.

In 'base editing', dCas9 is coupled to enzymes that subsequently lead to the conversion of certain bases (the primary unit of information in DNA) into another without the introduction of DNA

double strand breaks at the target sequence (Gaudelli et al., 2017; Komor et al., 2016). This has been used on plants (Lu and Zhu, 2017; Zong et al., 2017) and animals (Kim et al., 2017; Zhang et al., 2017b) in ‘proof of concept’ studies. In the first generation of base editors the specificity was not reliable as surrounding bases of the same type at the target sequence were also changed in a 5 base-pair window (Komor et al., 2016). In an attempt to reduce unwanted side effects, these systems are being further revised (Gehrke et al., 2018). Nevertheless, two recently published findings showed, independently of one another, increased occurrence of off-target mutations using cytosine base editors (CBEs) in rice and mouse embryos (Zuo et al., 2019; Jin et al., 2019). Surprisingly, the off-target mutations, induced by CBEs, occurred at genomic positions, predominantly in actively transcribed genic regions, that were not depicted by off-target prediction software (Jin et al., 2019). Another study, in two human cell lines, showed that base editors generated transcriptome-wide editing of off-target RNA in addition to DNA editing. These effects occurred independently of both the guide RNA used and off-target DNA editing and were found both in CBEs and adenosine base editors (ABEs). These results show that base editor off-target effects are multi-dimensional and illustrate the importance of an adequate assessment of off-target effects, not only of DNA, but also RNA in such cells. These off-target effects can result in missense and nonsense mutations resulting in an altered protein content or generation of splice variants (Grunewald et al., 2019).

Dead Cas9 can also be used to introduce changes in the epigenome of a target organism. The epigenome is shaped through biochemical modifications of the DNA sequence itself or associated proteins called histones (Jenuwein and Allis, 2001). These epigenetic modifications regulate the gene expression in all tissues of an organism (Berger, 2007; Margueron and Reinberg, 2010). In epigenome editing applications dCas9 is connected to epigenetic modifiers (for example, methyltransferases or acetyltransferases) intending to change epigenetic markers at the target sequence and consequently shaping gene expression (Hilton et al., 2015; McDonald et al., 2016). These applications need to be further improved as it was recently shown that they can induce unspecific genome wide changes of epigenetic modifications (Galonska et al., 2018) which could lead to an altered gene expression in these cells. Thus, the specificity of these dCas9-epigenetic modifiers cannot be predicted properly so far (Enríquez, 2016).

RNA can also be edited using the Cas-variant Cas13 (Abudayyeh et al., 2017). Cas13 is structurally different to Cas9 and can be targeted to a specific mRNA. Analogous to CRISPR/Cas9-approaches, Cas13 is recruited to a target mRNA using a crRNA (CRISPR RNA), leading to the binding and cutting thereof (Abudayyeh et al., 2017). Cutting of the mRNA leads to the down-regulation or even prevention of the formation of the corresponding protein. An enzymatically inactive form of Cas13 (‘dead’ Cas13, dCas13) was developed to recruit dCas13 to a target mRNA without cleavage. This enables the editing of bases at the target sequence of the respective mRNA (Cox et al., 2017) without altering the underlying DNA-sequences. Thus, Cas13-edited mRNA and the corresponding proteins become naturally degraded in the organism without leaving traces behind.

In bacterial cells, where a Cas13-variant was initially investigated and characterized, it was found that after its initial binding and cutting of a specific target RNA, Cas13 remains in an enzymatically ‘active’ state. This leads to the cutting of unspecific RNAs in the cells and is thought to be a kind of programmed cell death in bacteria to prevent the spreading of a viral infection throughout the entire bacterial population (Abudayyeh et al., 2017). In mammalian and plant cells this nonspecific RNA cutting activity was not yet observed but should be considered as an off-target risk.

5. Unintended effects of genome editing

Although genome editing techniques are often described as ‘precise' (see, e.g. Duensing et al., 2018; Sauer et al., 2016; Voytas and Gao, 2014; Hartung and Schiemann, 2014), in reality there is potential for unforeseen genomic interactions, genomic irregularities and unintended biochemical alterations, as described below. These can produce unexpected effects in the resultant GMO.

Unintended effects associated specifically with genome editing fall into two main categories:

- off-target effects where the nuclease unintentionally cleaves DNA at a site in addition to the target site;
- unintended on-target effects such as the insertion of template DNA into the genome.

5.1 Off-target effects

Off-target effects are a major concern of genome editing techniques (Yin et al., 2017; Wolt et al., 2016; West and Gill, 2016). Off-target effects occur when genome editing introduces a change at an additional, unintended site of the genome in addition to the intended (target) location. The main cause of off-target effects is a lack of specificity (precision) over the location where the nuclease cuts the DNA because there is a degree of tolerance for mismatches between the target DNA and the guide RNA (Wolt et al., 2016). Some types of sgRNA have a high degree of specificity, whilst some are promiscuous (Wolt et al., 2016). In addition, the delivery of the complex, cell type and duration of exposure to the nuclease can also affect the number of off-target events (Cameron et al., 2017).

Many crops, e.g. maize, wheat and sugar beet have multiple sets of genomes (are polyploid) and multiple copies of genes organized in so called gene clusters (Nutzmann and Osbourn, 2014). This means there are similar and/or repeated genes, making it more likely that they are also subject to cleavage (Jung et al., 2017; Zhu et al., 2017). Hence, in contrast to first generation genetic engineering technologies, repeated or similar sections of DNA might be unintentionally changed during the genome editing process. Such unintended changes may not be close to the target gene, but could be at distant locations within the genome.

Overall, the tendency is for more off-target effects with the CRISPR-Cas9 technique in comparison to other SDN techniques, such as zinc finger and TALEN as these other systems use long recognition sequences (see, e.g. Zhu et al., 2017). Therefore, attempts are being made to make the CRISPR system more reliable, e.g. it appears the CRISPR-Cpf1 system has a higher specificity than CRISPR-Cas9, which also increases the possibilities to target more genes (Begemann et al., 2017; Mahfouz, 2017; Wang et al., 2017).

Off-target effects have been detected during experiments with several crop plants, including rice, soy and barley (Modrzejewski et al., 2019; Wolt et al., 2016; Zhu et al., 2017) and in farm animals such as pigs (Ryu et al., 2018), as well as model animals, rats and mice (Anderson et al., 2018; Shin et al., 2017). However, the detection of off-target effects can be confounded by genetic variation (Wang et al., 2018).

5.2 Unintended on-target effects

Even though the intended change to the target sequence of the genome may be achieved through genome editing, its impact might differ or there may be additional, unintended impacts to those

expected, i.e. it may cause unintended on-target effects. These unintended on-target effects relate to deletions and rearrangements of DNA, production of altered mRNA and proteins, and also interactions with other genes (including their regulation).

Large deletions and complex rearrangements of DNA have been reported during the CRISPR/Cas9 process (Kosicki et al., 2018). Even small insertions or deletions can lead to altered reading frames via disruption to alternative splicing mechanisms, resulting in exon skipping (Lalonde et al. 2017, Kapahnke et al. 2016). This misreading of DNA has the potential to produce aberrant proteins, confirmed by the detection of an aberrant protein resulting from the application of CRISPR/Cas9 to a laboratory culture of human cells (Kapahnke et al. 2016). In addition, large deletions induced by a single guide RNA were found to delete whole exons causing exon skipping in cell lines (Mou et al., 2017; Sharpe and Cooper, 2017). It is evident that CRIPSR/Cas9 is prone to causing inadvertent genomic abnormalities.

An example of how the application of CRISPR can unintentionally disturb the signalling pathways and gene regulation stems from medical research: it was recently shown that CRIPSR in cells can interfere with a ‘security-system’ of the cells. In general, DNA double strand breaks lead to the activation of the DNA damage response pathway and induce a cell cycle arrest in order to allow the cell to repair the damage. The DNA damage response pathway is activated and regulated by the tumour suppressor gene called p53. If the DNA damage causes severe alterations in the genome, p53 can induce apoptosis (programmed cell death), which leads to the elimination of damaged cells. Mutations in p53 lead to an increase in unwanted mutations within the genome (Rivlin et al., 2011). In humans, mutations in p53 are one of the main causes for the formation of cancer.

CRISPR-mediated double strand breaks also lead to the activation of p53 causing an arrest in the G1-phase of the cell cycle. Generally, the cell cycle can be divided in different phases: The mitosis which means the division of one cell into two daughter cells, and the G1-phase where the cells are growing and are producing RNAs and protein. This is followed by the so called S-phase where the DNA is duplicated and the G2-phase where the cells are prepared for the next mitosis. In human cell lines, mutations in p53 lead to a significant increase in the efficiency of the integration of DNA templates after cutting of CRISPR/Cas9 (Haapaniemi et al., 2018; Ihry et al., 2018). Under normal conditions the integration of foreign DNA only takes place during S-phase of the cell cycle (Symington and Gautier, 2011), which severely reduces the efficiency to introduce changes to the genome when the cells are stuck in the G1 arrest. It is under discussion whether p53 mutations can be used in general to increase the efficiency of CRISPR/Cas9, but this is a high-risk approach as harmful mutations might accumulate.

5.3 Detection of off-target and unintended on-target effects at the genomic level

With genome editing, unintended genomic changes are not limited to flanking regions of any insert but may also occur at distant locations to the targeted genes. Thus, detection of off-target effects and unintended on-target effects which occur at the level of the genome requires investigating the whole genome (Agapito-Tenfen et al., 2019). Whilst whole genome sequencing (WGS) can now be performed more-or-less routinely, this may miss some of the off-target effects if they are small changes. As yet, there is no validated, reliable test for detecting small off-target effects. A validated test requires a reference genome for the plant, and few exist at the level of detail required to use in a test for off-target effects (Wolt et al., 2016). Similarly, computational (or bioinformatic) approaches lack verification that their predictions of both target specificity and off-target locations for cleavage

by nucleases are correct (Wolt et al., 2016). The problem is compounded by the polyploidy of many crop genomes and duplication of genes, making it harder to detect off-target effects. However, advancement in detection methodologies for off-target effects from genome editing have recently been reported (Urnov, 2017; Zischewski et al., 2017) and further developments could make detection of off-target effects more robust. Guidance documents on test methodologies to detect off-target activity and associated unintended effects have yet to be developed (Agapito-Tenfen et al., 2019). The vast majority of studies using genome editing applications are looking for off-target effects in a biased way investigating solely at predicted *in silico* sites of the genome, while a sparse minority of these studies are using unbiased WGS approaches to identify off-target effects (Modrzejewski et al., 2019).

5.4 Unexpected effects at the organismal level

As with plants developed through first generation genetic engineering technologies, both intended and unintended changes can be important in terms of protein production and metabolism. Such unexpected effects can also occur in genome-edited animals. Thus, it is possible, even likely that, like first generation techniques of genetic engineering, ODM and other genome editing techniques can give rise to GM plants and animals displaying unexpected and unpredictable effects with implications for food, feed and environmental safety. Therefore, a risk assessment requires robust techniques for assessing unexpected effects in genome-edited organisms. A suite of techniques, collectively known as '-omics', could assist with the detection of unexpected effects (see Risk assessment related to the genome editing process).

5.5 Genome-edited farm animals

Currently, there are no commercial GM farm animals, and the only GM animal approved for food use is limited to a GM salmon in Canada and the U.S. (Bruce, 2017). The production of GM animals is thought to be limited by difficulties with first generation genetic modification techniques for animals (Bruce, 2017; West and Gill, 2016). In contrast, CRISPR is reported to have high efficiencies in animals (Ishii, 2017), meaning that there may be applications to market genome-edited farm animals as food.

Genome editing, particularly CRISPR/Cas has been applied to farm animals (e.g. pigs, cows, sheep, goats and chicken) in 'proof of concept' studies (Ishii, 2017). Examples of genome-edited animals include pigs resistant to a respiratory disease (Burkard et al., 2018), cattle without horns and ultra-muscular cows and pigs (Ishii, 2017). Problems remain with genome editing of livestock: if cloning is involved, this can lead to birth defects, abortions and early postnatal death and CRISPR creates a mixture of edited and unedited cells (mosaicism) in embryos (Tan et al., 2016). In addition to concerns regarding unexpected effects (see Unintended effects of genome editing) in the resultant genome-edited animals (which are in common with plants) there are specific ethical concerns relating to livestock.

In general, ethical concerns of genome editing animals are largely similar to those that have been raised for genetic engineering and/or cloning (Eriksson et al., 2018; Ishii, 2017; Bruce, 2017; Group of Advisers on the Ethical Implications of Biotechnology to the European Commission, 1996) and include:

• harm to animal health and welfare;

• impact on human health;

• that animals are being used as mere instruments for human benefit and interests;

• the infringement of animal ‘integrity’ or of the ‘intrinsic’ or ‘inherent’ value of animals;

• the viewpoint that it is ‘unnatural’ for example because it transgresses species boundaries;

• taking environmental risks - the consequences of which are difficult to calculate;

• the potential for a slippery slope towards eugenic applications on human beings.

These societal concerns suggest that many people are unlikely to accept products from genome-edited animals (Eriksson et al., 2018; Ishii, 2017).

EFSA have issued two guidance documents for GM animals. One on the environmental risk assessment (EFSA, 2013) and another on the safety aspects of food and feed derived from GM animals and their welfare (EFSA, 2012b). Neither guidance specifically covers farm animals, but are more related to GM fish and insects, whose applications for commercial use were regarded as looming at the time of the assessment. Similarly, neither guidance considers ethical concerns.

The environmental risk assessment (EFSA, 2013) considers that GM farm animals are likely to be in confined or semi-confined (e.g. unfenced pasture) conditions. However, contamination of food or animal feed with GM animals can, and has, occurred through accidental co-mingling or mislabelling (Price and Cotter, 2014). In the event that GM farm animals become commercialised, it is possible they could escape and either join or form new feral populations. The GM trait could spread through these populations, which then act as a gene pool with the potential to mate with farm animals causing GM contamination. Indirect effects on ecological systems are expected to be considered by applicants, but GM animals could also impact the environment via changes in the demands of the GM animals (e.g. increased percentage of protein in the diet) or human behaviour (e.g. the consumption of increased amounts of meat). These too would be indirect effects of GM animals.

Considerations of the welfare of GM animals is included in EFSA’s guidance (EFSA, 2012b). It stipulates that a wide range of assessment measures may be necessary to capture any unintended side effects (e.g. behavioural abnormality or an increase in disease susceptibility in a GM animal with enhanced). However, the definition of a “better” welfare for GM animals is of particular concern because the creation of GM animals (facilitated by genome editing) can help perpetuate, or even increase poor animal management. For example, if genome editing for disease resistance allows pigs to be kept in less hygienic or cattle without horns to be kept in more crowded enclosures (Bruce, 2017). Thus, what might appear a trait that improves animal welfare may, in practice, be detrimental to animal welfare.

6. Gene drives

The intention of gene drives is that a specific and artificial genetic condition is spread throughout a population of plants or animals, particularly insects (National Academies of Sciences, Engineering, and Medicine, 2016; Esvelt and Gemmell, 2017). They involve the release of a GMO population, carrying novel (inserted) genes, that is intended to mate with the non-GMO population to produce GMO offspring, carrying the novel genes and driving them through a population. 'Gene drives' are genetic elements that do not follow the Mendelian pattern of inheritance as they increase the probability that a specific genetic condition is being transmitted to the next generation above the normal 50% for sexual reproduction. With gene drives, contrary to most other applications of genetic engineering, the GMOs are not intended to be contained within in the laboratory or restricted to a single generation of hybrid plants. They are intended to genetically engineer wild (uncultivated) populations of animals and plants. In this backdrop, new layers of risk-related issues emerge including a lack of spatio-temporal control and disruptive processes that can affect whole species and/or associated ecosystems.

6.1 Potential applications of gene drives and current status

There are several potential applications for gene drives in wild populations (for an overview, see Critical Scientists Switzerland et al., 2019; Champer et al., 2016). Broadly, there are two types of gene drive applications. The first one aims to suppress or drive a population into extinction. The second can be summarized as 'population replacement' and aims to engineer specific biological characteristics in populations.

Gene drives are not necessarily new, but genome editing (see Genome editing techniques), particularly CRISPR, greatly facilitates their mechanisms. There are 'proof of concept' studies regarding the possibility of gene drives for yeast (Di Carlo et al., 2015), mosquitoes (Gantz and Bier, 2015; Hammond et al., 2015), flies (Champer et al., 2017; KaramiNejadRanjba et al., 2018) and mice (Grunwald et al., 2018). However, no gene drive has, as yet, been released into the environment, even as field trial. To date, the aims are to control diseases such as malaria, to drive invasive species to local extinction or to supress populations of weeds and pest insects in agriculture (National Academies of Sciences, Engineering, and Medicine, 2016). Most concepts attempting to make a whole population extinct are targeting one sex within the target population. This result (for example) can be achieved by gene drives that specifically target female fertility or the survival of female offspring to reduce populations or attempt to drive them into extinction (see Galizi et al., 2014; Gantz and Bier, 2015; Hammond et al., 2016; McFarlane et al., 2018).

It is generally considered that it will still take years before technological developments might get to the point of considering specific applications but there is considerable investment in this very active field of research (see, e.g. Courtier Orgogozo et al., 2017).

6.2 Risks of gene drives

Gene drives, no matter if supposed to replace or suppress a population, can give rise to genetically engineered populations that persist in the environment with little or no opportunity for recall. If persistence of genetically engineered organisms goes along with lack of spatio-temporal control, it becomes difficult or largely impossible to predict either the short-term or the long-term ecological

impact. Long-term, evolutionary processes make it possible for hazards with a low probability of ever happening to turn into events that may feasibly happen (Breckling, 2013). Consequently, performing a robust risk assessment may no longer be possible (see also Bauer-Panskus et al., 2013).

It is either very difficult or impossible to control any unintended effects or manage risks with gene drives. Classical methods in risk assessment such as a comparative approach or a step by step process cannot be applied successfully: for example, existing methods for biocontrol by using Wolbachia in mosquitos (Shaw et al., 2016) or conventional sterile insect technique (SIT) (see Reeves and Phillipson, 2017), cannot be considered as suitable comparative systems to predict long-term effects of synthetic gene drives.

There is a broad range of further negative or adverse impacts that require consideration, such as spontaneous transboundary movements, introgression into organic production systems in agriculture, socio-ecological and ethical considerations (see for example Critical Scientists Switzerland et al., 2019; Courtier Orgogozo et al., 2017; Esvelt and Gemmell, 2017; National Academies of Sciences, Engineering, and Medicine, 2016). As a consequence, there are many serious and valid concerns regarding uncontrolled spread of organisms with synthetic gene drives (see, e.g. Esvelt and Gemmell, 2017; National Academies of Sciences, Engineering, and Medicine, 2016; Taning et al., 2017).

Such is the concern over the potential adverse impacts of gene drives on the environment and agricultural systems, there have been calls for a moratorium on field trials and some laboratory research (Callaway, 2016; 2018). Because any GMO released as gene drives will inevitably cross international boundaries, the need for regulation at the international level, as well as the national level is recognised (Anon, 2017; Esvelt and Gemmell, 2017; National Academies of Sciences, Engineering, and Medicine, 2016). In 2018, the United Nations Convention on Biological Diversity agreed that, prior to any gene drive release (including experimental releases), a thorough risk assessment should be carried out and safety measures put in place to prevent potential adverse effects (Convention on Biological Diversity, 2018; Callaway, 2018). EFSA is currently assessing whether current guidelines for the environmental risk assessment of GMOs are sufficient for GM insects engineered with gene drives and what additional considerations may be necessary (EFSA, 2019b). This consideration is due to be finalized by March 2020 (EFSA, 2018b).

Prior to considering any release of gene drives, governments must also seek or obtain the approval of potentially affected indigenous peoples and local communities (United Nations Convention on Biological Diversity, 2018; Callaway, 2018). In Europe, the risk assessment for GMOs is science-based and conducted by EFSA, whose remit does not include societal aspects. Therefore, it is not clear how the approval of local communities could be sought as at present, there is no mechanism for societal consultation on GMOs in the EU. Since 2015, national EU governments can ‘opt-out’ of cultivation of a GM crop on societal grounds (European Commission, undated), but it is not yet clear whether this ‘opt out option’ would extend to gene drive GMOs.

Gene drives are intended to achieve permanent genetic changes to the make-up of wild populations of animals and plants. They also have potential to cause disruption to ecological and food production systems (National Academies of Sciences, Engineering, and Medicine, 2016). Therefore, it is difficult to envisage how the scrutiny required to deliver a risk assessment for gene drives can be fulfilled, especially considering long term impacts. Application of the precautionary principle, as enshrined in EU law (European Commission, 2000), would preclude the release of GMOs as part of a gene drive system.

7. Risk assessment for organisms developed through genome editing techniques

In the EU, genome-edited organisms are required to undergo both environment and food and feed risk assessments, as is required of first-generation GMOs (European Court of Justice, 2018). Risk assessment guidelines for GM plants and animals have been developed by EFSA for the environment (EFSA, 2010; 2013) and also for food and feed (EFSA, 2011, 2012b). Once current deficiencies in the risk assessment guidelines outlined in other RAGES chapters on the current risk assessment of GM crops have been rectified, these would, in general, be applicable to genome-edited organisms as many of the concerns associated with first generation GMOs also apply to organisms developed through new genetic engineering techniques. However, the risk assessment guidelines, both for environment and food/feed will have to be revised and expanded to ensure they capture all hazards associated with genome-edited organisms (Agapito-Tenfen et al., 2019; European Network of Scientists for Social and Environmental Responsibility, 2018). The risk assessment guidelines will have to undergo regular review and revision as genome editing techniques and their applications develop and as knowledge of the risks (e.g. of unintended effects) is gained.

In general, the risk assessment procedure falls short of identifying and quantifying risks to the environment, animals and humans because of incomplete knowledge of the organismal effects of genetic modification (intended or unintended); the receiving environment (e.g. ecology of the agricultural environment) and interactions between the GMO and the receiving environment. In such cases, the precautionary principle needs to be utilised as all GMOs potentially have adverse effects, but data are limited and scientific uncertainty remains high.

There are two broad categories of hazards relating to the risk assessment of GMOs. These are:

1) those related to the genetic engineering process and
2) those related to trait.

Both these categories require additional elements to be considered to include hazards specifically associated with genome-edited organisms.

7.1 Risk assessment related to the genome editing process

Genome editing can lead to unintended effects at the molecular level (as detailed in Unintended effects of genome editing above). These effects can arise as a consequence of two principal undesirable actions of genome editing: off-target effects and unintended on-target effects. As genome editing is such a new field, other ways in which unintended effects could arise may yet be discovered.

EFSA has, as yet, only issued an opinion on the risk assessment for SDN-3 genome-editing in plants (where genes are inserted) (EFSA, 2012c). EFSA has recently received a mandate to produce an opinion on whether the risks and hazards identified for a safety assessment of plants developed using SDN-3 are applicable in whole or in part to plants developed with SDN-1, SDN-2 and ODM as well. This scientific opinion is expected in 2020 (EFSA, 2019c).

EFSA's opinion on SDN-3 (EFSA, 2012c) considers that "*The main difference between the SDN-3 technique and transgenesis is that the insertion of DNA is targeted to a predefined region of the genome. Therefore, the SDN-3 technique can minimise hazards associated with the disruption of genes and/or regulatory elements in the recipient genome. Whilst the SDN-3 technique can induce off-target changes in the genome of the recipient plant these would be fewer than those occurring with most mutagenesis techniques. Furthermore, where such changes occur they would be of the same types as those produced by conventional breeding techniques*". However, in light of recent publications (since 2012), the opinion that genome-editing can "*minimize hazards*" or that changes would be "*of the same types as those produced by conventional breeding techniques"* requires revision as it is now clear that both off-target and unintended on-target effects can be far reaching, and possibly with important consequences for environmental, food and feed safety.

The consequences of these unintended effects for the risk assessment cannot be assessed *a priori* and are likely to be highly dependent on the actual unintended effect itself. Like genomic irregularities in GMOs produced by first generation genetic engineering methods, unintended effects in genome-edited crops could lead to a variety of unexpected effects. For example, the functioning of a particular gene may be compromised if its component DNA has been cleaved by the nuclease. This could lead to changes in the organisms' chemistry, including its metabolic and protein profile which, in turn, could affect its toxicity and allergenicity. As this would impact food, feed and environmental safety, it's important that any genome-edited organism is screened genome-wide for off-target effects and that any such effects that are detected are evaluated for their potential consequences prior to any deliberate release to the environment (including field trials) and placing on the market as food or feed (Fig. 1). This may involve further development of genome sequencing techniques (see Detection of off-target effects).

For both genome-edited and first generation GMOs, it is now apparent that genomic irregularities can occur at several levels, not only at the DNA level, but also the epigenome and RNA levels. Thus, a risk assessment requires information, not only of the whole genome and epigenome, but also of the products of that genome, i.e. RNA, protein and metabolites to assess the consequences of any genomic irregularities (Fig. 1). There are several techniques that can be used to assist assessment of the risks of genome-edited GMOs and improve the risk assessment of GMOs created by first generation techniques. These are collectively summarized as 'omics'-approaches and include profile analyses of the DNA (genomics), the RNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics). These techniques are either being, or could be, further developed to refine their capabilities to be used to analyse GMOs (Heinemann et al., 2011; van Dijk et al., 2014; EFSA et al., 2018). The first layer of interest is changes introduced to the DNA, which can be investigated by a multiple set of methods called whole genome sequencing (WGS). In order to analyse the transcribed RNA profile of a GMO, applications like RNA sequencing (RNA-seq) or microarray analyses can be applied (transcriptomics). Transcriptomics are particularly relevant as genome editing might lead to an altered reading frame of a gene, resulting in the production of changed mRNAs (e.g. via exon-skipping) or the disruption of regulatory elements such as non-coding RNAs (see Unintended on-target effects). The subsequent formation of altered proteins can be investigated by using multiple mass spectrometry approaches (proteomics).

Plants are constantly obtaining and using energy for the synthesis of biomolecules. Metabolism is the sum of all these reactions and needs to be balanced to maintain the life of an organism. Hence, unintended changes that act on metabolic pathways might cause an imbalance and could have a severe impact on the GMO itself or on an organism consuming it. Several techniques can be used to adequately analyse and compare the metabolic profile of a GMO and its unchanged counterpart in order to assess metabolic changes. Metabolic profiling characterizes the current status of all molecules involved in the metabolism using methods combining chromatography and spectrometry (Hong et al., 2016).

Genome-edited GM plants should also be analyzed in regard to the composition of their microbiome as the microorganisms colonizing the surfaces and inner tissues of plants play an important role for functional traits of the plant such as crop yield and nutrient quality. Furthermore, the microorganisms provide defense pathways influencing the coexistence of different species and consequently whole ecosystems (Berg et al., 2014). More research needs to be done further investigating the host-microbiome interaction and defining host-microbiome systems for crop plants with standardized microbial culture collections and reference genomes (Busby et al., 2017). Bioinformatics brings all these approaches together. However, computational evaluation of the resulting data has to be robust. Additionally, a profound computational analysis of already existing data sets regarding published knowledge in databases would be invaluable.

7.2 Unexpected effects arising from the insertion of genes, even if subsequently removed

Genome editing techniques, in common with reverse breeding and RdDM techniques typically insert genes through first generation genetic engineering techniques and then remove them after their function has been performed via backcrossing. In theory, the resultant plant therefore does not contain any novel genes, although the novel genes have enacted a change in the genomic material (usually DNA) of the plant. Nevertheless, in these cases it is important to verify that any inserted genes have actually been fully removed, including any backbone sequences (Eckerstorfer et al., 2019) and there has been no unintended integration of DNA from any plasmids containing the CRISPR complex (Kim and Kim, 2017).

Some developers of CRISPR-edited plants (see, e.g. Strauss and Sax, 2016), have claimed that risk assessment is not necessary because the resultant plant does not contain any novel genes. However, as discussed in Cisgenesis and Intragenesis, concerns arise from the act of inserting genetic material, which can cause irregularities in the genome. These include large genomic rearrangements and epigenetic alterations in the vicinity of the integration site (Jupe et al., 2019; Wilson et al., 2006). These irregularities can disrupt normal functions of the affected genes, e.g. potentially producing novel or altered proteins. The findings of Jupe et al. (2019) highlight the need to search for irregularities in both the genome and epigenome of a GMO, particularly in regions flanking integration sites. At present, EFSA does not require applicants to submit data on any epigenetic alterations for GMOs.

The concept of genomic irregularities arising from the insertion of genetic material is evident in the current risk assessment for first generation GMOs, as EFSA do not allow negative segregants to be used as sole comparators (EFSA, 2011):

"*For the Food & Feed risk assessment and the ERA of GM plants containing single events the EFSA GMO Panel confirms that the risk assessment must include a conventional counterpart. The EFSA GMO Panel also indicates the possible use of additional comparators, such as negative segregants, if deemed useful to support the risk assessment*." (EFSA, 2011)

It cannot be excluded that any genomic irregularities would remain in the organism after any inserted genes have been removed. Therefore, it would be important to include the possibility of genomic and epigenomic irregularities, including the production of unintended or changed RNA, proteins and metabolites also in the risk assessment of those GMOs (Fig. 1) where inserted genes may be subsequently removed, *inter alia* genome editing, reverse breeding and RdDM techniques.

7.3 Risk assessment related to the trait

Risk assessment related to the trait of a genome-edited organism is broadly similar to that which exists for GMOs developed using first generation techniques (see EFSA, 2010, 2011, 2012b). That is, the trait will need to be assessed for its environmental safety (e.g. *inter alia* toxicity to non-target organisms, potential changes to invasiveness) and human and animal safety (e.g. *inter alia* allergenicity). First generation GMOs generally consist of herbicide tolerant and insect resistant crops, and this is where the experience of assessing GMOs lies. By contrast, the traits that can, at least theoretically, be conferred by genome editing are highly varied (Eckerstorfer et al., 2019; Agapito-Tenfen et al., 2019) and the possibilities to alter the genome resulting in novel genetic combinations are more numerous (Kawall 2019). Thus, there is a requirement for studies to assess the potential impacts of traits other than herbicide tolerance and insect resistance (Fig. 1).

Eckerstorfer et al. (2019) reviewed the novel traits of GM plants developed by new genetic engineering techniques. Traits were grouped into three classes: 1) those related to traits in conventionally bred plants; 2) those with traits similar to established first-generation GM plants and 3) those which have been established neither in conventional nor other biotechnological methods. Prior knowledge may be insufficient and available information limited for many of these traits, and particularly those with no safe history of use. Eckerstorfer et al. (2019) suggested that, for each trait, it is important to consider, not only the modification itself, but the impact of the modification and the novel trait on the physiology and phenology of the GM plant. This suggests that, whilst it's important to both detect and assess unexpected effects at the organism level (see Unexpected effects at the organismal level), it may also be important to attribute these changes to either unintended genomic irregularities, or consequences of the novel trait.

Indirect effects may also arise from the trait itself. For example, what might be the implications to biodiversity from delayed flowering of a genome-edited plant, or the implications to the environment from super-muscly pigs that may require increased amounts of feed? These too need to be taken into consideration in a risk assessment.

7.4 Broadening the risk assessment

The risk assessment will require broadening to encompass the additional challenges posed by genome-edited plants and animals, as summarized in Fig. 1. The additional types of unintended genomic errors require expansion of the current examination of DNA to encompass examination of epigenetic changes and changes in the transcriptome, proteome and metabolome of the GMO. Such examinations will require further development, including protocols of WGS and -omics, and may be assisted by new analytical tools in the future (Fig. 1). Political will and research funding may be required to develop appropriate analytical tools. Importantly, guidance on the requirements of molecular characterisation for the risk assessment would need to be developed before any gene-edited organisms could be considered by EFSA.

Risk assessment related to traits will require additional knowledge of their consequences, which would be aided by further research. This may particularly be necessary for traits where experience with either current GM plants or conventional plants are lacking. There is a complete lack of experience in the risk assessment related to any GM traits in farm animals as there have not yet been applications for the marketing of GM animals, developed by either first-generation or genome-editing techniques. This too may require further research. Unlike the molecular characterisation, risk assessment related to the GMO trait may necessitate evaluation as applications with new traits

are made. However, it is difficult to see how a full risk assessment can be made within the six month time scale given to EFSA to issue an opinion on a GMO if there is no prior experience of the trait.

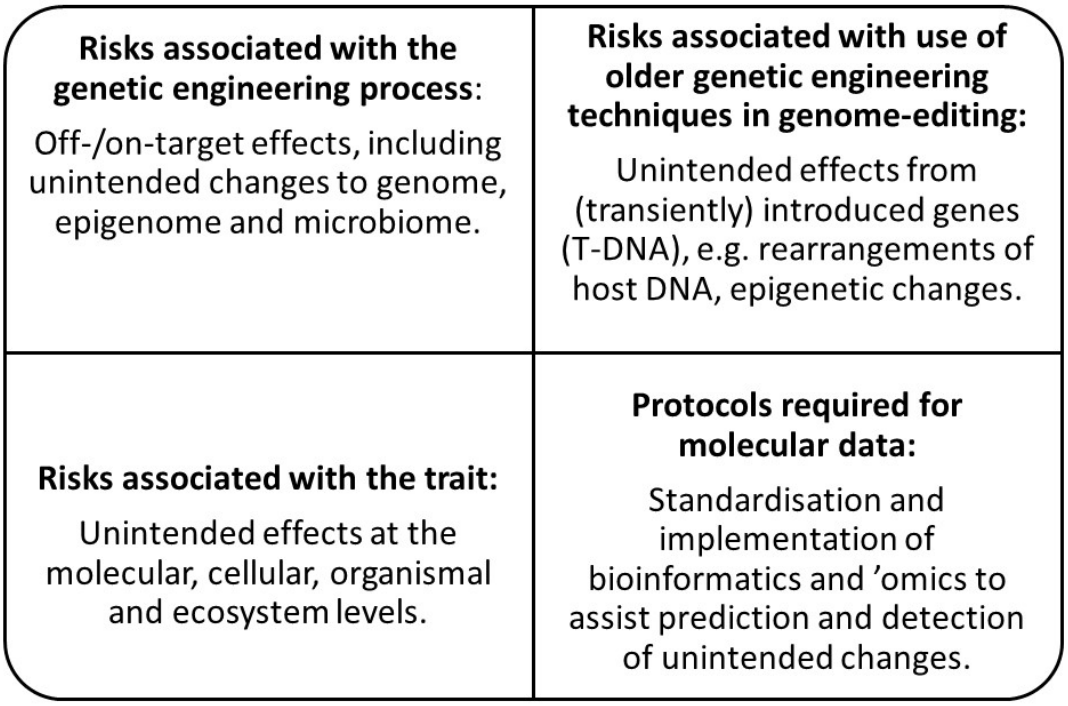

Figure 1. Elements of a risk assessment for genome-edited organisms.

7.4 Detectability of GMOs developed with new techniques

As with current GMOs, labelling of GMOs created by genome editing is necessary to enable consumer choice (Helliwell et al., 2017), and to protect agricultural systems that exclude GMOs, e.g. organic agriculture (IFOAM, 2016; Wickson et al., 2016). Detection of GMOs is a prerequisite to their labelling and also necessary to detect any contamination of plants or animals with GM varieties (Price and Cotter, 2014).

GMOs developed by genome editing are detectable, provided prior information is available regarding the intended genomic changes (European Network of GMO Laboratories, 2019; Scientific Advice Mechanism, 2017). However, the lack of common interest genetic elements, e.g. CaMV35S, which form the backbone of PCR screening for unauthorised GMOs (European Network of GMO Laboratories, 2019; Price and Cotter, 2014), in genome-edited organisms make it imperative that a detection protocol is needed for any environmental release, i.e. at the field trial stage for crops, rather than the commercialisation stage. This allows for third party verification that GM contamination has not taken place (Price and Cotter, 2014).

The development of further protocols (including advances in the robustness of WGS) and techniques is likely to facilitate better, cheaper and more reliable detection of small changes (e.g. one base pair changes) in genome-edited organisms (Bertheau 2019; Boutigny et al., 2019; Dobnik et al., 2018; Liang et al., 2018). These include bioinformatic tools for the analysis of DNA sequence data (Boel et al., 2016; Garst et al. 2017), and spectroscopy methods for differentiating between genome-edited and conventionally bred plant varieties (Liu et al., 2016). WGS approaches are being further improved to reduce background mutations that can accumulate during the production of the sequencing library in the lab and can be interpreted as false-positive mutations (Boutigny et al., 2019; Stahlberg et al., 2016). Another approach for plant identification is not to look for specific DNA sequences, but for the pattern of genetic changes within the genome (see, e.g. Nielsen and Voight, 2018). For example, Duensing et al., 2018 state: *"(…) genome editing is adept at knocking out genes present in multiple copies. Thus, whenever a crop is found with multiple copies of the same gene knocked out, it will be almost certain that genome editing was used.*" In consequence, they state: "*For most products of genome editing, there is a clear signature in the DNA, for instance the exact stretch of nucleotides erased. If that signature is revealed by the developer, the same PCR technology used for detecting GMOs can be applied to the detection and monitoring of genome-edited products in most cases."*

It is evident that advances in detection technologies are needed, not only for genome-edited organisms, but for other new genetic engineering techniques such as RdDM. Already networks of laboratories exist that coordinate and develop techniques to detect GMOs. In Europe, there is the European Network of GMO Laboratories (ENGL). ENGL has already discussed the issues surrounding detectability of new GMOs created by genome editing (European Network of GMO Laboratories, 2019) and concluded that further consideration is necessary. Therefore, there needs to be political will to develop suitable detection technologies. Regulatory requirements of traceability and labelling would be likely to spur research into developing new detection technologies.

8. Conclusions

Novel types of GMOs (e.g. RNAi-based GM plants) and synthetic genomics can give rise to new risks to environment, animal and humans health. Grafting, cisgenesis and intragenesis, reverse breeding and RdDM either utilise GMOs created using first generation techniques as an intermediary stage or can, in the case of agro-infiltration, unintentionally give rise to GMOs. Just like first generation techniques, new genetic engineering techniques described here (RdDM and genome editing) can produce unexpected and unpredictable effects in the resultant GMOs, even if any inserted genes are subsequently removed prior to commercialisation.

All genetic engineering techniques have the potential to induce unforeseen genomic interactions, genomic irregularities and unintended biochemical alterations. Therefore, it is important that any applications for cultivation (including field trials) and marketing of GMOs produced by these techniques undergo full environmental and health risk assessment.

For RNAi-based GM crops, major uncertainties and knowledge gaps exist, resulting in open questions remain on how to assess the risks of RNAi-based GM crops to both the environment and food and feed. Despite the lack of EFSA guidance on the risk assessment of RNAi-based GM crops, two RNAi-based GM crops have been approved for food use in the EU. This is not acceptable, and

it is strongly recommended that the issue of risk assessment guidance for new GMOs, particularly those developed by genome editing, precedes any consideration of applications to cultivate or market.

The current risk assessment guidance in the EU would need to be expanded in order to assess the additional unintended effects that genome editing can cause. The molecular characterisation element of the risk assessment will need to be expanded to include analysis for unintended changes at the genomic level, including off-target effects, unintended on-target effects and effects on genomic regulation. It will also require analysis of the transcriptome, proteome and metabolome. Such analysis would also improve the risk assessment of first generation GMOs. The risk assessment will need to consider a broader range of traits conferred by the genetic engineering process, for some of which there may be a lack of experience. It will need to consider direct and indirect implications for agricultural practices and ecological impacts caused by any changes in animal diets.

Gene drives represent a special case of GMOs, with the spectre of severe consequences for biodiversity. Performing a robust risk assessment, particularly in respect of long-term ecological effects would be highly problematic and it would be difficult, if not impossible to give safety assurances for any environmental release. In addition, it is not clear how the approval of local communities could be sought as at present (as required under the Conventional for Biological Diversity) there is no mechanism for societal consultation on GMOs in the EU. Application of the precautionary principle, as enshrined in EU law would preclude the release of GMOs as part of a gene drive system.

Further developments in technologies to detect off-target effects and unintended on-target effects caused by genome editing are needed, as are developments in technologies and to detect the resultant GMO organisms may be necessary in some cases. However, the technological problems are not insurmountable, and techniques can be developed if there is political will to do so.

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