Vitenskapskomiteen for mat og miljø
Norwegian Scientific Committee for Food and Environment

Genome editing in food and feed production – implications for risk assessment

Opinion of the Steering Committee of the Norwegian Scientific Committee for Food and Environment

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Preparation of the opinion

The Committee, by the Scientific Steering Committee appointed specifically for the assignment, assessed and approved the final opinion.

The Norwegian scientific Committee for Food and Environment (VKM) appointed six project groups consisting of ten VKM members, nine external experts, and six project leaders from the VKM secretariat to answer the Terms of Reference of this VKM self-initiated project. Three referees commented on and reviewed the draft opinion.

Authors of the opinion

The authors have contributed to the opinion in a way that fulfils the authorship principles of VKM (VKM, 2019a). The principles reflect the collaborative nature of the work, and the authors have contributed as members of the project groups and the VKM Scientific Steering Committee appointed specifically for the assignment.

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Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third-party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

Table of contents

Summary

The Norwegian Scientific Committee for Food and Environment (VKM) initiated this work to examine the extent to which organisms developed by genome-editing technologies pose new challenges in terms of risk assessment.

This report considers whether the risk assessment guidance on genetically modified organisms, developed by the European Food Safety Authority (EFSA), can be applied to evaluate potential risks of organisms developed by genome editing.

Background

Gene technology has allowed for the transfer of genes between organisms and species, and thereby to design altered genotypes with novel traits, i.e. GMOs. A new paradigm started in the early 2000s with the development of genome-editing techniques. Unlike traditional genetic modification techniques resulting in insertion of foreign DNA fragments at random locations in the genome, the new genome-editing techniques additionally open for a few single nucleotide edits or short insertions/deletions at a targeted site in an organism's genome. These new techniques can be applied to most types of organisms, including plants, animals and microorganisms of commercial interest.

An important question is how the novel, genome-edited organisms should be evaluated with respect to risks to health and the environment. The European Court of Justice decided in 2018 to include genome-edited organisms in the GMO definition and hence in the regulatory system already in place. This implies that all products developed by genome-editing techniques must be risk-assessed within the existing regulatory framework for GMOs. The European and Norwegian regulatory frameworks regulate the production, import and placing on the market of food and feed containing, consisting of or produced from GMOs, as well as the release of GMOs into the environment.

The assessment draws on guidance documents originally developed by EFSA for risk assessment of GMOs, which were drawn up mainly to address risks regarding insertion of transgenes. The new genome-editing techniques, however, provide a new continuum of organisms ranging from those only containing a minor genetic alteration to organisms containing insertion or deletion of larger genomic regions.

Risk assessment of organisms developed by genome editing

The present discourse on how new genome-editing techniques should be regulated lacks an analysis of whether risk assessment methodologies for GMOs are adequate for risk assessment of organisms developed through the use of the new genome-editing techniques. Therefore, this report describes the use of genome-editing techniques in food and feed production and discusses challenges in risk assessment with the regulatory framework.

Specifically, this report poses the question as to whether the EFSA guidance documents are sufficient for evaluating risks to health and environment posed by genome-edited plants, animals and microorganisms. To address these questions, the report makes use of case examples relevant for Norway. These examples, intended for food and feed, include oilseed rape with a modified fatty acid profile, herbicide-tolerant and pest-resistant crops, sterile salmon, virus-resistant pigs and hornless cattle. The report considers all aspects of the stepwise approach as described in the EFSA guidance documents.

Conclusions

The inherent flexibility of the EFSA guidance makes it suitable to cover health and environmental risk assessments of a wide range of organisms with various traits and intended uses. Combined with the embedded case-by-case approach the guidance is applicable to genome-edited organisms. The evaluation of the guidance demonstrates that the parts of the health and environmental risk assessment concerned with novel traits (i.e. the phenotype of the organism) may be fully applied to all categories of genome-edited organisms.

The evaluation of the guidance demonstrates that the parts of the health and environmental risk assessment concerned with the genetic modification (i.e. the genotype of the organism) may be fully applied to genome-edited organisms with inserted genes or long fragments of DNA. However, these parts are not fully applicable for genome-edited organisms with minor insertions, deletions or single mutations.

In summary, VKM finds that the EFSA guidance on risk assessment of genetically modified organisms provides a functional framework for risk assessment of genome-edited organisms. However, inclusion of specific considerations in the guidance regarding different properties of genome-edited organisms would be beneficial to ensure a common understanding between product developers and risk assessors regarding the type and extent of data needed to perform a risk assessment.

Determining how genome-edited organisms should be risk assessed and understanding how the regulatory landscape will form under the broad technological opportunities described in this report seems essential but fragile because of a range of uncertainties. The report offers some perspectives on topics that will benefit from further attention.

Key words: VKM, risk assessment, Norwegian Scientific Committee for Food and Environment, GMO, genome editing, food production, feed production, CRISPR, TALEN, ZNF, biodiversity

Sammendrag på norsk

I denne rapporten vurderer Vitenskapskomiteen for mat og miljø (VKM) utfordringer knyttet til helse- og miljørisikovurdering av genomredigerte organismer til mat- og fôrproduksjon. VKM har gått gjennom veiledningen for risikovurdering av genmodifiserte organismer (GMO) som Den europeiske myndighet for næringsmiddeltrygghet (EFSA) har utviklet, og vurdert om veiledningen også kan brukes til å vurdere risiko ved organismer som er utviklet ved genomredigering. VKM har selv tatt initiativ til denne rapporten.

Bakgrunn

Genteknologi har gjort det mulig å overføre gener mellom organismer og arter, og dermed designe organismer med nye egenskaper, det vil si genmodifiserte organismer (GMO). Tidlig på 2000-tallet førte utvikling av genomredigeringsteknikker til et paradigmeskifte. I motsetning til genmodifisering, hvor man stort sett setter inn artsfremmede DNA-fragmenter på tilfeldige steder i arvestoffet til en organisme, brukes de nye genomredigeringsteknikkene til å gjøre målrettede endringer i organismenes eget arvestoff. Dette gjøres ved å redigere, slette eller sette inn DNA-fragmenter. Disse nye teknikkene kan brukes i de fleste typer organismer, inkludert planter, dyr og mikroorganismer.

Et sentralt spørsmål er hvordan genomredigerte organismer bør vurderes med hensyn til risiko for helse og miljø. EU-domstolen besluttet i 2018 å inkludere genomredigerte organismer i GMO-definisjonen, og derved i regelverket for genmodifiserte organismer. Det innebærer at risiko ved genomredigerte organismer vurderes innenfor samme regelverk som produkter utviklet ved genmodifisering. Både europeisk og norsk regelverk regulerer produksjon, import og markedsføring/salg av mat og fôr som inneholder eller består av genmodifiserte organismer, og utsetting av genmodifiserte organismer til miljøet.

Ved hjelp av genomredigeringsteknikker kan man utvikle organismer med alt fra små genetiske endringer, tilsvarende endringer som oppstår spontant i naturen, til organismer som inneholder artsfremmed DNA. EFSAs veiledning for risikovurdering av GMO ble utviklet før de ny genomredigeringsteknikkene var tilgjengelige, og er derfor først og fremst utviklet for å vurdere risiko ved organismer som inneholder artsfremmed DNA.

Risikovurdering av organismer utviklet ved genomredigering

Den pågående debatten om hvordan genomredigerte organismer bør reguleres, har hittil i liten grad omfattet eventuelle utfordringer knyttet til risikovurdering.

For å vurdere om EFSAs veiledning er egnet for risikovurdering av genomredigerte organismer, har VKM tatt utgangspunkt i eksempler på genomredigerte planter, dyr og mikroorganismer som er relevante for Norge. Eksemplene inkluderer oljeraps med modifisert fettsyreprofil, jordbruksplanter som er resistente mot skadedyr og tolerante for ugressmidler, steril laks,

virusresistent gris og hornløse kyr. VKM har vurdert alle aspekter av den trinnvise tilnærmingen som beskrives i EFSAs veiledning.

Konklusjoner

Fleksibiliteten i EFSAs veiledning gjør den egnet til å dekke helse- og miljørisikovurdering av et bredt spekter av organismer med ulike egenskaper og bruksområder. Kombinert med veiledningens sak til sak tilnærming, fungerer den også for genomredigerte organismer. Evalueringen av veiledningen viser at de delene av helse- og miljørisikovurderingen som tar utgangspunkt i egenskap (dvs. fenotypen til en organisme), kan benyttes for alle kategorier av genomredigerte organismer.

Videre viser evalueringen at de delene av helse- og miljørisikovurderingen som tar utgangspunkt i genetisk endring (dvs. genotypen til en organisme), kan benyttes for risikovudering av genomredigerte organismer hvor det er satt inn gener eller lange fragmenter av DNA. Disse delene kan imidlertid ikke benyttes fullt ut for genomredigerte organismer med små innsettinger, slettinger eller enkeltmutasjoner.

Konklusjonen er at EFSAs veiledning for risikovurdering av genmodifiserte organismer er et funksjonelt rammeverk for risikovurdering av genomredigerte organismer. Imidlertid ville det være gunstig å inkludere aspekter som er spesifikke for genomredigerte organismer, for å sikre at produktutviklere og risikovurderere har en felles forståelse av type og omfang av data som trengs for å utføre en risikovurdering.

Det er fortsatt mye usikkerhet knyttet til forholdet mellom mulighetene de nye teknologiene gir, hvordan organismer utviklet med de nye teknologiene skal risikovurderes og det regulatoriske landskapet. Til slutt i rapporten diskuteres derfor noen temaer relatert til dette.

Stikkord: VKM, risikovurdering, Vitenskapskomiteen for mat og miljø, genomredigerte organimser, GMO, genomredigering, matproduksjon, fôrproduksjon, CRISPR, TALEN, ZNF, biodiversitet

Abbreviations and glossary

Abbreviations

ASIP	-Agouti signaling protein
BCE and CE	-Before Common Era and Common Era
BCO2	-Betacarotene oxygenase 2
BMPR-IB	-Bone morphogenetic protein receptor type IB
bp	-Base pair
BSE	-Bovine spongiform encephalopathy
Cas	-CRISPR-associated system
Cas9	-CRISPR associated protein 9
CRISPR	-Clustered regularly interspaced short palindromic repeats
CRISPRa	-CRISPR activation
CRISPRi	-CRISPR interference
crRNA	-CRISPR RNA
DNA	-Deoxyribonucleic acid
DSB	-Double-stranded breaks
EFSA	-European Food Safety Authority
ERA	-Environmental risk assessment
FAO	-Food and Agriculture Organisation of the United Nations
FDA	-United States Food and Drug Administration
FGF5	-Fibroblast growth factor 5
GxE	-Genotype-environment interaction
GM	-Genetically modified
GMO	-Genetically modified organism

HDR	-Homology directed repair
INDEL	-Insertions or deletions
IPN	-Infectious pancreatic necrosis
IR	-Insect resistance
KO	-Knockout
MAS	-Marker assisted selection
MCE	-Ministry of Climate and Environment
miRNA	-MicroRNA
MSTN	-Myostatin gene
NEA	-Norwegian Environment Agency
NFSA	-Norwegian Food Safety Authority
NHEJ	-Non-homologous end-joining
ODM	-Oligonucleotide-directed mutagenesis
ORF	-Open reading frame
PAM	-Protospacer-Adjacent-Motif, usually having the sequence 5'-NGG-3'
PGC	-Primordial germ cell
Prp	-Prion protein
PMEM	-Post-market environmental monitoring
PRNP	-Prion protein gene
PRRS	-Porcine reproductive and respiratory syndrome
PRRSV	-Porcine reproductive and respiratory syndrome virus
PUFA	-Polyunsaturated fatty acid
QTL	-Quantitative trait locus
RNA	-Ribonucleic acid
RNAi	-RNA interference

SCNT	-Somatic cell nuclear transfer
SDN	-Site-directed nuclease
sgRNA	-Single guide RNA
shRNA	-Short hairpin RNA
siRNA	-Small interfering RNA
SRY	-Testis-determining factor
TALENs	-Transcription activator-like effector nucleases
ToR	-Terms of reference
tracrRNA	-Trans-activating CRISPR RNA
UCP1	-Uncoupling protein 1
VKM	-Norwegian Scientific Committee for Food and Environment
ZNF	-Zinc-finger site-directed nucleases

Glossary

Backcrossing	Selection of a donor allele while converging to a genome identical to the recipient genome, usually within 10 generations of crossing.
Bacteriophage	A virus that infects bacteria.
Base editing	The process of producing single nucleotide changes without introducing double-strand breaks in the genome.
Breeding diagram	The scheme used to visualize the crossing or breeding that results in the final organism from the initial ancestors.
Callus	Undifferentiated plant tissue.
Cartagena Protocol on Biosafety	A global protocol for ensuring the safe transfer, handling and use of living modified organisms created through modern biotechnology.
Case-by-case approach	An approach that allows case-specific assessments to be made and data requirement to depend on the context. The case-specific assessments relate to all aspects regarding the organism, e.g. species, modification/edit, trait, environment etc.
Cisgene	A naturally occurring gene with its regulatory sequences that has been transferred between variants of the same species.
Cisgenesis	A genetic modification process whereby alleles of cisgenes are transferred from a sexually compatible species into a recipient organism.
Clone	An exact genetic copy.
Comparator	The non-modified conventional counterpart used as control to detect characteristical differences due to the modification.
Conspecifics	A member of the same species.
Event(s)	The inserted DNA that leads to a new trait after insertion into the genome of an organism in a single transformation process.

Ferals	Animals or plants existing in a wild state after escaping from domestication.
Founder F0	The ancestor or parental organism, where F1 is the first offspring generated from F0 and F2 the offspring from F1.
Gene knockout	Inactivation of a gene as a result of genetic changes (insertion/deletion/substitution).
Genetic introgression	The genetic exchange from one gene pool to another through hybridisation and backcrossing.
Genetic modification	The process of inserting novel DNA/genes from the same or foreign species or deleting genes. Common to all is the use of recombinant DNA technology.
Genome editing	The process of editing DNA with techniques such as CRISPR, ZNF and TALEN to target genetic changes to a specific location in a genome. Most often with the aim to change single nucleotides or produce short insertions/deletions (indels).
Genotype	The sum of all genes in an organism.
GM crops	Genetically modified crops - cultivated plants whose genetic characteristics have been changed usually by the insertion of modified and recombined genes from the same or from a different species using the techniques of genetic engineering.
Gonad	Reproductive gland.
Guidance	In this report the term is used to refer to one or several documents published by the European Food Safety Authority (EFSA) that outline specific approaches and considerations for risk assessment.
Herbicide	A chemical or other substance that is toxic to plants, used to destroy unwanted vegetation, e.g. weeds on agricultural land.
Hemizygous	A gene in a diploid organism is hemizygous when only one copy is present in the genome. The cell or organism is called hemizygote for the gene. In genetic modification, e.g. in transformation of plants, a transgene may be inserted on one DNA strand without an allelic counterpart. Designation (+/-).

Hybridisation	The act or process of mating organisms of different varieties or species to create a hybrid.
Hybrid vigor	Increased biological fitness in the offspring as a result of outbreeding two genetically different yet strong parental lines.
Inbreeding depression	Reduced biological fitness of the offspring as a result of low genetic variation and accumulation of homozygous undesired alleles.
Insecticide	A chemical or other substance that is toxic to insects, e.g. used to control pests that infest cultivated plants in agriculture.
Intragenesis	A genetic modification of a recipient organism with gene fragments from donor organism(s) of the same species as the recipient.
Keystone	An organism that other species depend on and that defines an entire ecosystem.
Line breeding	Specific breeding of animals with special characteristics to enhance this trait over several generations.
Locus/loci	A defined area of the genome that encodes a gene.
Loss of function	Change in phenotype as a result of a gene knockout.
Mosaic	Mosaicism in genetics is defined as the presence of two or more cell lineages with different genotypes arising from a single zygote in a single individual.
Null (negative) segregant	Plants that are negative segregants lack the transgenic event and can be produced, for example, by self-fertilisation of hemizygous GM plants, or from crosses between hemizygous GM plants and non-GM plants.
Off-target effects	Unintended effects when DNA is cleaved/altered at sites in the genome not intentionally targeted.
Oligonucleotide-directed mutagenesis	Genome editing technique using oligonucleotides to introduce one or a few nucleotide changes in the genome
Operon	A functioning unit of DNA containing a cluster of genes under the control of a single promoter.

ORF	Open reading frame, ORFs are roughly defined as spans of DNA sequence between start and stop codons, that encode an amino acid sequence (protein) according to the genetic code.
The PiggyBac transposon system	A mobile genetic segment used for integration of foreign DNA via a "cut and paste"-approach. It comprises two main elements; a donor plasmid carrying the gene of interest and a helper plasmid, expressing the enzyme piggyback transposase that facilitates movement of genetical content.
Pesticide	A chemical or other substance used to control target pest(s), e.g. weeds, insects and fungi.
Phenotype	The composite of an organism's observable characteristics or traits. An organism's phenotype results from two basic factors: the expression of an organism's genetic code, its genotype, and the influence of environmental factors.
Ploidy	The number of complete sets of chromosomes in a cell, which gives the number of allelic variants for each gene.
Post-market monitoring	A predefined strategy to monitor for possible adverse effects on human health and the environment. This monitoring is a key feature of the legislative framework on GM plants which is an important part of the cycle of measures in place to detect and limit possible adverse effects, including those that may occur over a long period of time.
Recombinant DNA	DNA that is formed by laboratory methods and combined by different sources.
Receiving environment	The environment that interacts with the organism upon release.
Site-directed nucleases	Enzymes that cleave double-stranded DNA at a targeted sequence.
SDN1	A category of genome-edited organism where the edited genome contains a single or a few base-pair

	changes after random repair of targeted double-strand breaks in the genome.
SDN2	A category of genome-edited organism where the edited genome contains single or a few defined base-pair changes after template-based repair of targeted double-strand breaks in the genome.
SDN3	A category of genome-edited organism where the edited genome contains longer DNA fragments inserted after template-based homologous repair of targeted double-strand breaks in the genome.
Stacked event(s)	Combination of two or more lines of genetically modified plants resulting in combination of transgenes in the same organism, e.g. both herbiced tolerance and insect protection.
Tailored RNA-guided alterations	Specifically altering gene expression, by e.g. of changing adenosine to inosine by the help of enzymes or RNA correctors. This leads to temporary genetic alterations that might be used in e.g. autoimmune disease therapy.
Traits	Characteristics, examples in plants are herbicide tolerance and insect/pest protection.
Transgene	A gene that is transferred from an organism of one species to an organism of another species by genetic engineering.
Transgenesis	A genetic modification of a recipient organism with gene fragments from donor organism(s) of different species as the recipient.
Trioploid	An organism with three sets of chromosomes, compared to the more usual diploid organism with two sets. Triploids are sterile due to impaired chromosome pairing at meiosis. Fish, like salmonids can become triploids by pressure or heat.
Unintended effect	Predicted or unpredicted effects from the genetic modification occurring at the genetic, organismal and/or environmental level

Vector	A vehicle, often a virus or a plasmid carrying desired DNA into a host cell and can also assist in multiplying or expressing the insert.
Volunteers	Plants not intentionally grown or cultivated, but self-sown plants from seeds or crop plants in the same area.
Zygote	Formation of a cell following fusion of two gametes, usually an egg cell and a sperm cell, in organisms that reproduce sexually.
Isogenic	Of similar origin, genetically near identical.

Background

VKM initiated this project to examine the extent to which organisms developed by genome-editing technologies pose new challenges in terms of risk assessment, detection and monitoring compared to genetically modified organisms.

The use of gene technology in food and feed production

Humans have always influenced the genetic composition of other species through selective hunting and harvesting, breeding and cultivation. Through time, we have sophisticated our techniques and become increasingly more efficient in shaping domestic organisms to better serve our needs. Selective breeding of animals and plants is a central aspect of human cultural history, and today, feeding the world population relies heavily on a few domestic animals and eight selectively bred crop species.

Recently, we have seen how new molecular understanding and technological advances have facilitated the production of genetically modified organisms (GMOs) (Box 1). Gene technology has allowed us to transfer genes between organisms and species, and thereby to design altered genotypes with novel traits. However, until very recently, this type of genetic modification was technically demanding and untargeted, resulting in insertions of recombinant DNA at random sites in the genome. A new paradigm emerged in the early 2000's with the development of genome-editing tools, e.g. TALENs (transcription activator-like effector nucleases) and ZFNs (zinc finger site-directed nucleases). The focus on the types of nucleases used quickly changed after 2012 with the discovery of the applied potential of the clustered regularly interspersed short palindromic repeats (CRISPR) and the associated protein (Cas9) system (Doudna and Charpentier, 2014) (Figure 1).

Since its discovery in bacteria (where it serves as a natural protection against viral attacks), the CRISPR system has been repurposed to facilitate targeted engineering of the genome in a wide variety of organisms. The CRISPR/Cas9 technology and other site-directed nuclease- (SDN) based techniques, such as TALENs and ZFNs, now represent the pinnacle of targeted genome-editing approaches. These methods combine the precise DNA-cutting abilities of different enzymes and the intrinsic DNA repair system of all cells to perform tailored alterations in the genome. The significantly reduced time, effort and costs associated with this method have changed genome editing from a niche technology into a mainstream method used in basic and applied life science research (Pramanik et al., 2021).

These new SDN-based technologies and CRISPR/Cas9 in particular can be applied to most types of organisms, including those of commercial interest. This report directs attention to the genome editing of plants and animals intended for food production. Scientists have now produced salmon without germ cells, virus-resistant pigs, hornless cattle, herbicide and pest resistant crops, amongst others (Box 7). In addition, altered nutritional composition profiles can be obtained by genome editing of e.g. rapeseeds with modified fatty acid profiles (Box 7).

Several crops with improved resistance to disease have been developed by knocking out-susceptibility genes, like apples resistant to fire blight disease. In Norway, scientists are now testing disease resistance in strawberries with a CRISPR-mediated knock-out of a gene involved in grey mould susceptibility. These examples show the potential for increased food and feed production or to promote environmental protection and animal welfare.

The power of this new approach and the ease with which it can be applied to various systems come with some potential risks to humans and the environment. This concerns the potential for an imbalance between rapid technological developments versus the possible health and environmental implications of novel genome-edited organisms. Understanding the risks to biodiversity and food safety and developing a consensus on assessing them is thus a key to ensure safe food and sustainable agriculture and aquaculture.

Unlike traditional genetic modification techniques that have relied heavily on the insertion of foreign DNA fragments, the new genome-editing technologies are primarily used to change the phenotype through a few single nucleotide edits or short insertions/deletions in an organism's genome. However, new phenotypes may also be produced by introducing targeted deletions (gene knockouts) or even without nucleotide changes to the genome at all, through epigenetic changes. The repertoire of SDN techniques also allows for larger DNA insertions that resemble the outcome of traditional genetic modification techniques (Pramanik et al., 2021). The boundaries between genetic modification and genome-editing are thus becoming increasingly hard to discern.

Prior to the development of site-directed nucleases, the term genetically modified organism was typically used to describe an organism that would carry foreign DNA introduced at random locations in their genome. Today, site-directed nuclease techniques can change as few as a single nucleotide at a targeted site in an organism's genome (Jinek et al., 2012). However, site-directed nucleases can also be used efficiently to introduce the same extensive changes as those achieved through established genetic engineering techniques, thereby excluding a concise definition of products produced by genome editing (Pramanik et al., 2021).

The broad opportunities for various forms of genome-engineering and -editing offered by site-directed nucleases have triggered an international debate on how they fit into the regulation of GMOs or if certain uses warrant a different regulatory approach (Box 3 and 4).

This report describes the use of genome-editing techniques in food and feed production and discusses challenges regarding risk assessment approaches concerning effects on human and animal health and the environment. The report will be of interest to those concerned with or involved in risk assessment, as well as regulatory bodies and risk managers relying on scientific advice. Key concepts used in the report are listed in box 2.

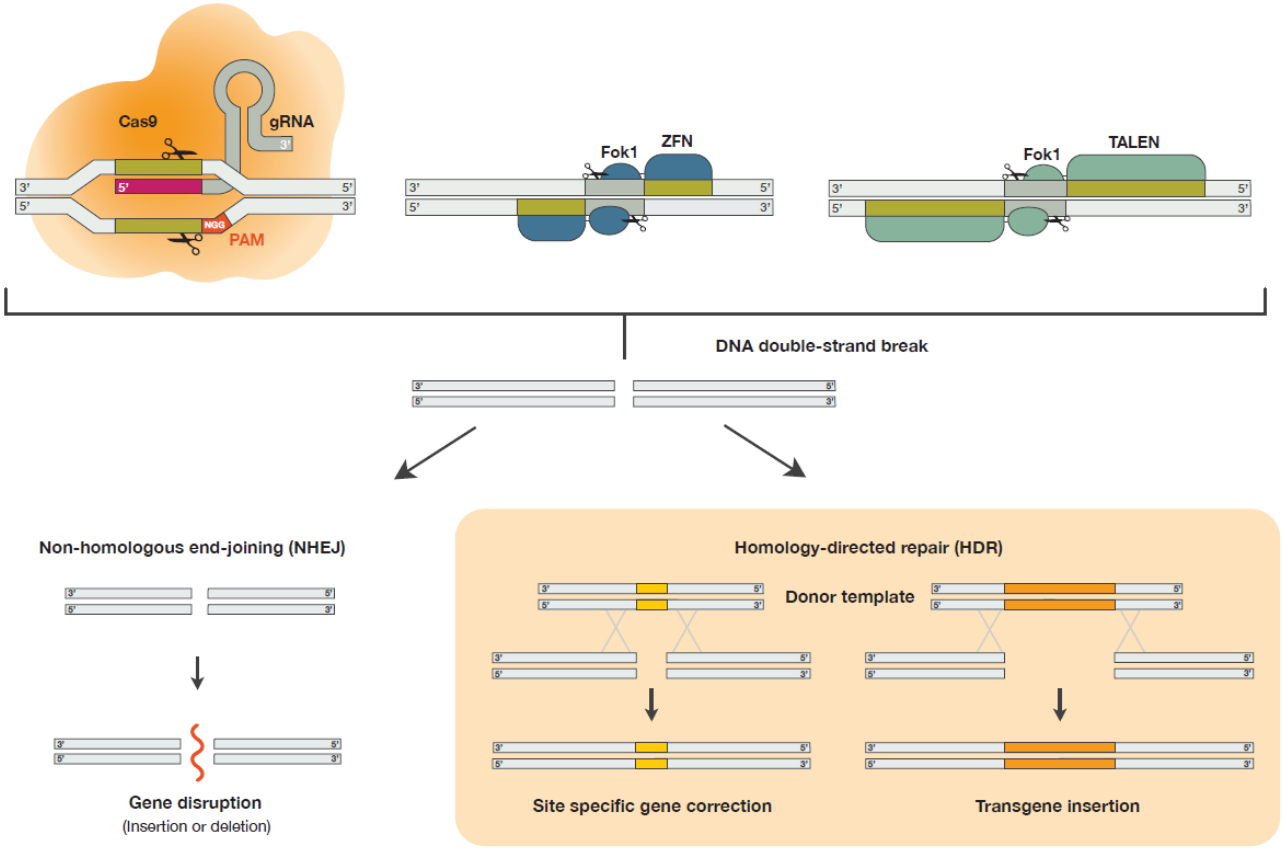

Figure 1. The outcome of genome editing with engineered site-directed nucleases (CRIPSR, ZFNs and TALENs). The starting point for each genome editing is that site-directed nucleases (SDN) - "molecular scissors" - cut DNA at specific sites directed by their DNA-binding moiety, introducing a double-strand break (DSB) which triggers cellular DNA repair mechanisms. If no template (donor DNA) is added, the induced break is repaired using NHEJ (non-homologous end joining) pathway leading to gene disruption. If a homologous repair template containing one or several single nucleotide variants is added, the break is repaired using HDR (homology directed repair) pathway resulting in gene correction. If the added template contains DNA insertions flanked by sequences homologous to the target DNA site, the construct is inserted by either HDR or NHEJ resulting in transgene insertion.

Box 1.

The definition of a genetically modified organism (GMO)

It is recognized that there is no single universal definition of a GMO and that different entities and national institutions offer different definitions. Key definitions important in a Norwegian context are those provided by the Norwegian Gene Technology Act, the EU regulatory framework with its directives and regulations, the Cartagena Protocol on Biosafety under the Convention on Biological Diversity, and the UN Environment Program.

Cartagena Protocol

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

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

"Modern biotechnology" means the application of a) *In vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or b) Fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection (CBD, 2021).

EU regulatory framework on GMOs

The key definition can be found in article 2 of Directive 2001/18/EC: ”Genetically modified organism (GMO) means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination” with further elaboration in annexes.

Directive 2001/18/EC on the deliberate release into the environment of Genetically Modified Organisms (EC, 2001).

For a more extensive overview of the definitions and regulatory context see (EC, 2010; EC, 2021c).

Norwegian Gene technology Act

(Government.no, 1993)

§ 4 Definitions

For the purposes of this Act, the following terms shall have the following meanings:

a. microorganism: any microbiological entity, cellular or non-cellular, that is capable of replication or of transferring genetic material

b. genetically modified organism: a microorganism, plant or animal in which the genetic material has been altered by means of gene or cell technology

c. gene technology: techniques that involve the isolation, characterisation and modification of heritable material and its introduction into living cells or viruses

d. cell technology: techniques for the production of living cells with new combinations of genetic material by the fusion of two or more cells

e. cloned animal: an animal that is genetically identical or almost identical to another animal

f. animal cloning: any technique for producing animals with identical or almost identical genetic material.

Box 2.

Key concepts used in the report

Genetic modification

The process of inserting novel DNA/genes from the same or foreign species or deleting genes. Common to all is the use of recombinant DNA technology.

Genome editing

The process of editing with techniques such as CRISPR, ZNF and TALEN to target genetic changes to a specific location in a genome. Most often with the aim to change single nucleotides or produce short insertions/deletions (indels).

Base editing

The process of producing single nucleotide changes without introducing double-strand breaks in the genome. The technique can also be used to make changes in the epigenetic pattern (e.g. methylation) at targeted genome sites.

Site-directed nucleases

Group of enzymes that are capable of targeted cleavage of a double-stranded DNA molecule/genome, based on recognition of a defined nucleotide sequence. The main site-directed nucleases (SDN) are ZFNs, TALENs and Cas of the CRISPR system (Figure 1). They are usually engineered forms of enzymes found in bacteria. The outcome of their use has been categorised in 3 groups (EFSA, 2012c):

SDN1

Category of genome-edited organism where the edited genome contains a single or a few base-pair changes after random repair of targeted double-strand breaks in the genome.

SDN2

Category of genome-edited organism where the edited genome contains single or a few defined base-pair changes after template-based repair of targeted double-strand breaks in the genome.

SDN3

Category of genome-edited organism where the edited genome contains longer DNA fragments inserted after template-based homologous repair of targeted double-strand breaks in the genome. This edit may resemble classic transgene-based modification but avoids issues with random DNA insertions, vector sequences and unintended foreign DNA.

ODM Oligonucleotide-directed mutagenesis (ODM) can be used to insert minor edits into the nucleotide sequence (Figure 10). Various versions of ODM have been developed. In the field of agriculture, it is often referred to as Rapid Trait Development System (RTDS) technology.

Off-target activity

The use of site-directed nucleases may in some cases cause DNA cleavage at sites in the genome not intentionally targeted. Such unintended effects are called off-target effects. The occurrence of such effects mainly depends on the enzymatic characteristics and cellular context of the SDN technology used. Causes of off-target activity include the presence of similar nucleotide motifs elsewhere in the genome, lack of 100% specificity of the SDN used, as well as mechanistic aspects of the nuclease delivery technology used and how it will control nuclease concentration etc. Double-stranded breaks occurring off-target may be repaired through normal cell repair mechanisms and can result in nucleotide changes, rearrangements or indels at those sites (Modrzejewski et al., 2019).

Cisgenesis, intragenesis and transgenesis

Cisgenesis is the genetic modification of a recipient organism with a gene from a crossable sexually compatible organism (same species or closely related species). This gene includes its introns and is flanked by its native promoter and terminator in the normal sense orientation (EFSA, 2012b).

Intragenesis is a genetic modification of a recipient organism that leads to a combination of different gene fragments from donor organism(s) of the same or a sexually compatible species as the recipient. These may be arranged in a sense or antisense orientation compared to their orientation in the donor organism.

Transgenesis is a genetic modification introducing an exogenous or modified gene (transgene) into a recipient organism of a different species from which the gene is derived.

The word "guidance" in this report

There are several EFSA guidance documents for risk assessment of GMOs available. These guidance documents are developed by the EFSA GMO Panel and provides a set of both requirements and recommendations of experimental data needed for a comprehensive risk assessment. The areas covered include molecular characterisation, toxicity, allergenicity, nutrition and environmental risk assessment. In this report the five main EFSA guidance documents for GMOs have been considered; 1) Guidance on risk assessment of food and feed from genetically modified plants, 2) Guidance for risk assessment of food and feed from genetically modified animals and on animal health and welfare aspects, 3) Guidance for environmental risk assessment of genetically modified plants, 4) Guidance on the environmental risk assessment of genetically modified animals, and finally 5) Guidance for risk assessment of genetically modified microorganisms and their products intended for food and feed use. These are the core set of EFSA guidance documents referred to in the report, outlining the main areas of concern and principles behind the assessments. It is acknowledged that the approaches developed in these guidance documents are continually refined/amended through subsequent Opinions and Technical notes published by EFSA. As of October 2021, more than 20 documents are available and applicable (EFSA, 2021b).

The case-by-case approach in risk assessment

One of the fundamental concepts in the EFSA guidance documents is the case-by-case approach. This approach allows case-specific assessments to be made and data requirements to depend on the context. In the case of risk assessment of genetically modified or genome-edited organisms, the organism, derived product and intended uses can vary substantially. It is not realistic to develop a detailed guidance that can cover all aspects for all possible uses. Hence, the guidance will necessarily have to be generic. The various areas of concern presented in the guidance may then be considered for their relevance on a case-by-case basis. The case-specific assessments relate to all aspects regarding the organism, e.g. species, modification/edit, trait, and uses etc.

The regulation of GMOs in the EU and Norway

The following sections concerning regulation on GMOs for food and feed uses, briefly reiterates aspects of the complex regulatory landscape of GMOs in the EU and in Norway. Comprehensive information can be found in the referred documentation and links throughout the text.

The European and Norwegian regulatory frameworks regulate the production, import and placing on the market of food and feed containing, consisting of or produced from GMOs, as well as the release of GMOs into the environment (box 3 and 4). The frameworks are interdependent and are all process oriented. The use of certain gene technologies to develop a product will trigger the regulatory framework and the regulated status, inter alia that authorization is required before placing on the market. The Court of Justice of the EU decided in 2018 that organisms obtained by genome editing techniques are also defined as GMOs (EU, 2018). Hence, organisms developed by new genome editing techniques are also subject to the obligations laid down by the EU legal framework.

The EU legal frameworks on GMOs secure that no genetically modified organism or products from GMOs, can be placed on the market before it has been granted an authorisation. To this end, a scientific risk assessment is performed by the European Food Safety Authority (EFSA), in cooperation with the scientific bodies of the Member States. EFSA evaluates the safety of GMOs on a case-by-case basis before they can be authorised for use as food or feed and/or for import and processing, or cultivation in the EU. Authorisations are granted for a ten-year period by the European Commission through a centralized procedure under Regulation No. 1829/2003 (EC, 2003a) or Directive 2001/18/EC (EC, 2001). The frameworks regulate genetically modified plants, microorganisms, and animals. GMOs are assigned a unique identifier, and food or feed consisting of, containing, or produced from GMOs are mandatory labelled to ensure traceability and enable consumers to make informed choices.

The EU Directive 2001/18/EC on deliberate release into the environment of genetically modified organisms is implemented in the EEA Agreement (European Economic Area Agreement) and transposed into the Norwegian Gene Technology Act. Norway is therefore affiliated with the GMO authorisation process in the EU for applications submitted under the directive (mainly products other than food and feed). The Regulation 1829/2003/EC concerning genetically modified food and feed is currently not a part of the EEA Agreement. However, in preparation for a legal implementation of the Regulation in Norwegian law, Norway adheres to the EU proceedings for GMO applications.

Current debate on regulation of GMOs in the EU and Norway

The European Court of Justice decision in 2018 (Van der Meer et al., 2021), that included genome edited organisms in the GMO definition and hence the regulatory system in place, sparked a debate about the suitability and continued use of the regulatory system for GMOs. The debate often emerges from various perceptions on the suitability of process- versus product-based approaches to safety assessments. Further, the current lack of international

harmonization has resulted in national decision making with various assessment provisions in e.g. the US, Japan, Argentina, Australia and others (Menz et al., 2020; Thygesen, 2019; Van der Meer et al., 2021). This heterogeneous landscape of regulatory approaches taken at the national level combined with a rapidly developing technology, new commercial opportunities, and lack of standardized terminology for new product categories, currently represent a substantial uncertainty for developers, producers and consumers. At the core, international trade requires transparency and consistent regulations. In this context, the Norwegian Biotechnology Advisory Board, on its own initiative, presented in 2018 its proposal for how GMOs could be regulated (Bioteknologirådet, 2018a). The board suggested that the requirements for risk assessment and approval could be differentiated in a tiered system based on the genetic change made. However, details enabling a regulatory categorisation, as well as other topics including the relationship to EU legislation, definitions and terms, and risk assessment, were not fully addressed in the board's proposal.

The principles for regulation of GMOs in both EU and Norway were developed in the 1990s (Van der Meer et al., 2021). Now in 2021, there are ongoing processes for considering possible regulatory amendments to the GMO frameworks, both in the EU and in Norway. In December 2020, the Norwegian Ministry of Climate and Environment assigned a Public Committee to assess questions related to gene technology. The mandate of the Committee is to prepare an updated knowledge base in the field of gene technology, and to consider amendments to the legal national framework. The report is expected in June 2022 (Government.no, 2020) . In late April 2021, the European Commission informed the public that processes for discussing a new legal framework for new genomic techniques will be put in motion (EC, 2021d).

Box 3.

The GMO legislation in the EU

The following sections concerning regulation on GMOs for food and feed uses, briefly reiterates aspects of the complex regulatory landscape of GMOs in the EU. Comprehensive information can be found in the referred documentation and links throughout the text.

Extensive legislation pertaining to GMO is in place in the EU with the aim to protect health and the environment. The EU legislation has several legal instruments for authorisations of GMOs and products from GMOs (EC, 2021c), three of these are:

1) Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms (EC, 2001),
2) Regulation (EC) No 1829/2003 on genetically modified food and feed (EC, 2003a), and
3) Regulation (EC) 1830/2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms (EC, 2003b).

These instruments specify the authorisation process behind GMOs or derived products intended for placing on the market in the EU, and requirements for labelling and traceability of GMOs. The basis for authorisation is a scientific assessment of the risks to health and the environment. Once authorised, GMOs and derived products can be placed on the whole EU market. A full overview of the EU GMO legislation can be found at the European Commission's GMO webpages (EC, 2021c).

The regulatory process under both frameworks (for products intended to be placed on the EU market) starts with the applicant (the notifier) submitting a dossier containing the material specified in the regulatory framework to the national competent authority. These specifications have been further articulated in the extensive set of EFSA guidance documents available (see Box 6), assessing potential risk to human or animal health, animal welfare and the environment.

Public engagement in the GMO authorisation process is secured by public consultation, and all EFSA risk assessments are publicly available. Overview over GMO applications can be found online, here (Register 2001/18/EC) for applications under the Directive and here (Register (EC)1829/2003) for applications under Regulation 1829/2003.

After the risk assessment procedure is finalised, the Commission prepares a draft implementing decision of authorisation to the Member States, and this draft decision is voted over by the Member States in Committee meetings under the applicable framework. When no qualified majority is obtained, is it up to the Commission to decide on authorisation of the GMO. In many cases it takes several years from an application is submitted to a decision is made.

Directive 2001/18/EC

Directive 2001/18/EC regulates the deliberate release of GMOs into the environment (field trials for research purposes, cultivation, and commercial use of GMOs). The term "release" is understood broadly and covers all aspects of GMO applications that do not have specific containment measures

Directive 2001/18/EC is, as mentioned above, implemented in the EEA Agreement and transposed into the Norwegian Gene Technology Act. Applications submitted under the Directive are therefore processed in Norway and a decision is made by the Norwegian Government (more details in Box 4).

Regulation 1829/2003

Regulation 1829/2003 regulates GM food and feed for placing on the EU market, and applicants can also apply for cultivation purposes under this regulation.

This regulation is not implemented in the EEA Agreement, But, as mentioned above, Norway follows the EU proceedings, and VKM performs risk assessments of GMOs applied for under the Regulation /commissioned by the Norwegian Food Safety Authority and the Norwegian Environment Agency.

Today, GMO applications for placing on the market are in large received under Regulation 1829/2003. Most GMO risk assessments performed by VKM fall under Regulation 1829/2003.

Regulation 1830/2003

"This regulation puts in place rules to ensure products containing GMOs and food and animal feed derived from them can be traced at all stages of the production and distribution chain. The rules cover labelling, monitoring environmental and health risks, and the ability to withdraw products where necessary." (EC, 2003b).

Possibility to prohibit cultivation of GMOs nationally

In March 2015, the European Parliament and Council adopted new EU rules allowing Member States to prohibit or restrict the cultivation of genetically modified seed or plant propagating material on their territory, even if such plants have already been authorised for cultivation in the EU. Provisions are laid down in Directive (EU) 2015/412 (EC, 2015)(EC, 2015). This so-called opt-out measure is meant to resolve the current Member States' conflict about gene technology by facilitating differences of states concerning cultivation regulations.

Several countries have used the opportunity to restrict the scope of GMO authorisations in Europe (EC, 2021e).

Box 4.

The GMO legislation in Norway

The following sections concerning regulation on GMOs for food and feed uses, briefly reiterates aspects of the complex regulatory landscape of GMOs in Norway. Comprehensive information can be found in the referred documentation and links throughout the text.

In Norway, the use of GMOs and derived food and feed are regulated under these two acts with associated regulations:

1) The Gene Technology Act (Government.no, 1993), and
2) The Food Act (Government.no, 2003).

The purpose of the Gene Technology Act is to ensure that the production and use of GMOs and the production of cloned animals take place in an ethically justifiable and socially acceptable manner, in accordance with the principle of sustainable development and without adverse effects on health and the environment. The provisions of the Act also apply to substances and products that consist of or contain GMOs. Additionally, there are requirements for labelling and traceability of GMOs.

The purpose of the Food Act is to ensure safe and wholesome food, to promote health, quality and consumer concerns along the whole production chain, and to provide for sustainable production. The Act is also intended to promote sound plant and animal health. Processed and derived genetically modified products for food and feed are regulated by different provisions founded on the Food Act. The provisions lay down authorization and labelling requirements, were the labelling requirements concern both derived and living GMOs for food and feed. These requirements are more or less harmonized with the requirements in Regulation (EU) No 1829/2003 on genetically modified food and feed. In addition, specific provisions prohibit GMOs for food and feed containing genes from genetically modified organisms coding for resistance to antibiotics.

Application processing

The Norwegian Environment Agency has the overall responsibility for processing applications for the deliberate release of GMOs. This entails *inter alia* coordinating the approval process, and to make an overall assessment and recommendation to the Ministry of Climate and Environment regarding the final authorization process in Norway. The Agency is responsible for assessing environmental risks upon the deliberate release of GMOs and to assess the product's impact on sustainability, benefit to society and ethics under the Gene Technology Act.

The Norwegian Food Safety Authority is the Competent Authority for processing applications for derived GMOs for food and feed. The Authority is responsible for assessing risks to human and animal health for derived GMOs for food and feed and upon the deliberate release of GMOs pursuant to the Food Act and Gene Technology Act, respectively. Furthermore, the Authority is responsible for assessing co-existence and environmental risks related to changes in agricultural practice when cultivating GMOs.

Box 5.

GMOs currently approved for food and feed uses and cultivation in EU and Norway

More than 60 genetically modified plants are authorised for the use as food or feed or for derived products in EU. These are 34 variants of maize, 21 soybean, 13 cotton, 5 oilseed and one variant of sugar beet (registered as of Feb 2021 in the GMO register by the European Commission) (Register 2001/18/EC; Register (EC)1829/2003). The introduced traits in these plants are primarily heightened tolerance towards herbicides, notably glyphosate and glufosinate-ammonium, and resistance to foraging larvae of certain crop pests of beetles and butterflies. Practically all genetically modified plant material imported in the EU are used as animal feed. Only one genetically modified plant is authorised for cultivation in the EU, the insect (European corn borer) resistant maize MON 810, for which an application for continued authorisation (renewal) is currently ongoing under Regulation 1829/2003. Several EU member-states have used Directive (EU) 2015/412 to restrict and prohibit cultivation of genetically modified plants in their territories (EC, 2015).

At present in 2021, only cut flowers of six variants of genetically modified carnation for ornamental purposes are authorised in Norway. No GMOs are authorised for food or feed uses nor cultivation (NEA, 2021).

The risk assessment process of GMOs in EFSA and VKM

EFSA is funded by the European Union to provide independent scientific advice and communication on risks associated with the food chain. It is working through its Scientific Committee and various standing Scientific Panels. The GMO Panel provides advice through its opinions on the health and environmental safety of specific genetically modified organisms for placing on the market.

EFSA assesses possible risks from GMOs to human and animal health, and the environment, in collaboration with member states. EFSA's risk assessment of a GMO is based on the documentation presented by the applicant and other relevant scientific information. EFSA has prepared several guidance documents for the risk assessment of GMOs (Box 6).

EFSA applies the criteria laid down in the EU regulatory framework as decided by the European Commission when evaluating the safety of a GMO. The GMO risk assessments consider the following aspects: molecular characterisation, comparative analysis, evaluation of potential toxicity and allergenicity and evaluation of potential environmental impact. Under EU legislation, applications for import and processing, cultivation or breeding of GMOs must contain a plan for detailed post-market environmental monitoring (PMEM). This plan should describe how the GMO will be monitored for possible adverse effects on the environment. Taken together, environmental risk assessment and PMEM are important parts of the measures in place to protect the environment. In addition, a validated protocol for detection is needed, and reference material must be provided to the EU reference laboratory for GM food and feed (EC, 2021b).

In Norway, VKM carries out health and environmental risk assessments of GMOs and products containing, consisting of, or produced from GMOs applied for approval in the EU under Directive 2001/18/EC or Regulation 1829/2003/EC. The risk assessments are performed on behalf of the Norwegian Food Safety Authority and the Norwegian Environment Agency (VKMs assignment, 2020). The VKM assessments form a key part of the documentation supporting the national GMO approval process (approval process only for GMOs applied for under the Directive, as Regulation 1829/2003 is not yet implemented, see Box 3). The Norwegian Biotechnology Advisory Board evaluates ethics, societal benefit and sustainability according to the Norwegian Gene Technology Act.

The VKM GMO Panel evaluates GMOs with reference to its intended use in the EEA, and according to the principles described in relevant national and EU frameworks. VKM also takes into account the appropriate principles described in the EFSA guidance documents for risk assessment of GMOs and derived food and feed, and the environmental risk assessment of GMOs, as well as other supporting documents developed by EFSA (Box 6).

Box 6.

The Guidance documents developed by the European Food Safety Authority (EFSA)

The GMO Panel develops general guidance documents that detail what type of scientific data and other information that must be considered when preparing applications (EFSA, 2021b).

These documents (Guidance and Opinions) are regularly updated. Some key examples are provided below:

- **Guidance on risk assessment of food and feed from GM plants**
- Human dietary exposure assessment to newly expressed proteins in GM foods
- Guidance on allergenicity assessment of genetically modified plants
- **Environmental risk assessment of GM plants**
- Guidance on the agronomic and phenotypic characterisation of genetically modified plants
- Guidance for renewal applications of genetically modified food and feed authorised under Regulation EC 1829/2003
- Guidance for the risk assessment of the presence at low level of GM plant material in imported food and feed
- **Risk assessment of GM microorganisms and their products intended for food and feed use**
- Risk assessment of GM plants used for non-food or non-feed purposes
- **Risk assessment of food and feed from GM animals and on animal health and welfare aspects**
- Guidance on Post-market environmental monitoring (PMEM) of GM plants
- Guidance on the selection of comparators
- Opinion on potential impacts on non-target organisms
- Opinion on the assessment of allergenicity of GM plants and microorganisms
- Opinion on statistical considerations including field trials
- Report on animal feeding trials
- **Guidance on the environmental risk assessment of genetically modified animals**

It is important to acknowledge the dynamic and continually evolving nature of the guidance provided by EFSA. Various documents update and amend comprehensive and broad guidance documents developed at an early stage. These main guidance documents (marked in bold script above) outline the risk assessment approach, the areas of concern, as well as the stepwise and case-by-case nature of assessments. Hence, they are the main focus of this report. Other relevant documents by EFSA in this context include:

- Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function (EFSA, 2012c)
- Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis (EFSA, 2012b)

- Applicability of the EFSA Opinion on site-directed nucleases type 3 for the safety assessment of plants developed using site-directed nucleases type 1 and 2 and oligonucleotide-directed mutagenesis (EFSA, 2020b)

As well as recent EFSA GMO Panel publications:

- Adequacy and sufficiency evaluation of existing EFSA guidelines for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of genetically modified insects containing engineered gene drives (EFSA, 2020a)
- Evaluation of existing guidelines for their adequacy for the microbial characterisation and environmental risk assessment of microorganisms obtained through synthetic biology (EFSA, 2020c)
- Evaluation of existing guidelines for their adequacy for the molecular characterisation and environmental risk assessment of genetically modified plants obtained through synthetic biology (EFSA, 2021a)

Is the EFSA guidance adequate for risk assessment of genome-edited organisms?

The present discourse on how new genome-editing techniques will be regulated lacks an analysis of whether current risk assessment methodologies are adequate to organisms arising from these new techniques. Therefore, this report aims to provide an overview of the new techniques, and to examine whether current risk assessment methodologies are adequate to evaluate potential risks from organisms developed by targeted genome editing. The report is intended for those concerned or involved with risk assessment of new genome-editing techniques as well as regulatory bodies and risk managers relying on scientific advice.

Terms of reference (ToR)

VKM has initiated a project to address the following terms of reference (ToR):

1. **Describe the various methods that constitute the genome editing technologies.** Different methods and their technologies, including the variation within these and the genomic alterations they result in, should be described.

2. **Describe the use of genome-editing technologies today, including future perspectives.** The main applications of new genome editing technologies within plant breeding, animal breeding (including farmed fish) and microorganisms should be described, and examples relevant for Norway should be highlighted.

3. **Discuss implications for risk assessment regarding genome-edited organisms.** Potential challenges for risk assessment of genome-edited organisms (and products thereof) with the EFSA guidance for genetically modified organisms should be investigated and described.

4. **Discuss possible implications for biodiversity in Norway**. Potential effects stemming from the spread and establishment following the use or production of genome-edited organisms should be discussed.

In considering the ToR, VKM decided not to include assessment of insects for food and feed production. Insects for food and feed production are not expected to have any substantial impact on the Norwegian market within the next ten years. There are a few examples of market-ready genome-edited insects for food and feed uses (Xu et al., 2019).

How to read this report

The continual development of methods to enhance food and feed production has resulted in the use of a wide range of techniques. The first part of this report (chapter 3-6) starts with an overview of the numerous techniques that give rise to genomic alterations. Established and new techniques used to alter plants, animals and microorganisms are described and examples of genome-edited plants, animals and microorganisms that may have relevance for Norway are presented.

The second part of this report (chapter 7-10) continues with an evaluation of the extent to which the established framework for risk assessment of genetically modified organims (GMOs) can be used for genome-edited organisms. Specifically, the report asks whether EFSA guidance for GMOs are sufficient to evaluate the risks to health and environment posed by genome-edited plants, animals and microorganisms. Possible implications for biodiversity in Norway are also discussed. The chapters on plants, animals and microorganisms can be read separately; therefore, some duplication of information will occur (chapters 8–10). These chapters are organised in accordance with the EFSA guidance.

Finally, the report provides a conclusion on the applicability of the EFSA guidance and offers some perspectives on topics that may need to be addressed as part of the further scientific and regulatory approach to genome-edited organisms. The main conclusions regarding applicability of the EFSA guidance is presented in chapter 11. The last chapter, chapter 12, extends beyond the mandate and highlights some topics for further considerations.

Key concepts and additional facts are highlighted throughout the text in the form of text boxes. Examples of genome-edited plants, animals and microorganisms relevant for Norway are highlighted in a separate box.

1 Introduction

In 2020, the Nobel Prize in chemistry was awarded "for the development of a method for genome editing". The Royal Swedish Academy of Sciences elaborated on this simple phrase, lauding the discovery of "one of gene technology's sharpest tools: the CRISPR/Cas9 genetic scissors" that can be used "to change the DNA of animals, plants and microorganisms with extremely high precision".

This breakthrough in genetic engineering came almost 50 years after it was discovered that genetic material from one bacterium could be cut and spliced into another (Cohen et al., 1973). This was the first genetically modified organism (GMO), and shortly thereafter the same technique was adopted for plants and animals.

Since its discovery, the use of genetic modification has been particularly successful in plant breeding, enabling introduction of novel traits considered impossible to achieve through conventional breeding. After the initial commercialisation of a genetically modified tomato more resistant to rotting (Flavr Savr) in 1994 (Kramer and Redenbaugh, 1994), the industry has grown exponentially. Today major crops such as maize and soybean are grown almost exclusively as genetically modified variants in major parts of the world, e.g. in the US, Brazil and Argentina. Herbicide tolerance is by far the primary introduced trait (VKM, 2019b), as well as other important traits for crop management and enhanced yields, e.g. resistance to insect pests and drought resistance.

In 2019, 190.4 million hectares of genetically modified crops were grown in 29 countries (ISAAA, 2019). Most of the cultivated area used for GM crops worldwide is primarily committed to maize, soybean, cotton and oilseed rape.

Following the approval for cultivation of insect resistant maize MON 810 in Europe in 1998 the European Union ceased all approvals of new GMOs for food, feed and cultivation until the European Food Safety Authority (EFSA) was established in 2002, and new and more stringent regulatory laws were passed in 2003. Today, all new GMO products for food, feed and cultivation are assessed by EFSA on a case-by-case basis in accordance with scientific guidance documents for risk assessment, before a market authorisation. EFSA has developed several guidance documents from 2003 until present that detail the type of scientific data and other information that should be available to risk assessors.

The Norwegian Scientific Committee for Food and Environment (VKM) carries out risk assessments of genetically modified organisms for the Norwegian Food Safety Authority and the Norwegian Environment Agency. As a response to the rapidly developing field of genome-editing techniques, and the new challenges that may emerge for risk assessors, VKM initiated the present report.

The main question to be discussed in this report is whether the EFSA guidance documents can also be applied to organisms developed with new and refined genome editing techniques such as the one that led to the Nobel Prize award in 2020.

The purpose of the project is defined by the terms of reference given by the VKM. The resulting report presents a consensus scientific assessment prepared by six appointed project groups.

1.1 History of gene technology until the latest CRISPR technologies

Ever since humans began cultivating around 12,000 BC, human activity has influenced evolution and biodiversity through domestication and selection of favorable traits and phenotypes of wild crops and animals. However, the time needed for such changes was drastically reduced by the introduction of techniques that enhance the natural process of random mutations in the genomes of target organisms. Muller (Muller, 1928) and Auerbach (Auerbach and Robson, 1946) demonstrated that the rate of mutagenesis could be enhanced with radiation or chemical treatment.

The next major technological development was the introduction of genome engineering in the 1970s, enabling the introduction of exogenous DNA from the same or other species. The first targeted genomic changes were produced in yeast in the 1970s and in the laboratory mouse in the 1980s. Methods to achieve the same transfer of DNA into domestic animals and plant cells were developed soon thereafter. The initial genome engineering techniques rely on delivery of foreign DNA (transgenic DNA) for random incorporation into the genome. GMO was established as the term to use for organisms produced by the first generation of genome engineering techniques. Today, genetically modified animals are mainly used for research purposes, while genetically modified plants are used in the large-scale production of commodity crops in some countries.

The next development in genome engineering was the introduction of DNA enzymes that created DNA double-stranded break (DSB) at a specific genomic location. Studies had already in the 1980s revealed that double-strand breaks enhance the frequency of homologous recombination events, but tools to generate double-strand breaks at specific locations were difficult to engineer. From around 2000, parallel techniques for generation of double-strand breaks in DNA were developed. These methods for targeting DSB-inducing nucleases to specific genomic sites relied on protein-based systems with customisable DNA-binding specificities, such as meganucleases, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). ZFNs are hybrids between a DNA cleavage domain from a bacterial protein (*Fok 1*) and sets of zinc fingers that were originally identified in sequence-specific eukaryotic transcription factors. TALENs employ the same bacterial cleavage domain but are linked to DNA recognition modules originating from transcription factors produced by a plant pathogenic bacterium (Sander and Joung, 2014).

These methods advanced genome engineering by eliminating several of the laborious steps required to perform site-specific genome engineering with homologous recombination. Although these new techniques demanded very laborious cloning steps to generate the site-specific cleavage tools, these were found, for a limited time, to represent a large step forward and were considered to replace the currently used homologous recombination (Sander and Joung, 2014). However, these technologies were rapidly outdated with the introduction of the CRISPR technology, which is characterised by efficiency, specificity, versatility, and simple design and ease of use. The conversion of this natural bacterial defense system into a genome-editing tool has revolutionised genome engineering (Doudna and Charpentier, 2014) and represents a classic example of the importance of numerous small advancements in basic science (primarily within microbiology), and how this can lead to fundamental breakthroughs in science and medicine, and breeding.

Genome-editing or engineering tools are the newest in the expanding toolbox available for gene technology. As for most of the older techniques used for the same purpose, they are based on engineered versions of biological systems which exist in nature. Humans have found a way of exploiting these systems to our own benefit. By building on the knowledge generated through the last decades of rapidly developing genome-sequencing techniques, an emerging goal is to edit and engineer new genotypes with favorable phenotypic traits. Organisms produced by these new genome-editing techniques are termed genome-edited organisms.

1.2 Genome-editing technologies in food and feed production

Genome-editing techniques like TALENs and CRISPR-Cas9 have been used for research purposes for several years already, especially for knocking out genes to study gene function. However, genome sequencing of crop species, better transformation methods and increasing understanding of the molecular mechanisms regulating agriculturally important traits, have allowed the genome-editing technology to be used for development of new commercially important crop varieties for food and feed. Most of the market-oriented traits under development these days are point mutations or indels (SDN1, box 2) knocking out gene function to improve nutritional value or stress tolerance, while a much smaller fraction are plants containing insertions of whole genes or gene fragments (SDN3, box 2) (Menz et al., 2020). A herbicide-resistant canola and a high oleic acid soybean developed by genome editing have already reached the market, and many more are expected in the coming 3–5 years. Genome-editing can also be employed to increase resistance against important diseases such as potato late blight (*Phytophthora infestans*), and consequently reduce fungicide usage (Kieu et al., 2021).

Genome editing has a broad range of potential applications in production animals, including making livestock more adapted to farming or environmental conditions, increase disease resistance, improvements in growth, fertility, nutritional enhancements and better animal welfare. Tools, such as ZNFs, TALENs and in particular CRISPR, have been used to alter targeted genes to be either active or inactive, both for research purposes and direct applications (Van Eenennaam, 2017).

Fish, in particular farmed fish such as salmon, are marine sources of PUFA (poly unsaturated fatty acids) in human diets, and by using genome-editing technology it is possible to increase the endogenous synthesis of these health-bringing marine fatty acids. CRISPR/Cas9 technology has been used to target genes for gonad development to obtain sterility in farmed fish (Wargelius et al., 2016). Producing fish that are sterile also opens up opportunities for genome editing of other traits.

Genome-editing can also be used to enhance food safety by targeting the production of specific proteins, such as the bovine prion protein, resulting in resistance to Bovine Spongiform Encephalopathy (BSE) in cattle (Bevacqua et al., 2016). Also, the improvement of milk quality free of major allergens has been the focus of many genome engineering projects (e.g. (Sun et al., 2018b)). The hen's egg is a widely consumed protein source and genome editing could be used for production of allergen-free or allergen-reduced hen's eggs by disrupting ovalbumin and ovomucoid genes in parent generations (Oishi et al., 2016).

The use of bacteria and yeasts in fermented foods is common world-wide and in this respect, genome-editing technologies have opened new possibilities for more rapid development of probiotics and starter strains/cultures for the food and feed industry.

1.3 Risk assessment of genome-edited organisms within the framework of the EFSA guidance for GMOs

An organism developed by genome-editing techniques is today classified as a GMO and will be risk assessed according to the EFSA guidance for risk assessment of GMOs. A company that wants to apply for authorisation of a GMO in the EU and EEA has the responsibility to provide sufficient information in an application according to Regulation 503/2013 (EC, 2013a) and (EC, 2003a), and/or directive 2001/18/EC (EC, 2001) (Box 6). The problem formulation in the application is based on hazard identification and characterisation, exposure assessment and risk characterisation. The aim is to ascertain whether the GMO is associated with a higher risk to human or animal health or to the environment compared to the closest unmodified relative to the organism (comparator).

There are several EFSA guidance documents available (Box 6). In this report, five of the main EFSA guidance documents have been considered.

1. Guidance on risk assessment of food and feed from genetically modified plants (EFSA, 2011a),
2. Guidance for environmental risk assessment of genetically modified plants (EFSA, 2010a),
3. Guidance for risk assessment of food and feed from genetically modified animals and on animal health and welfare aspects (EFSA, 2012a),
4. Guidance on the environmental risk assessment of genetically modified animals (EFSA, 2013), and

5. Guidance for risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA, 2011e).

These guidance documents outline a structured approach to risk assessment including the areas of concerns and the various steps of the assessment process. It is emphasised that the assessment process that is guided by these documents also draws strong support from subsequent opinions and technical notes developed by EFSA. The total of these documents constitutes the basis for current and updated assessment practices. As seen from Box 6, numerous documents have been developed to take into account developments in technology and the evolving knowledge base.

VKM has used the five core guidance documents, and case examples that are relevant for Norway (Box 7) , to describe the methodology and assess whether the EFSA guidance is adequate for environmental risk assessments of genome-edited organisms in general.

2 Data collection and literature searches

For each of the questions in the terms of reference, separate literature searches were performed. The project group discussed and agreed on the search terms and databases to be used together with a senior librarian at the Norwegian Institute of Public Health, who performed the searches. The literature searches are further described below. Full search strategies are included in Appendix I.

2.1 Literature search on genome-edited plants

For this topic, literature searches were performed in Medline, Embase, ISI Web of Science and Scopus. These databases were chosen to ensure comprehensive study retrieval. The comprehensive search strategy is presented in Appendix III. The search was performed by senior librarians at the Norwegian Institute of Public Health (NIPH) on two separate occasions: on 3 July 2018 and an updated search on 14 June 2019.

The main searches resulted in a total of 1833 records after duplicates were removed, both automatically and during primary screening of the Endnote bibliography. In the primary screening, titles and abstracts of all publications retrieved were independently screened against the inclusion criteria.

Inclusion and exclusion criteria:

- Inclusion criteria:
    - Publication type – primary research studies, review papers, systematic reviews, editorials and meeting abstracts addressing livestock/aquaculture, veterinary medicine or basic research with indirect applicability.
    - Publications period for the records were from 2013 to 2019.
    - Only examples relevant for Norway were included
    - Only English records were included.
- Exclusion criteria:
    - Pathway analyses (e.g. gene function studies)
    - Not relevant for the use in Norway as stated in the terms of reference
    - Papers/opinions addressing ethical or socio-economical aspects of genome editing on plants.

Articles that did not appear to meet the inclusion criteria were excluded from further analysis. In situations where it was unclear whether the publication was of relevance to the study, it was retained for further screening. Full text articles that passed the primary screening were retrieved and compared against the inclusion criteria and assessed for relevance and quality.

The primary and secondary screenings as well as quality assessment of papers were performed independently.

The primary screening resulted in 704 full text articles, of which 36 papers passed the secondary screening and were included in the opinion.

To strengthen the data basis of the opinion, additional manual searches for papers and relevant grey literature were performed. Manual searches included snow-balling, i.e. articles that were referred to in papers found in the main literature, searches via Google, Google Scholar, and PubMed via EndNote. The manual searches resulted in 24 relevant papers and documents included in the opinion (Figure 2).

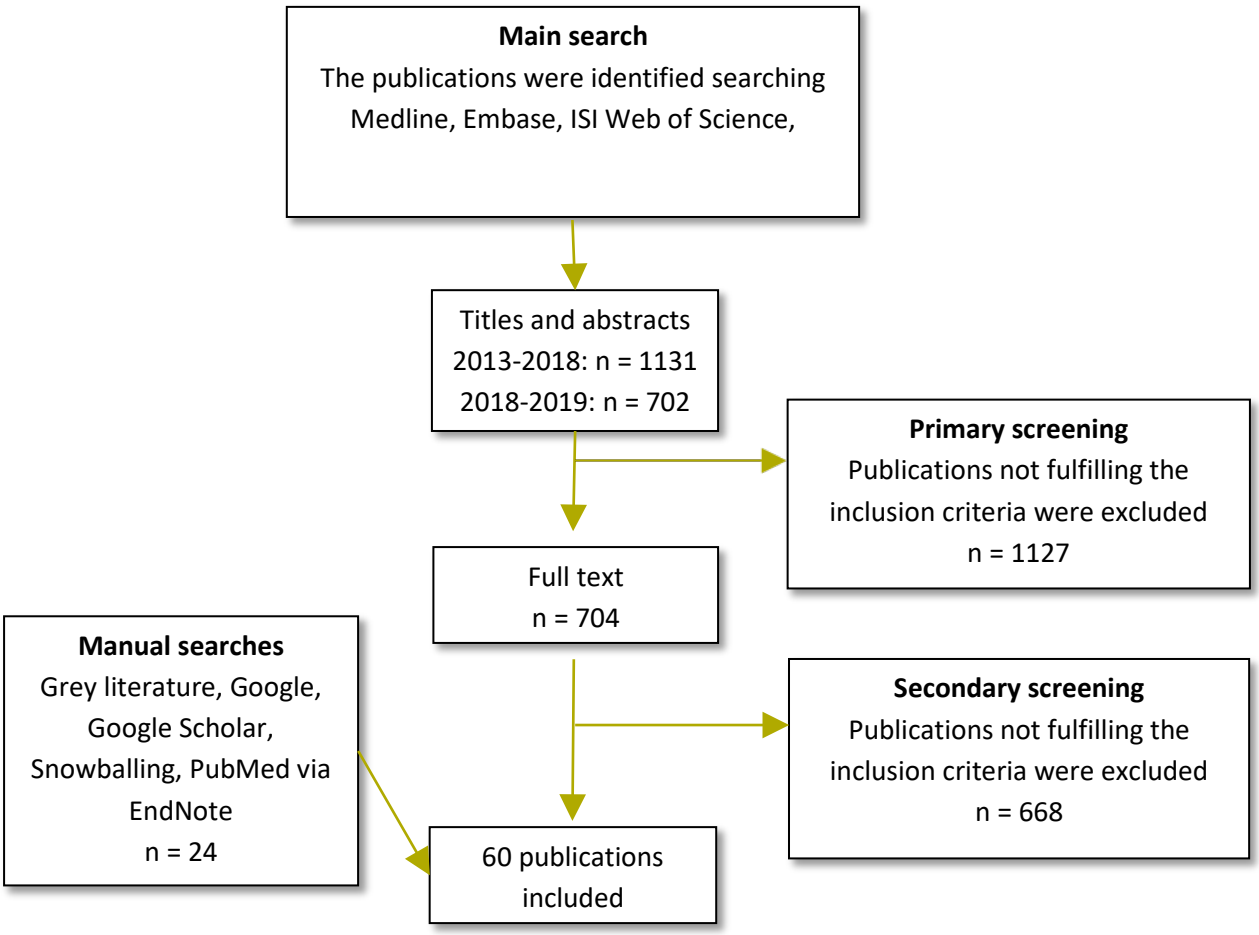

Figure 2. Flowchart for the literature search on genome editing in plants.

2.2 Literature search on genome-edited animals

For this topic, literature searches were performed in Medline, Embase, ISI Web of Science and Scopus. These databases were chosen to ensure comprehensive study retrieval. The comprehensive search strategy is presented in Appendix III. The literature search was performed by senior librarians at the Norwegian Institute of Public Health on two separate occasions: on 6 July 2018 and an updated search on 20 June 2019.

The main searches resulted in a total of 1729 records after duplicates were removed, both automatically and during primary screening of Endnote bibliography. In the primary screening, titles and abstracts of all publications retrieved were independently screened against the inclusion criteria.

Inclusion and exclusion criteria:

- Inclusion criteria:
    - Publication type – primary research studies, review papers, systematic reviews, editorials and meeting abstracts addressing livestock/aquaculture, veterinary medicine or basic research with indirect applicability.
    - Publications period for the records were from 2013 to 2019.
    - Only English records were included.
- Exclusion criteria:
    - Using animals for human biomedicine, ranging from human disease modelling to xenotransplantation.
    - Genome-edited animals for companionship (i.e. pets).
    - Papers/opinions addressing ethical or socio-economical aspects of genome editing on animals.

Articles that did not appear to meet the inclusion criteria were excluded from further analysis. In situations where it was unclear whether the publication was of relevance to the study, it was retained for further screening. Full text articles that passed the primary screening were retrieved and compared against the inclusion criteria and assessed for relevance and quality.

The primary and secondary screenings as well as quality assessment of papers were performed.

The primary screening resulted in 423 full text articles, of which 32 papers passed the secondary screening and were included in the opinion.

In order to strengthen the data basis of the opinion, we manually performed additional, more targeted searches for papers and relevant grey literature. Manual searches included snowballing, meaning inclusion of articles that were referred to in papers found in the main

literature sources, searches via Google, Google Scholar, and PubMed via EndNote. The manual searches resulted in 28 relevant papers and documents included in the opinion (Figure 3).

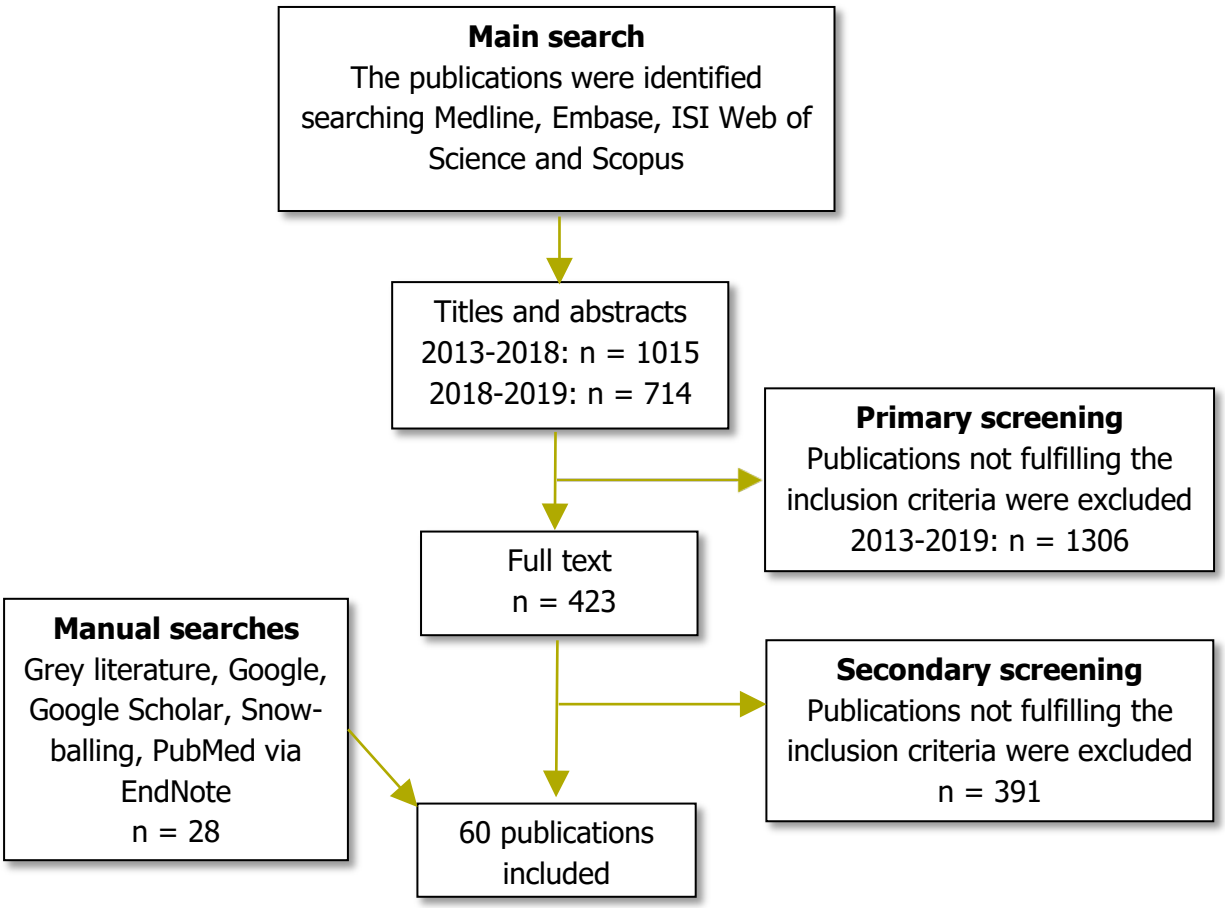

Figure 3. Flowchart for the literature search on genome editing on animals.

2.3 Literature search on genome-edited microorganisms

For this topic, literature searches were performed in PubMed, EmBase, ScienceDirect, and ISI Web of Science. These databases were chosen to ensure comprehensive study retrieval. The comprehensive search strategy is presented in more detail in Appendix IV. The literature search was performed by senior librarians at the Norwegian Institute of Public Health on two separate occasions: on 27–29 June 2018 and an updated search on 24 June 2019.

The main searches resulted in a total of 1299 records (both review and original articles) after duplicates were removed, both automatically and during primary screening of Endnote bibliography. Using the same databases and terms resulted in 763 studies (both review and original articles) on 24 June 2019.

Titles and abstract were screened for relevance.

Inclusion and exclusion criteria:

- Inclusion criteria:
    - Only review articles regarding nuclease-based genome-editing methods were included in the microbiological part of this literature study, since they were considered to give a sufficient overview of the original articles and state of the field.
    - Only beneficial microorganisms with potential use in food/animal feed including probiotics and starter culture microorganisms were included.
    - Publications period for the articles were from 2013 to 2018 and from 2018 to 2019.
    - Only articles published in English were included.

- Exclusion criteria:
    - Original articles and articles dealing viruses and pathogenic species bacteria and fungi were excluded.
    - Editorials and commentaries

Articles that did not appear to meet the inclusion criteria were excluded from further analysis. In situations where it was unclear whether the publication was of relevance to the study, it was retained for further screening. Full text articles that passed the primary screening were retrieved and compared against the inclusion criteria and assessed for relevance and quality.

The primary and secondary screenings as well as quality assessment of papers were performed.

The primary screening resulted in 94 full text articles, of which 20 papers passed the secondary screening and were included in the opinion.

We identified seventy-nine (79) review articles in the search from 2018. The titles and abstracts of all review articles were screened manually and those that did not relate to the terms of reference were excluded. Of those having potential relevance (13 articles), the full text was obtained and assessed whether it was of relevance to this Opinion.

In a search in 2019, we identified 7 articles, three review and four original, which were included.

To strengthen the data basis of the opinion, we performed additional, manual searches for papers and relevant grey literature. Manual searches included snowballing, meaning articles that were referred to in papers found in the main literature sources, searches via Google, Google Scholar, and PubMed via EndNote (Figure 4).

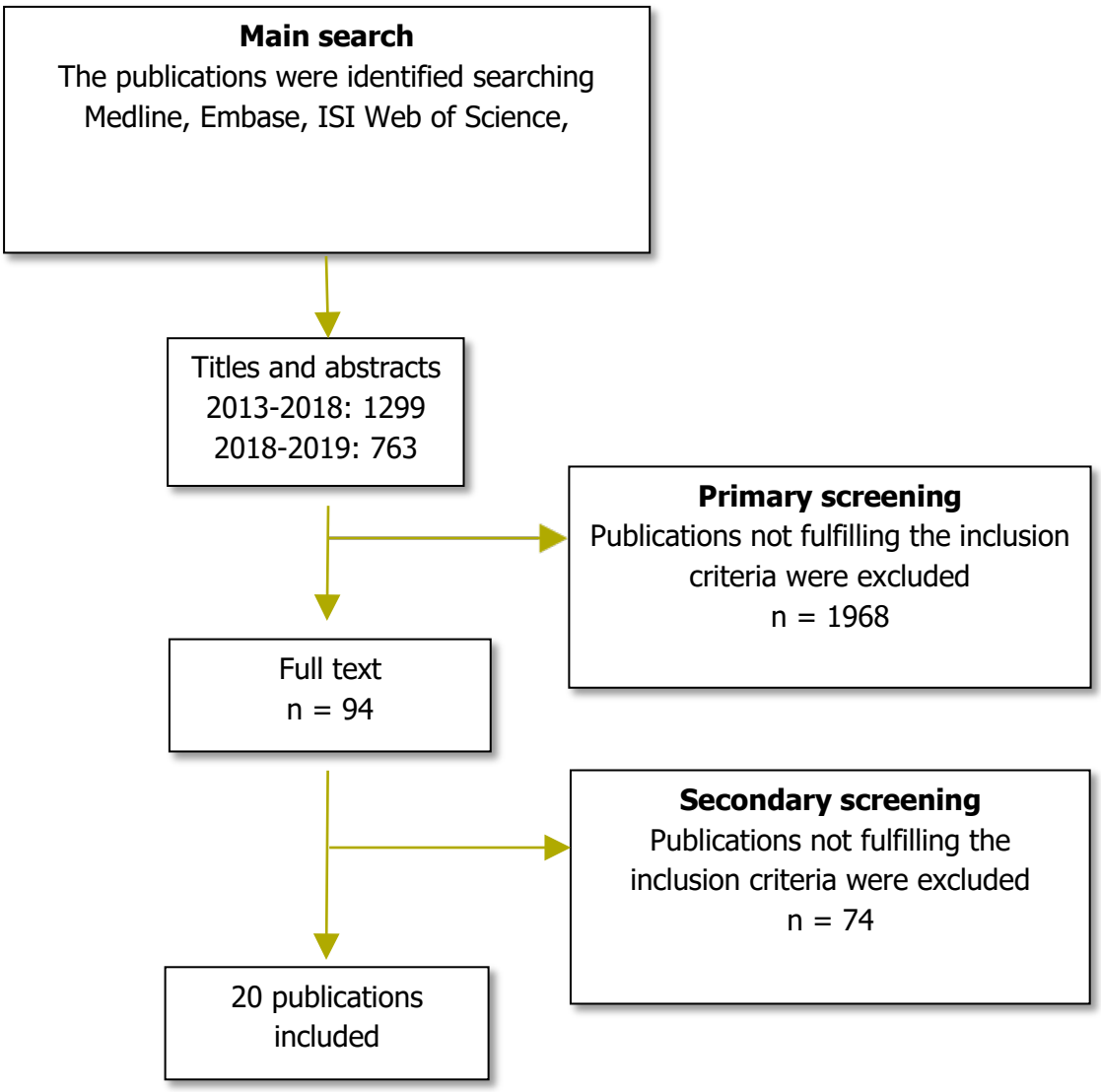

Figure 4. Flowchart for the literature search on microorganisms.

3 Conventional breeding and genome-editing techniques (ToR 1)

3.1 Conventional breeding techniques (1st generation)

As a natural part of evolution, all species evolve over time. As mentioned earlier, strategies to select for and accumulate favourable phenotypic traits in crops and livestock have been used by humans for a long time. The overall intent of these strategies is to select for traits that are desired by interfering with the random evolution of a species.

In the first sections of this chapter, we will describe the less advanced strategies that may be used to enhance phenotypic traits in species and then explain the terminology used to distinguish these various breeding strategies. Importantly, most of these approaches can be dated far back in history and they are often used in combination. In the final sections of this chapter, we will describe the genome modifying methodologies, with a special focus on the new genome-editing techniques.

3.1.1 Breeding

Sexual reproduction in nature for a given species is often referred to as **non-selective breeding**. Even non-selective breeding is not all random but influenced by positive and negative selection of fitness. As a result, weak favouring of offspring with improved phenotypical traits tends to improve overall fitness of the species in relation to the surrounding environment. Various **selective breeding** strategies have been developed to select for desirable phenotypical traits in order to maintain, enhance or remove these traits in future generations. The most commonly used breeding techniques are described below.

3.1.1.1 Selection

Selection is the most basic and the first means of breeding. This method involves selection of individuals based on preferred phenotypical traits as the basis for the next generation. In crop and livestock production, typical traits to select and bring to the next generation will be those that result in e.g. higher yield e.g. number or size of seeds, better taste, or better resistance against weeds, pests or abiotic stresses.

Today, selection of individual animals or plants containing desired traits can, for some species, be done through "marker assisted selection" (MAS). This method involves mapping of single nucleotide polymorphisms (SNPs) occurring in a species that is known to be linked to one or more desired phenotypic traits. By genotyping for these, one can select the most preferable parents for crossing and production of the next generation. "Genomic selection" is an extension

of MAS which is based on prediction models that take a large number of SNPs into consideration simultanously in order to estimate the breeding value of individuals.

3.1.1.2 Inbreeding

Inbreeding is an extreme form of selective breeding. Animals are set up for mating between siblings for typically 20 generations or more. For crops, self-fertilisation or mating between related individuals are arranged for the same number of generations. Inbreeding minimises genetic diversity since chromosome pairs merge towards being isogenic with identical nucleotide sequences. With only one allelic variant represented in the genome for all genes, both recessive and dominant allelic variants of each gene will contribute to the phenotype. Strict inbred lines that are homozygous on all loci, where recessive alleles are not compensated for, often have many phenotypic traits that distinguish the line from the species in nature. In general, inbred lines are therefore often more vulnerable to diseases and inbreeding depression. This tends to reduce fitness in a diverse environment, but not necessarily in a strictly controlled environment where the sum of phenotypic traits may favour fitness of the inbred line over individuals from the natural population. Importantly, inbred lines are nearly identical clones and therefore genetically very stable, where all produced offspring have a phenotype identical to the parents. Hence, the phenotypic traits will remain the same over many generations, resulting in high predictability for the producer.

3.1.1.3 Crossbreeding

Crossbreeding (similar to inbreeding) is the process of combining parental lines with two or more unique, desired, traits. These traits may be represented as variation within the species in the wild population, or alternatively, have been enhanced individually through human selection of parallel lines of the same species. If successful, the resulting offspring will share the traits of both lines, which is often referred to as heterosis effect or hybrid vigour.

Crossbreeding has been, and still remains in some developing countries, an important technique to enhance livestock genetics (Amdam et al., 2003; Mueller et al., 2015). Important crops like wheat and maize are still being enhanced through this method, where produced lines show promising results in terms of producing more drought-resistant varieties (Gilbert, 2014).

3.1.1.4 Hybridisation

Hybridisation is traditionally used to describe sexual reproduction between two related, but distinct species. In relation to breeding techniques, it is often used to describe an animal or crop variant produced from two distinct inbred lines of the same species. Crosses of two parallel inbred lines can be done to minimise the contribution of recessive allelic variants to the phenotype and thereby improve fitness, reduce susceptibility to disease and avoid inbreeding depression, which often occurs after strong selection towards an inbred line.

Similar to crossbreeding, hybridisation includes crossing of two distant inbred lines. However, the two methods differ in terms of the genetic background of the two lines. When referred to as hybridisation, the two lines have the same traits, but do not have the same alleles, and the resulting offspring will be heterozygous on all loci. The hybrid offspring often show enhanced phenotypic traits compared to each of the individual parental lines (hybrid vigour). On the other hand, a second cross between hybrids will result in lines that segregate with the same homozygote recessive alleles, meaning that the hybrids will not yield good results if used to produce new seeds. This benefits the provider of seeds, since farmers will need to buy new first-generation hybrid seeds every year to achieve the best result, including value, of their production. Maize is typically grown as a hybrid stock, and wheat hybrids are being developed.

3.1.1.5 Backcrossing

Backcrossing is a technique where a desirable favorable trait occurring in a one individual (donor) is fixed into the genome of a related individual lacking the same trait (recipient). The generated offspring from the first cross is subsequently crossed again with another representative of the recipient. Resulting offspring from the second cross carrying the donor trait is then selected for a third cross into a recipient. The same procedure is carried out for multiple generations, typically ten or more. For each generation, the offspring will be selected for the presence of the donor allele but will otherwise converge to a genome identical to the recipient genome.

Backcrossing is only meaningful if the recipient is an inbreed line with (nearly) isogenic chromosomes. Otherwise, genetic drift in the recipient population will never enable chromosomes to converge towards being isogenic, since some level of genetic variance will be reintroduced by the recipient for each generation. Backcrossing is widely used in research to introduce and fixate one selected allele (phenotype trait or specific genetic modification) into an inbred strain that has a certain genetic background carrying isogenic chromosomes.

3.1.1.6 Double haploids

The production of double haploid offspring is a technique used to double the number of chromosomes originating from only a single parent. For plants, this technique starts with gametes (pollen or other haploid cells), which are treated with colchicine that destroys the spindles during mitosis so that there is no separation of the chromosomes after the first round of DNA replication. The techniqueis also used on animal cells, predominantly fish, where either a heat shock, pressure shock or UV radiation (Komen and Thorgaard, 2007) is used to stop the cell from dividing. The resulting cell has a double DNA content (2n) with two identical alleles for all traits. The genetic variation is therefore like that of meiotic cells, but without the variation from combining two different gametic cells, and thus much lower than for regular vertical inheritance from sexual reproduction. The process is known as gynogenesis if the fertile gamete represents the female sex (egg), and androgenesis if the sperm is the untreated starting point. This method is beneficial, for instance, in genome sequencing, as a haploid genome is much

easier to assemble correctly, compared to a diploid genome. The Atlantic salmon (*Salmo salar*) genome sequence is based on the double haploid individual "Sally" (Lien et al., 2016).

3.1.1.7 Other ploidy manipulations

The production of double haploids is one of several methods of ploidy manipulations. Another alternative is monoploids (or monohaploids), which are derived from diploid species and have been developed through hybridisation of distant relatives, hormone or chemical treatment, or X-ray radiation (Das et al., 2018). Tobacco was the first full-fledged haploid plant produced *in vitro*. Today, many propagated crops start out as haploids before being crossed or treated to become polyploid.

Gamete manipulation can also be used to create organisms with extra sets of chromosomes, known as polyploids. Most common are tetraploids (4X), which occur in several of the most common cultivated crops, such as some varieties of wheat, cotton, potato, rapeseed, tobacco and peanut, among others. However, other ploidy levels, like triploid (3X, e.g., different apple varieties), hexaploid (6X, e.g. oat and kiwifruit) and octaploid (8X, e.g., strawberry and sugar cane) organisms are also important in crop and fruit production world-wide. These organisms with altered polyploid levels, such as farmed fish, crops or fruits, are often sought after due to their sterility which inhibit spreading to the natural environment. Many fruit varieties are also preferred in their polyploid state since this often result in sterility and loss of seeds, a trait which appeal to the consumers.

3.1.1.8 Cloning

The term cloning has different meanings depending on whether one refers to biotechnology or to reproduction. In biotechnology, cloning means a process whereby one or more DNA fragments are inserted into a recipient DNA sequence, often a plasmid. In plants and animal reproduction, cloning refers to a process without sexual reproduction whereby genetically identical individuals are formed naturally or by artificial intervention. Although the genome is identical to the parental genome for all produced clones, they have variable alleles at most loci. Cloning is beneficial in cases where the parental genotype is superior to other genotypes of the same species, and if this yields a uniform, predictable production.

One widespread example of cloning in agriculture is the production of potatoes from seed potatoes. In general, plants can more easily be proliferated as clones as most cells are pluripotent and thus able to develop into a new individual. Animal cells, however, become developmentally determined at an earlier stage, and reprogramming the cells to being embryogenic is more challenging. It was done, however, with the sheep Dolly as a proof of principle (Evans et al., 1999), and later in several other livestock species. Cloning of farmed animals can be beneficial if an individual has exceptional genetic value, and superior traits. The efficiency is rather low, which makes the technique economically unattractive to implement for mammals.

3.2 Non-targeted genome-editing techniques

3.2.1 Induced mutagenesis

Mutations in the DNA occur with a certain frequency in all organisms as a result of erroneous DNA replication, DNA repair errors and exposure to mutagenic compounds. The vast majority of these are repaired by various DNA repair mechanisms, but occasionally these errors are not repaired correctly, and the resulting mutations will persist in the genome. Mutations can be transmitted to the progeny if this occurs in the germline. Most such single nucleotide mutations have no effect; some mutations are harmful, and occasionally they turn out to be favourable. These rare favourable mutation events are the backbone of evolution, as they generate phenotypic diversity. Mutation frequencies differ between species. In humans, whole-genome sequencing of parent–offspring trios, suggests an average human mutation rate ranging from $1.1–1.7 \times 10^{-8}$ per nucleotide for each generation for base-substitution mutations, while small insertions or deletions occur at ~8% of the base-substitution rate. Large structural changes involving mobile-element insertions and inter-chromosomal exchanges arise at a rate of ~0.08 per haploid genome per generation. The human diploid genome size of ~6 billion bases predicts that an average newborn contains approximately 100 *de novo* mutations, whereof very few will occur within the coding sequences of genes and affect gen functions (Lynch, 2016).

Minor, spontaneously introduced mutations are the basis for evolution. To induce mutation frequency and speed up evolution, several methods have been developed. These can be divided broadly into two or three categories depending on the method and the agent used to induce mutations: chemicals, radiation and transposons (see 3.2.1). Compared to transposon mutagenesis (see below), both chemical and UV-induced mutagenesis can potentially generate gain-of-function mutations, as well as loss-of-function mutations (Bose, 2016) .

3.2.1.1 Chemical mutagenesis

Some chemicals can induce DNA lesions that ultimately lead to single nucleotide changes and/or deletions. The different chemicals differ in the type of mutation they tend to induce (shown in parenthesis below). The most commonly used laboratory mutagens include Acriflavine (intercalations), ethyl methanesulfonate (point mutations), diethyl sulfate (point mutations) and N-methyl-N'-nitro-N-nitrosoguanidine (point mutations).

3.2.1.2 Radiation mutagenesis

Ionising radiation is an effective method by which to introduce genetic variability and generate loss-of-function alleles. The physical properties of ionising radiation (γ-rays, X-rays, UV light, α-particles, β-particles, and neutrons) are different, and therefore, their potential uses in breeding programs differ. At the beginning of the twentieth century, ionising radiation started with X-rays (Muller, 1928), whereas γ-rays and neutrons were introduced later. X-rays or γ-rays have been widely used in biological systems. γ-rays have high penetrating potential and various crops have been improved through γ-radiation followed by screening of generated mutants.

The mutagenic effects of ionising radiation result mostly from generated DNA double-strand breaks (DSBs). The mutagenic effect of radiation can be caused by direct destruction of random nucleotides or indirectly through the generation of reactive oxygen species (Bose, 2016). Structural and functional changes in DNA caused by radiation with subsequent DNA repair may result in base alterations, base substitutions, base deletions, and chromosomal aberrations, all of which may contribute to phenotypic alterations. A wide range of genetic variability has been induced by mutagenic treatments for use in plant breeding and crop improvement programmes. Irrespective of type of ionising agent, it is necessary to map the generated mutations afterwards. In this modern genomics era, random mutagenesis using ionising radiation can be performed to isolate mutations of interest when combined with whole-genome sequencing (Bose, 2016).

3.3 Established genome modification or editing techniques (2nd generation)

Random mutations introduced by mutagenic chemical agents and radiation result in a large number of offspring with unfavorable phenotypic traits and only a few individuals carrying a potentially favourable phenotypic trait. Considerable effort is needed to screen and identify the few best suitable individuals for further breeding. Genetic changes or variations are key to crop improvement and for centuries, naturally occurring mutations have been used.

Once DNA and genes were recognised as major contributors to how all life is shaped, it became obvious that changing DNA sequences induces phenotypic variations. A more sophisticated approach is to link a favourable phenotypic trait to a particular gene and subsequently specifically mutate or introduce this gene in an organism. Achievements in molecular biology in the 1980s were first restricted to introducing a gene of interest, but soon thereafter, more sophisticated techniques were developed that enabled deletion of a certain gene of interest. The discovery of *Agrobacterium tumefaciens* (*A. tumefaciens*) as a tool for introducing foreign DNA into an organism was one of the major achievements in genetic engineering. This chapter describes these various molecular techniques.

3.3.1 Transformation (Agrobacterium)

Agrobacterium tumefaciens is a ubiquitous soil bacterium containg a plant viral pathogen that causes crown gall tumours in infected plants (Schell and Van Montagu, 1977) (Figure 5). The infectious mechanism of *A. tumefaciens* is extensively used as a means to introduce genetic material and desired traits into frequently cultivated domesticated crops, e.g. soybean, maize and oilseed rape. Due to a simplified and better controlled integration of the donor DNA into the host genome, the technique has with time replaced the gene gun to generate genetically modified plants. The technique has therefore had a profound impact on agriculture and commerce of crops.

A. tumefaciens is an example of applied horizontal gene transfer (HGT) from bacteria to eukaryotes. During infection of a plant, *A. tumefaciens* transfers a linear DNA sequence – a part of a circular DNA molecule called the tumour-inducing plasmid (Ti plasmid) to the plant cells. The transferred DNA sequence (T-DNA) can randomly integrate into a plant nuclear genome. The transfer of the T-DNA to the nucleus of the plant cell is facilitated by a set of several virulence (*vir*) genes located on the Ti plasmid. When integrated into the host plant genome, expression of genes on the T-DNA will commence, some of which encode plant hormones promoting uncontrolled proliferation and tumour formation. The T-DNA sequence also encodes enzymes involved in synthesis of modified carbohydrates and amino acids (opines), which are sources of energy for the bacteria.

When used as a tool for crop improvement, a strain of *A. tumefaciens* that are disarmed by removal of the Ti plasmid regions that encode for genes that affect plant development and production of opines are used to deliver the DNA. The removed regions are replaced with genes that encode for new desired traits, e.g. introduction of insect resistance. Sequences encoding insect resistance and additional supportive genes, e.g. enhancers and promoters, are inserted in the T-DNA sequence. *Agrobacterium* is now the preferred mode for trans- gene insertions because the gene of interest is guided into the chromosomes without generating multiple copies or rearrangements, as opposed to gene gun techniques.

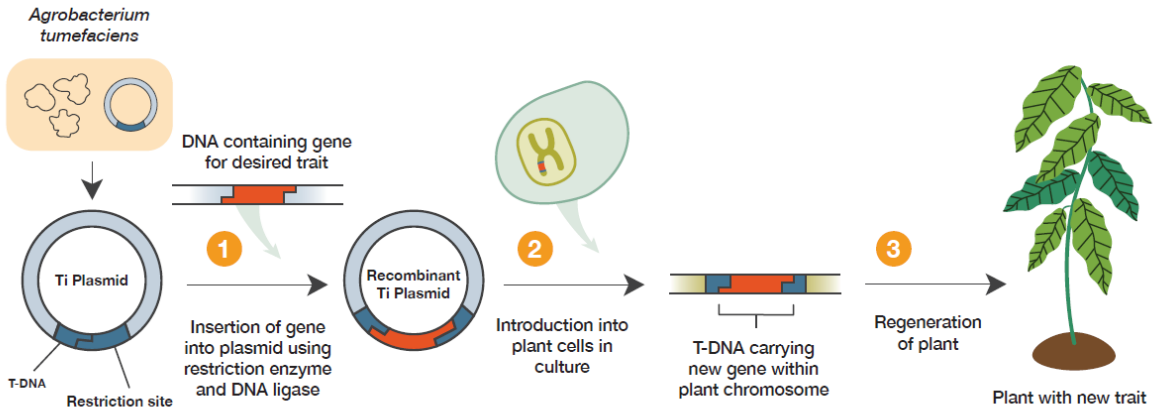

Figure 5. Transformation by *Agrobacterium tumefaciens*. The infectious mechanism of *A. tumefaciens* is extensively used as a means by which to introduce genetic material and desired traits. During infection of a plant, *A. tumefaciens* transfers a linear DNA sequence – a part of a circular DNA-molecule called the tumour-inducing plasmid (Ti plasmid) into the plant genome. This Ti plasmid can be modified with insertion of genes coding for desired traits.

3.3.2 Transposons/insertional mutagenesis

One of the most direct approaches to determine gene function is insertional mutagenesis. Insertion mutants offer one of the direct ways to relate a gene to its function by employing forward or reverse genetics approaches. Both T-DNA (transfer-DNA) and transposon insertional mutants are used to produce several crops.

Transposon mutagenesis is a powerful approach to producing random gene mutations in bacterial genomes. It allows genes to be transferred to a host organism's chromosome, interrupting or modifying the function of a gene on the chromosome and causing a mutation. In transposon mutagenesis, a foreign DNA fragment is randomly inserted into the genome to disrupt genetic elements. Transposons can disrupt the expression of a gene, alternatively alter expression of surrounding genes, by positional effects. Sometimes, the random insertion fails to knock out the open reading frame of a gene, leading to mutant plants with partial functions.

Transposon mutagenesis is much more effective than chemical mutagenesis, with a higher mutation frequency and lower lethality rates. Other advantages include induction of single hit mutations, incorporation of selectable markers, and the ability to identify affected genes after mutagenesis. Disadvantages include the low frequency of transposition in living systems and the inaccuracy of most transposition systems (Kulasekara, 2014).

Transposons or retro-transposons have been widely used for random incorporation of T-DNA inserts in plants. Transposon mutagenesis is the method of choice for mutagenesis of bacterial genomes and has been utilised extensively to study bacterial pathogenesis and biology. The

genetics and biochemistry of bacterial transposons have been characterised in detail, and transposon tools have been used since the 1980s. The most frequently used transposons in bacterial genetics are based on the Tn5 and Tn10 platforms (Kulasekara, 2014).

3.3.3 Gene gun/biolistics

A gene gun, also called a biolistic particle delivery system, is used to deliver DNA to cells. With this technique, metal particles are covered with a cloned DNA construct and the particles are fired into cells with a pressure-driven gene-gun that fires the DNA metal particles through the cell membrane. The technique was developed in the 1980s to circumvent the difficulties in delivering DNA to plant cells (Klein et al., 1987; Sanford et al., 1987). Due to the strong cell walls in plants, methods developed to deliver DNA into animal cells are often not suitable for penetrating plant cell membranes. Although the technique can be used for most cell types, it has mainly been used to produce GMO plants. The metal particles used are typically gold or tungsten, as these do not have any biological activity and will disappear from the plant as it is cultivated.

The gene gun technique has several limitations. The DNA is often fragmented in the process, and multiple deleted versions of the construct are often integrated in a single plant cell. Furthermore, the delivered DNA will be randomly incorporated into the genome of the target cells. With multiple insertion sites where alternative fragments are being inserted, screening of cells to identify cells containing only the desired modification is a tedious process that requires screening over several generations.

3.3.4 Nuclear transfer

Nuclear transfer is a technique whereby a nucleus is transferred from one cell to another cell. The nucleus is first removed from a newly fertilised embryo (the recipient cell) followed by either injection of the nucleus from the donor cell, or stimulated fusion of the two cells into one merged cell containing only the donor nucleus. The recipient cell is usually a fertilised one-cell embryo whereas the donor cell can originate from various cell types. In either case, the cytoplasmic environment in the recipient cell expresses the factors needed to re-program the genome of the donor nucleus. The resulting embryo will develop into an organism with a genome identical to the donor cell. Dolly, a sheep, was the first successful cloning of a mammalian organism (Evans et al., 1999). The procedure depends on several steps carried out in cells cultured *in vitro* and requires species-specific optimisation. Efficiency is rather low, and due to high costs, the technique is not widely used outside research. Nuclear transfer has been used for cloning of larger animals and production of transgenic mammals that overexpress a gene product (sheep, pig, and cattle).

3.3.5 Pronuclear injection

Pronuclear injection of DNA can be used for direct delivery of DNA to the nucleus of a recipient cell. A thin needle is introduced into the nucleus of the cell with the purpose of injecting a micro

volume of a solution containing DNA. To produce a transgenic animal, the recipient cell used is a newly fertilsed one-cell embryo (Capecchi, 2005; Demayo et al., 2012; Gordon and Ruddle, 1981). A laboratory mouse was the first genetically modified mammal produced with pronuclear injection. Pronuclear injection has been used to introduce DNA into the nucleus of mammalian cells to obtain stable permanent integration of the injected DNA into the genome (transgenes). The technique is efficient in most species, where a low percentage of the injected cells will stably integrate the injected DNA in the genome. A major disadvantage of the technique is that the injected DNA construct will integrate in the recipient genome at random, and expression from the injected DNA will vary depending on where in the genome the DNA is inserted. The injection stations developed for pronuclear injection may now conveniently be used to introduce various genome-editing reagents (e.g. CRISPR reagents) into the nucleus, which enable site-specific genome alterations (Doe et al., 2018).

3.3.6 Blastocyst injection

Blastocyst injection is a technique where pluripotent embryonic stem (ES) cells are fused into an early-stage, developing recipient embryo. The donor ES cells are delivered by microinjection of a smaller number of ES cells directly into the blastocyst inner mass of the recipient embryo. Alternatively, the donor ES cells can be fused to the recipient embryo at an earlier developmental stage (from the 8-cell stage) (Huang et al., 2008; Larson, 2020; Wang et al., 1997). In both cases, a relatively high number of the resultant offspring will be chimeric and consist of a mixture of cells from the recipient embryo and the injected donor ES cells. Usually, the injected ES cells and the recipient embryo have been selected based on having two different coat colours, which will favour identification of founders with a high degree of cellular contribution from the injected donor ES cells. The technique is seldom used alone, but usually in combination with upstream techniques that have been employed to alter the genome in the pluripotent ES cells being injected (Longenecker and Kulkarni, 2009; Wang et al., 1997). Blastocyst injection was first established in mice and has been widely used for site-specific genome editing in this species. The following three key steps are required to generate a genetically modified mammal with blastocyst injection: 1) establishment of conditions for culturing of pluripotent stem cells; 2) establishment of conditions for site-specific genetic modification through homologous recombination of the desired allele in pluripotent ES cells; and 3) Establishment of conditions for injection of the genetically modified pluripotent ES cells into recipient embryos that will fuse and generate a chimera. As described above, the technique depends on many steps performed *in vitro*, and rigorous optimisation for each of these steps is not only species-specific but is even found to vary between various strains of laboratory mice. Due to these demanding requirements, the technique is mostly used to generate mouse models for research proposes and it has been used less frequently in other species.

3.3.7 Chloroplast or mitochondrial transfer

In addition to their inherited genomes, eukaryotic cells contain small energy-producing organelles, called mitochondria in animal cells and chloroplast in plants, with their own small genomes(Leister, 2005; Martin et al., 1998). Instead of manipulating the host genome, certain

phenotypic alterations may be achieved by changing the genome of these small energy producing organelles. Organelles are predominantly uniparental inherited, usually maternal, meaning that the oocyte or fertilised embryo must be manipulated. In animal cells, the most often used procedure relies on the nuclear transfer technique described above, whereby a nucleus from a donor embryo is removed and replaced with a nucleus from a recipient embryo. Since no mitochondria are transferred in the process, the new embryo will contain mitochondria from the donor embryo and nucleus from the recipient embryo.

3.3.8 Gene knockout

Gene knockout (KO) techniques are part of the functional genomics toolbox and are used to study the function of genes, usually by investigating the effect of gene loss (Hertzog and Kola, 2001; Iredale, 1999). Gene KO is accomplished through a combination of techniques, starting in the test tube with a plasmid, bacterial artificial chromosomes (BACs) or other DNA constructs. Usually, the aim is to create a transgenic organism that has the altered gene. However, one needs to distinguish between heterozygous and homozygous KOs. In the former, only one of two gene copies (alleles) is knocked out; in the latter, both are knocked out.

Genes that are active during early development may not be knocked out without lethal or detrimental effects to the organism, especially in animals. Conditional knockout is a way to get around this and allows gene deletion in a tissue in a time-specific manner. The original conditional knockout method made use of a site-specific recombinase called Cre that recombines short target sequences known as LoxP. Other recombinases have since been developed and used for conditional knockout studies.

3.4 New editing techniques (3rd generation)

The genome-editing technologies mentioned in the previous chapters are laborious and typically have low specificity and/or efficiency.

From around 2000, new improved technologies were developed. These techniques circumvent several of the troublesome *in vitro* steps and moved genome engineering to an efficiency level whereby manipulations could be achieved directly in embryos. Most of these systems are designed to introduce site-specific DSBs in the genome, which stimulates repair by the endogenous DNA repair system (Gaj et al., 2013)

It is fundamental for a cell to repair a DSB. A generated DSB will divide one chromosome into two smaller fragments, which will result in genomic instability if the cell divides. Only one of the generated fragments will contain a centromere, the attachment site for the spindle, and if the cell divides only this part of the chromosome will be segregated to each of the daughter cells. The other fragment, without a centromere, will end up randomly. As a result, a considerable part of the genome will be lost during cell divisions unless the integrity of each chromosome is tightly controlled. Eukaryotic cells have therefore developed quite impressive strategies to repair a DSB, and these can be utilised for genome editing. The introduced DSB will activate a series

of cellular processes including DNA damage response and cell cycle checkpoints to ensure that the DNA damage is repaired prior to cell division. The repair of DSBs occurs primarily by one of two major pathways: homology-directed repair (HDR) or non-homologous end-joining (NHEJ) (Chiruvella et al., 2013; Symington and Gautier, 2011; Wyman and Kanaar, 2006). These two DBS repair mechanisms have several consequences: small insertions–deletions (indels) in the case of NHEJ repair, and substitution, gene disruption, insertion, correction and chromosomal rearrangements in the case of HDR repair. The HDR pathway depends on strand invasion of the broken end of DNA into a homologous sequence and subsequent repair of the break in a template-dependent manner. HDR is the more accurate mechanism for DSB repair due to the requirement of higher sequence homology between the damaged and intact donor strands of DNA. The process is error-free if the DNA template used for repair is identical to the original DNA sequence at the DSB site, or it can introduce very specific mutations into the damaged DNA. The homologous DNA template can be an exogenous DNA strand containing a desired genetic change introduced along with the DSB causing reagents, such as TALENs or CRISPR/Cas9.

NHEJ is a homology-independent pathway and functions to repair DSBs without a template through direct re-ligation of the cleaved ends. This DNA repair pathway is error-prone and often results in indels at the site of the break. Activation of NHEJ by site-specific DSBs can be used to disrupt target genes in a wide variety of cell types and organisms by taking advantage of these indels to shift the reading frame of a gene. Equipped with the ability to utilise the cell's endogenous DNA repair machinery, it is now possible to engineer a wide variety of genomic alterations in a site-specific manner. NHEJ is active throughout the cell cycle and has a higher capacity for repair, as there is no requirement for a repair template (sister chromatid or homologue) or extensive DNA synthesis. NHEJ is consequently the principle means by which genome-editing techniques such as CRISPR/Cas9-introduced breaks are repaired. The NHEJ pathway mediated DSBs repairing activity is two orders of magnitude higher than the HDR pathway. The lower efficiency of HDR-mediated gene targeting is an important obstacle of HDR-pathway mediated repair (Steinert et al., 2016).

The techniques described below differ in the way the DNA strand breaks are generated, except for the oligonucleotide-directed mutagenesis (ODM) technique which does not induce DNA breaks.

3.4.1 Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis (ODM) can be used to insert minor edits into the nucleotide sequence. Various versions of ODM have been developed. In the agricultural field, it is often referred to as Rapid Trait Development System (RTDS) technology. ODM relies on synthesis of a synthetic site-specific single-stranded oligonucleotide (20 – 100bp) that is largely complementary to the target sequence but contains a few nucleotide mismatches to be incorporated, combined with various strategies to enhance stability of the oligonucleotide. The oligonucleotide stability may be enhanced by RNA base-pairing or chemical modifications of the 5'- and 3'-end nucleotides (Sauer et al., 2016). Through homology-directed pairing between the

delivered ODM and the DNA target region, the cell's endogenous DNA repair machinery corrects the mismatch guided by the delivered oligonucleotide sequence without inducing DNA break. The technique has been used with success in bacteria, in eukaryotic yeast, in mammalian cells and plant cells (Sauer et al., 2016).

Modification efficiency is rather high, but the technique is limited to exchange of only a few nearby nucleotides. The technique is often used to insert a single nucleotide polymorphism (SNP) to generate a frame-shift mutation.

3.4.2 Meganucleases

Meganucleases are natural proteins encoded by mobile genetic elements, which are present in diverse species, including eukaryotes, archaea and bacteria. Meganucleases are highly specific DNA cleaving enzymes that recognise and cleave DNA at long nucleotide-specific target sites (>14 bp), resulting in site-specific digestion of the target DNA. Several families of meganucleases have been identified, which have been grouped based on protein sequence similarity and conservation of structural motifs. The LAGLIDADG family is best characterised DNA cleaving enzyme, and members of this family have been used for genome editing. The LAGLIDADG motifs contribute amino acid residues to both the protein-protein interface between protein domains or subunits, and to the enzyme's active sites. Compared to other genome-editing nucleases described below, meganucleases are difficult to engineer. They are still attractive to use due to their small size, which allows many different options for delivery into cells, and their high specificity, which is necessary for delivery of therapeutic or gene-drive constructs (Silva et al., 2011; Stoddard, 2014; Taylor and Stoddard, 2012) . The LAGLIDADG endonucleases make extensive sequence-specific contacts with the DNA target region (Stoddard, 2011). However, the cleavage and DNA-binding domains of LAGLIDADGs are not clearly determined. Further, the binding and cleavage domains in meganucleases are not modular, making their customization challenging and thereby limiting their utility as tools for routine genome-editing applications. To address these limitations, Boissel et al. developed a hybrid nuclease that combines the ease of engineerability of a TAL effector (TALE) with the cleavage sequence specificity of a meganuclease cleavage domain – called megaTALs (Boissel et al., 2014). The engineered megaTAL consists of a fusion of a TAL effector domain to the N-terminus of a LAGLIDADG endonuclease, which induces highly specific gene modifications (Boissel et al., 2014). Meganucleases have been used to edit plants such as arabidopsis, barley, tobacco and maize.

3.4.3 Zinc Finger Nucleases (ZFNs)

ZFNs are engineered nucleases that catalyse site-specific double-stranded DNA cleavage. A single ZFN consists of a sequence-specific zinc finger DNA-binding domain fused to the nonspecific bacterial DNA cleavage domain of the Fok1 endonuclease (Figure 6). The Fok1 nucleases only function when assembled as a dimer, and ZFNs are used in pairs. When two ZFN DNA-binding domains bind in close proximity in the genome, the two Fok1 domains are able to form a pair and function as a highly specific genomic scissor (Gupta et al., 2012). Combined,

the ZFN pair generates a DSB at a specific sequence, allowing site-directed genome editing and gene correction (Kim et al., 1996; Urnov et al., 2010). The modular structure of zinc-finger proteins makes them attractive for design of customized DNA-binding proteins. The ZFN domain typically comprises several ~30 amino acid modules, each organized in a conserved ββα configuration that specifically interacts with a nucleotide triplet. A customized ZFN is typically composed of three to six zinc finger modules that will recognize a 9 to 18 bp long specific DNA sequence (Onori et al., 2013; Urnov et al., 2010). A pair of adjacent binding ZNFs are designed to generate cleavage at a specific locus, where one ZFN recognizes the sequence upstream and the other ZFN recognizes the sequence downstream of the cleavage site. The Fok1 dimer generates a DSB within the five- to seven base-pair spacer-sequence that separates the two adherent ZFN binding sites (Bitinaite et al., 1998; Gaj et al., 2016).

The discovery and application of zinc-finger proteins was a great contribution to the genome-editing toolbox. Zinc fingers are the most common DNA binding domain found, and they are an ideal platform for the design of customized DNA binding domains. ZFNs have been used with high success in various organisms, including plants such as corn, tobacco, Arabidopsis and soybean (Zhang et al., 2018). The design and assembly of ZFNs are technically challenging, laborious and time-consuming. These shortcomings and toxicity have greatly limited their widespread adoption as a genome-editing tool for site-specific genome alterations.

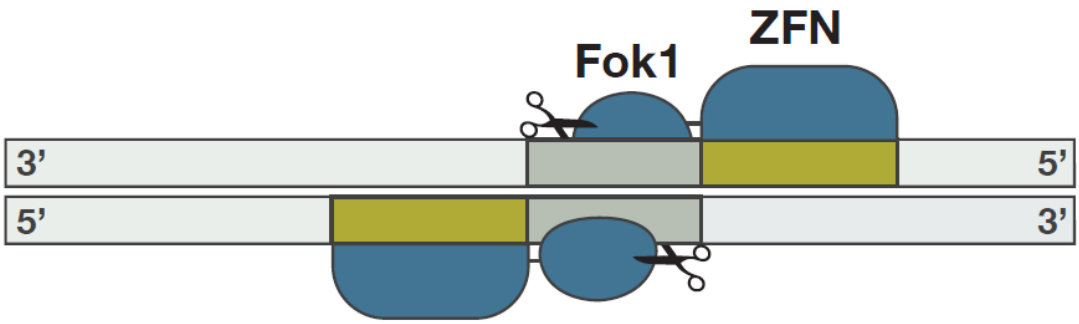

Figure 6. Zink Finger Nuclease consists of a sequence-specific zinc finger DNA-binding domain fused to the nonspecific bacterial DNA cleavage domain of the Fok1 endonuclease.

3.4.4 TALENs (Transcription Activator-Like Effector Nucleases)

TALENs are in most aspects similar to ZNFs, engineered nucleases consisting of a DNA-binding TALE protein fused to the endonuclease Fok1 (Figure 7). A pair of TALEN proteins directs a DNA endonuclease Fok1 dimer to cleave the genome at a specific site. TAL effector proteins (TALEs) originate from the plant pathogen *Xanthomonas sp.*, which secretes TALE proteins that alter transcription of certain genes in the invaded plant (Boch et al., 2009). The TALE protein is composed of a central DNA-binding domain, a C-terminal transcription-activation domain and an N-terminal translocation signal. The DNA-binding domain is composed of several tandems of nearly identical 33–35 amino acid repeats, which are highly conserved except for two adjacent residues (positions 12 and 13) named repeat variable di-residues. The repeat variable di-

residues determine the DNA binding specificity of each TALE repeat (Boch et al., 2009; Bogdanove et al., 2010). Compared to ZFN, construction of TALENs is easier because of the modular nature and the simplified and predictable protein-DNA recognition whereby each module recognises a single nucleotide (Miller et al., 2011). Since the Fok1 nucleases only function as dimers, a pair of TALENs is needed to make a double-strand break at a particular genome site. A TALEN pair is designed to bind adjacent at the target site separated by a short spacer (~14 – 18 bp) to stabilise formation of an active Fok1-dimer that catalyse DSB.

The simplified assembly of TALENs compared to ZFNs, has resulted in a wider use of this genome-editing tool in plants, including Arabidopsis, rice, barley, maize, tobacco, soybean, wheat, tomato, potato and sugarcane (Zhang et al., 2018).TALENs exhibit significantly reduced off-target effects and cytotoxicities compared with ZFNs, which makes them an attractive genome-editing tool (Ding et al., 2013). A disadvantage of TALENs is that the construct encoding a TALEN protein is around three times larger than a ZFN and consists of a highly repetitive structure. Customised TALENs are therefore difficult to clone and, depending on the delivery methods used, they are sometimes too large for efficient delivery into cells.

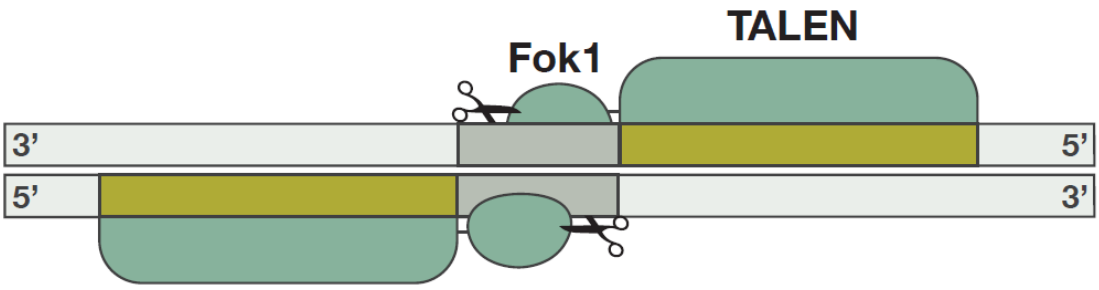

Figure 7. Transcription Activator-Like Effector Nucleases consisting of a DNA-binding TALE protein fused to the endonuclease Fok1.

3.4.5 CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)

Whereas DNA recognition with ZFNs and TALENs is based on protein-DNA interactions, the CRISPR system is based on RNA-DNA nucleotide pairing (Figure 8). As discussed below, this represents a great advantage. CRISPR was discovered as a prokaryotic immune system protecting cells by targeting and destroying foreign DNA, such as viruses or plasmids (Horvath and Barrangou, 2010; Marraffini and Sontheimer, 2010). Various CRISPR/Cas9 systems (Cas; CRISPR-associated protein) are found in several bacterial and archaeal species (Jinek et al., 2012). Some of these have been engineered into genome-editing tools that are described below.

3.4.5.1 CRISPR/Cas9

The first engineered and most often used CRISPR/Cas9-based system has been adapted from the type II CRISPR adaptive immunity system in the bacterium *Streptococcus pyogenes* (Jinek

et al., 2012). The system has two main components: a Cas9 protein and a guide RNA (gRNA or sgRNA). The gRNA binds to the specific target sequence directly upstream of a protospacer adjacent motif (PAM; 3-nucleotide sequence, which is an NGG-motif for the SpCas9 from *S. pyogenes*) (Figure 10). The single guide RNA (sgRNA) consisting of an approximately 20-nucleotide-long guide sequence that directs the Cas9 endonuclease to the target genomic (N_{20}NGG) sequence through base pairing. Two separate nuclease domains of Cas9 each cleave one of the DNA strands, which together generates a double-strand break in the targeted sequence.

The Cas9 HNH nuclease domain cleaves the DNA strand that is complementary to the guide RNA, while the RuvC-like nuclease domain cleaves the other strand. Both cleave within the proto-spacer about three to four nucleotides upstream of the PAM site. The short sgRNA defines specificity and guides the Cas9 nuclease to the desired site in the target DNA.

Shortly after its introduction, the CRISPR/Cas system was used for genome editing in many different organisms, including plants (Doudna and Charpentier, 2014). The CRISPR/Cas9 system is particularly attractive because of its simple design with higher efficiency in producing targeted mutations. The system relies on a universal Cas9 protein and only modifying the 20-nucleotides of the sgRNA sequence will retarget it to a different locus without the need to design a different protein for each DNA target. One can design multiple sgRNAs with different target sequences for simultaneous multiplex genome editing. The unparalleled simplicity, efficiency and multiplexibility makes the CRISPR/Cas9 technology a true breakthrough in genome editing.

Limitations of the CRISPR/Cas9 system include the large size of the Cas9 protein and its requirement of a GG dinucleotide in the PAM sequence. However, alternative Cas-endonucleases with other PAM specificities have been cloned from other species that can be used in the absence of an NGG-PAM sequence in the targeted sequence.

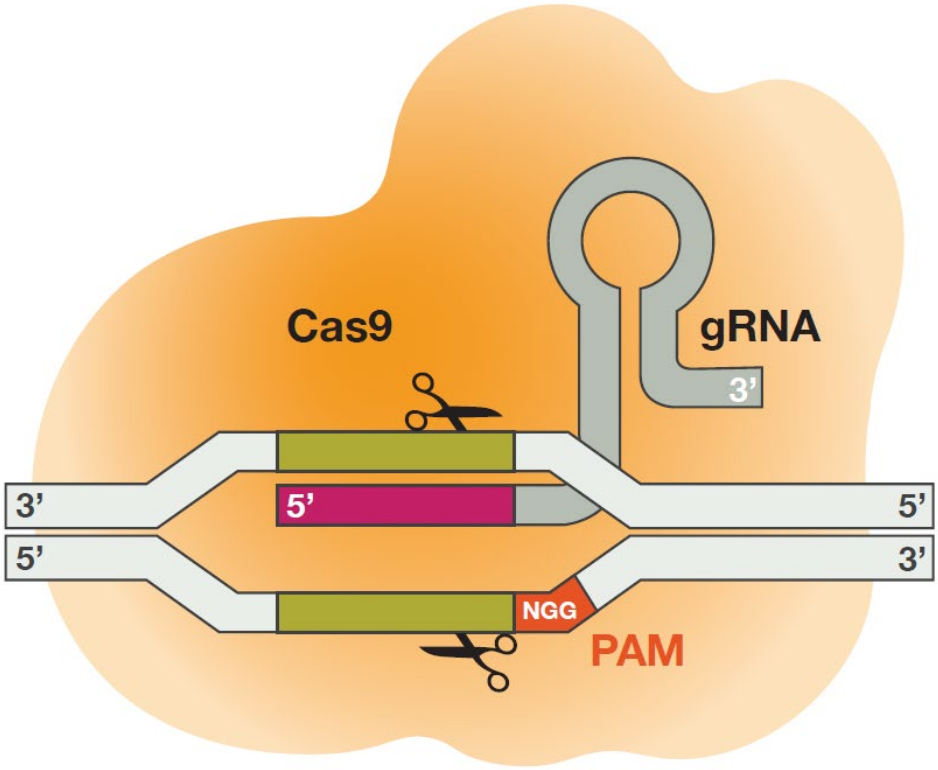

Figure 8. Cleavage of double-stranded DNA (dsDNA) by CRISPR/Cas9. The Cas9 protein attached to the dsDNA with guide RNA (gRNA) attached to the target sequence of the genome of the organism. The PAM site (containing the NGG-domain) is required to anchor the gDNA to the target sequence and successfully create clean DBS in the target genome sequence.

3.4.5.2 Modified CRISPR/Cas9

A major obstacle common to all the genome-editing technologies generating DSBs is unspecific off-target cleavage in the genome. When repaired, mutations are often introduced in the process, resulting in unwanted genome modifications. One approach to minimise off-target cleavage is to use a modified version of the native Cas9, the Cas9-nickase. Cas9-nickase is developed through a mutation in native Cas9. Cas9-nickase has one of these two mutations: a RuvC mutation – a D10A mutation abolishing the RuvC domain endonuclease activity or an HNH mutation – an H847A mutation abolishing the HNH domain endonuclease activity. A Cas9 with a RuvC or HNH mutation can create a nick, instead of a DSB at the target site when combined with a sgRNA. Instead of generating a DSB, Cas9-nikase is often used in combination with two separate sgRNAs with adjacent binding to opposite strands at a given locus, creating a double nick that will activate repair by the HDR or NHEJ pathways. A paired Cas9-nickase system, with two different gRNAs, can be used to extend the number of specifically recognised bases for target cleavage and improve the specificity of genome editing (by 50-fold over conventional Cas9) without sacrificing on-target cleavage efficiency (Khatodia et al., 2016). The use of Cas9-nickase may therefore improve the CRISPR/Cas9 specificity and minimise off-target effects.

3.4.5.3 CRISPR/Cpf1

A parallel CRISPR technology based on the Cpf1 (also known Cas12a) nuclease adds another option to the CRISPR toolbox. The CRISPR/Cpf1 system was employed as a genome-editing tool in 2015 (Zetsche et al., 2015). The CRISPR/Cpf1-system is largely similar to the CRISPR/Cas9 system, but with some differences. As for CRISPR/Cas9, the CRISPR/Cpf1-system consists of two components, the Cpf1 endonuclease enzyme and the crRNA which determines the specificity of the system. However, the CRISPR/Cpf1 system operates without a trans-acting crRNA (tracrRNA), which is necessary for crRNA maturity in the CRISPR/Cas9 system. The engineered crRNA in the CRISPR/Cpf1 system is about 42–44 nucleotides long (including a 19 nucleotide repeat and a 23–25 nucleotide spacer), compared to the ~100 nucleotides sgRNA in the CRISPR/Cas9 system. Unlike Cas9, Cpf1 endonuclease contains two RuvC-like domains and has no HNH domain. Cpf1 is guided by the single gRNA to a target site where it reorganises its PAM sequence on the opposite strand and cleaves the two strands downstream of the PAM sequence, resulting in sticky ends with 5-bp overhangs. The generation of these sticky ends enhance flexibility, and this trait is believed to establish Cpf1 as an even more robust genome-editing tool than Cas9.

Cpf1 is slightly smaller in size compared to Cas9 and Cpf1 derived from *Acidaminococcus* and *Lachnospiraceae* was shown to be effective in mammalian cells, which may also be applicable to plants (Zetsche et al., 2015).

3.4.5.4 CRISPR/CasX

CasX has quite recently been described as a new genome-editing system (Liu et al., 2019). The CRISPR/CasX system is a fundamentally distinct RNA-guided genome-editing system that differs

from the above described CRISPR-Cas systems. Like Cas9, CasX is an RNA-guided DNA endonuclease, but CasX generates a staggered DSB in DNA at sequences complementary to the 20-nucleotide segment of its guide RNA. The efficiency to generate DSB seems somewhat lower for CasX compared to Cas9. Still, the small size of the CasX protein (<1,000 amino acids), its unique DNA cleavage characteristics, its alternative PAM requirement, and its derivation from a non-pathogenic microorganism in sum offer important advantages compared to the other CRISPR–Cas genome-editing systems.

3.4.5.5 RNA-guided FokI Nucleases (RFNs)

Another example of a modified CRISPR/Cas9 system are dimeric RNA-guided FokI Nucleases (RFNs) (Tsai et al., 2014). RFNs are a fusion of dCas9 with a FokI-nuclease domain. Dimerisation of two RFNs is required for efficient genome-editing activity, which yields high genome-editing frequencies and reduced off-target mutations compared with the Cas9-nickase. The dCas9-RFNs cleavage activity depends strictly on the binding of two gRNAs with a defined spacing and orientation, which reduces the likelihood of target site occurring more than once in the genome (Havlicek et al., 2017). The dCas9 is a modified version of the native Cas9, which has a double-mutation at the two RuvC and HNH endonuclease domains of the Cas9 protein. These two catalytically inactive mutations do not affect the DNA binding ability and dCas9 still binds to DNA in a sequence-specific manner guided by a sgRNA. In the eukaryotic systems, the RNA-guided FokI Nucleases technique performs as expected in reducing off-target cleavage when compared to Cas9, however with a large trade-off in efficiency (Aouida et al., 2015; Paul and Qi, 2016). When a high frequency of mutations is not necessary over precise mutagenesis or gene targeting, it may be attractive to use the dCas9-FokI systems to take advantage of increased specificity.

3.4.5.6 Base editors

An attractive and alternative use of CRISPR technology is base editing. Unlike native Cas9, which results in DSBs and random indels at the target sites in the absence of a repair template, Cas9 based base editors are capable of precisely converting a single nucleotide base into another without causing DSBs. Base editors consist of three modules fused into one functional protein: a Cas9-nickase, a nucleobase deaminase enzyme, and an inhibitor of base-excision repair such as uracil glycosylase inhibitor (UGI) (Komor et al., 2016). Base editors have been developed to facilitate either C to T mutations or A to G mutations (Adli, 2018; Tan et al., 2019). Cas9-nickase fused to an APOBEC1 deaminase enzyme effectively converts C into T by catalyzing C to U deamination (Komor et al., 2016), whereas Cas9 fused to a modified transfer-RNA adenosine deaminase converts A to G by catalyzing A to I deamination (Gaudelli et al., 2017).

3.4.5.7 CRISPR prime editing

Prime editing is a recently described CRISPR-based genome-editing tool (Anzalone et al., 2019) that facilitates precise targeted insertions, deletions and all possible base-to-base conversions,

without requiring DSBs or donor DNA templates. The prime-editing method has been used to introduce point mutations, insertions and deletions in rice and wheat. However, the editing efficiency was relatively low (Lin et al., 2020; Tang et al., 2020).

Prime-editors consist of a prime editor (PE) protein, a Cas9-nickase fused to an engineered reverse transcriptase enzyme, and an engineered prime editor (pegRNA). The pegRNA is a guide RNA that is extended in the 3-end with a repair template that is partially homologous to the guide RNA sequence but is modified with the desired edit(s). This extended region will later function as a reverse transcriptase template and repair template. Prime editing starts by forming a complex between the prime editor (PE) protein and the pegRNA at the desired genomic location. Then, the Cas9-nickase nicks one of the DNA strands, generating a flap. The extended edited RNA sequence is used to synthesise the complementary single stranded DNA sequence by the reverse transcriptase enzyme and used to replace the original DNA strand with an edited DNA strand. This creates a DNA heteroduplex containing one edited strand and one unedited strand at the editing location. Lastly, replacement of the second unedited DNA strand at the editing location using the inserted edited DNA sequence as a repair template, completes the process. Currently, three types of prime editors have been developed. The first version developed was PE1, which could be used to introduce insertions, deletions, and base transversions at modest editing efficiencies. Then a new version of PE1 with improved editing efficiencies was developed. PE2 contains modifications that lead to improved DNA binding and thermostability. The most recent versions, PE3 and PE3b, include the ability to avoid insertion of mismatch sequences that sometimes occur with prime editing using PE1 or PE2.

Compared to base editors developed thus far that can only create a subset of changes (C->T, G->A, A->G, and T->C), prime editors are more precise and can generate all 12 possible base-to-base changes.

3.5 Non-intrusive techniques

In addition to the above-mentioned methods that cause permanent genomic alterations in the nucleotide sequence, there are other methods that do not cause heritable changes in the nucleotide sequence, but nevertheless influence the use of the inherited genome. These methods can be used to change phenotypic traits by engineering a desired phenotype by temporarily disrupting the signaling needed to translate genetic information into a functional gene product (RNA or protein) (Abudayyeh et al., 2017; Adli, 2018; Galizi and Jaramillo, 2019; Kamthan et al., 2015; O'Connell, 2019; Saurabh et al., 2014; Subbotina et al., 2016). In most cases, these changes will not persist across generations and must be reintroduced to each generation. In a few cases, epigenetic modifications may be inherited in the next generation. In the following sections we will briefly describe methods that can alter the phenotype without changes in the genome. Importantly, some of the methods below can be combined with methods that generate permanent changes in the genome.

3.5.1 RNAi

RNA interference (RNAi) is an RNA-mediated gene silencing pathway found widely in eukaryotes, including mammals. RNAi originally evolved as a defense system against foreign RNA molecules invading eukaryotic organisms. Many organisms encode for RNAi molecules that can target viral mRNA frequently infecting the host cell and help to inhibit gene expression or translation of these genes to minimise damage caused by host infection. Since its discovery in 1998, this naturally occurring system has been extensively studied and has been developed into one of the most powerful tools for temporal and reversible silencing of gene expression (Kamthan et al., 2015). RNAi specificity is based on sequence-specific recognition of a single stranded RNA target sequence through nucleotide base pairing. The short effector interfering RNA can either be delivered as exogenous double-stranded RNA (siRNA, ~22 nucleotides long) or endogenously expressed single stranded RNA (miRNA). Both of these can initially be expressed in the form of longer hairpin RNA (shRNA) that will be processed by the ribonuclease DICER (or Dicer-like enzyme) in the host cell into functionally active siRNAs. Binding of the siRNA to target RNA forms segments with double-stranded RNA (dsRNA), which may result in inhibition of transcription or translation or, alternatively, cleavage of the duplex RNA resulting in degradation of the RNA-transcript. The RNAi machinery has been incorporated into engineered and customised vector systems that nowadays represent a valuable research and crops engineering tool. When used in cell cultures or living organisms, the synthetic dsRNA introduced will selectively and robustly suppress expression of specific genes of interest (Saurabh et al., 2014).

3.5.2 CRISPR/Cas13

Cas13 (formerly C2c2) is an effector protein of class 2 type VI CRISPR/Cas systems that lacks homology to other known DNA nuclease domains. In comparison to the other RuvC domain-containing Cas proteins that recognise and cleave double-stranded DNA, CRISPR/Cas13 system can only effectively cleave RNA (O'Connell, 2019). When Cas13 assembles with a CRISPR RNA (crRNA), it forms a crRNA-guided RNA-targeting effector complex. Naturally occurring type VI CRISPR–Cas systems can be divided into four subtypes (A–D) based on Cas13 phylogeny. All Cas13 proteins possess two enzymatically distinct ribonuclease activities that are required for optimal interference. The first RNase is responsible for pre-crRNA processing to form mature Type VI interference complexes, while the second RNase activity is required for degradation of target-RNA during viral interference. The Cas13 systems are easier to design and have fewer off-target effects, compared to small RNAs (miRNA and siRNA). CRISPR/Cas13 system is therefore a promising new generation of RNA-targeting tools for applications in research, therapeutics and crop improvement.

3.5.3 Morpholinos

A morpholino oligomer consists of nucleic acids bound to a backbone of methylenemorpholine rings linked by phosphorodiamidate groups. These analogs are used in molecular biology to modify gene expression. Morpholinos are single stranded and bind sequence-specifically to

target nucleic acid molecules and block access of other molecules to small specific sequences (~25 nucleotides) when they base-pair with RNA. Unlike RNAi, they do not cause degradation of the target mRNA, but cause inhibition of splicing or prevent translation. Morpholinos are frequently used as research tools for reverse genetics to knock-out genes to investigate their function(s) (Subbotina et al., 2016).

3.5.4 Riboswitch

Riboswitches are naturally occurring genetic regulatory segments of messenger RNA (mRNA) molecules, found in all three domains of life (Archaea, Bacteria, and Eukarya). Riboswitch elements respond to a range of metabolites, including protein cofactors, amino acids, nucleobases, metal ions and some natural products, often with exquisite specificity. Most riboswitches are located in the untranslated regions (UTR) of metabolic genes and are usually involved in regulation of the associated pathway.

Naturally occurring riboswitches control gene expression through transcription, translation or by acting as cis-acting ribozymes. To date, only the glmS ribozyme is known to operate through this mechanism, although riboswitches that affect splicing in fungi, plants and algae are also known. Engineered riboswitches can be used to regulate gene expression by incorporating aptamers that can bind a particular ligand, which upon binding, produces a conformational change that modulates expression of a downstream gene. Ligand binding and the following conformational change in the expression platform will usually switch gene expression from "on" to "off" or from "off" to "on".

A sophisticated riboswitch approach can be employed to sense CRISPR-sgRNAs by attaching an aptamer domain into an allosteric structure, which prevents the binding of the gRNA-endonuclease complex to its target. An interaction with the ligand will stabilise the aptamer to create a conformational change and expose the guide sequence. This system has been tested in eukaryotes using Cas9 or Cpf1. Potentially, any ligand-responsive riboswitch could be adapted for sgRNA sensing. As an example, by replacing a naturally occurring aptamer with a modified aptamer, a light or a temperature sensing riboswitch which interacts with a photo-switchable molecule or responds to altered temperature can be created to regulate the initiation of translation or transcription termination by controlling binding of a sgRNA to its target sequence (Galizi and Jaramillo, 2019).

3.6 Future promising genome-editing techniques

3.6.1 DNA-guided genome editing

Argonaute (Ago) proteins bind different classes of non-coding RNAs, including miRNA and siRNA, and are involved in small-RNA guided gene-silencing processes. The Ago family members are highly conserved and found in all eukaryotes and some bacteria and archaea (Meister, 2013). The Argonaute protein from the thermophilic bacterium *Thermus thermophilus*

(*TtAgo*) has DNA-guided DNA interfering activity only at high temperatures (Swarts et al., 2014; Swarts et al., 2017), which may pose a challenge to their application in mammalian cells. So far, editing activity in human cells using TtAgo has not been reported.

A recent, and somewhat disputed work suggests the possibility of genome editing in mammalian cells using the Argonaute protein from *Natronobacterium gregoryi* (*NgAgo*) (Gao et al., 2016). Gao et al. used *NgAgo*, a haloalkaliphilic archaebacterium, with the help of a single-stranded DNA molecule (gDNA) as a guide for site-specific targeting. When NgAgo binds to a 5′- phosphorylated gDNA of ~24 nucleotides, it will create site-specific DNA double-strand breaks at the corresponding DNA target site. The advantage of using the NgAgo–gDNA system compared to the CRISPR-Cas system, is that NgAgo does not require a PAM sequence and will edit GC-rich regions within the genome with high efficiency. However, this paper has been retracted because another group was unable to reproduce the findings.

3.6.2 GONAD (rat Genome-editing via Oviductal Nucleic Acids Delivery)

Recently, Kobayashi et al. reported mproved genome-editing via Oviductal Nucleic Acids Delivery (GONAD) in rats (rGONAD)(Kobayashi et al., 2018). The new improvement is an *in vivo* genome-editing system using CRISPR/Cas9-system that does not require *ex vivo* handling of embryos. The *in vivo* genome-editing was applied in early preimplantation embryos present in oviducts of pregnant rats. The procedure does not require *ex vivo* handling of embryos, such as isolation of zygotes, zygote microinjection and transfer of the injected embryos to recipient females. Using this method, the authors demonstrated the feasibility of producing knockout (i.e. CRISPR/Cas9-mediated induction of an indel in the wild-type tyrosinase (Tyr) locus) and knock-in models (i.e. single-stranded-oligonucleotide-guided mutation in the Tyr-locus) in WKY rats.

3.6.3 STAGE (Sperm Transfection Assisted Gene Editing)

Generation of genetically modified mammals usually involves *in vitro* manipulation of oocytes or single cell embryos. Due to the comparative inaccessibility of avian oocytes and single cell embryos, alternative protocols have been developed to genetically modify birds. Sperm transfection assisted gene editing (STAGE) is a recently developed method where sperm are transfected with genome-editing vectors and subsequently used to deliver these genome-editing tools directly to a newly fertilised embryo (Cooper et al., 2017). STAGE will simplify genom editing in bird species, for which no efficient methodology currently exists. The STAGE approach was designed to utilise the ability of sperm to deliver nucleic acids together with genom-editing system such as CRISPR/Cas9. Exciting methods to develop transgenic and genome-edited birds take at least two-generations before reaching complete germline expressing transgenic or gene-edited birds. Using STAGE, the first generation of birds will carry the modification reducing both cost and time (Cooper et al., 2017). However, this method is still in the early stages, and adjustments are needed before this method can be used frequently. In the future, STAGE may be employed in other species including those for which no genome-editing methodology currently exists.

3.7 Outcomes of genome editing

The intention of all induced manipulations of crops, feed or livestock is to alter one or several phenotypic traits of the organism, either directly through permanent alteration of the genome, or indirectly through epigenetic changes or temporally altered transcriptional regulation of certain pathways that affect phenotypic traits. The desired phenotypic alterations can be grouped into loss of function or gain of function, both of which can be achieved through either permanent or transient genome modifications. These modifications can further be introduced at random or at a specifically selected location in the genome, depending on the techniques used.

Mosaicism is the presence of more than one genotype in one individual, i.e. variation among cells. Mosaicisms are present in nature. However, it may also be a consequence of genome editing, where the founder (F0) organism will be a mosaic because not all cells are edited. In addition to unintended off-target edits, mosaicism remains a challenge with genome-editing techniques. The mosaicism issue in the founder (F0) organism and unintended off-target edits in the F2-homozygous progeny, should be considered (Hennig et al., 2020; Mehravar et al., 2019; Su et al., 2019).

The various types of genome-editing outcomes, including off target edits, are described below.

3.7.1 Point mutations

Point mutations refer to any type of single nucleotide exchange, insertion or deletion. Point mutations arise very frequently in most cells, but the vast majority of these are quickly corrected by the DNA repair machinery and are therefore not inherited or passed on when cells divide. However, some mutations escape the DNA repair machinery and will remain uncorrected after cell division. Depending on the type of point mutation, and where the mutation is introduced, the outcome can range from having no importance to being lethal for the cell or the organism. Often, introduced uncorrected point mutations are "silent", meaning that the altered nucleotide does not interfere with regulatory cis-acting transcriptional elements or the protein coding sequence of a gene. However, some point mutations generate changes that affect phenotypic traits. Mutations will usually be deleterious or have effects on phenotypic traits when they arise within a regulatory locus, a gene regulatory region, or the protein coding region of a gene. When occurring within a gene, exchange of a nucleotide can lead to the expression of an alternative amino acid or introduce an early translation stop codon resulting in expression of a truncated protein. Mutations where a nucleotide is inserted or deleted often lead to missense transcription, meaning that the reading frame of the gene is altered, and the genetic code is transcribed incorrectly after the site where the mutation is inserted (often referred to as frameshift mutations). Occasionally, random mutations introduced in nature will be beneficial and result in an improved phenotypic trait, but this outcome is more common when the mutation is purposely introduced by humans.

Introduction of point mutations that results in frameshift is a useful method in the development of new and improved lines of crops and farmed animals, as the desired phenotype can often be

manifested through knocking out a gene. Mushrooms that do not go brown, and thus have a longer shelf life, is an example where one of the genes encoding polyphenol oxidase has been silenced by applying the CRISPR/Cas9 method (Waltz, 2016b). This has also been done with apples and potatoes, although with different technologies (Waltz, 2015a; Waltz, 2015b). Cattle born without horns is another example, where the naturally occurring knock-out mutation of the *POLLED* locus has been replicated with CRISPR technology (Carlson et al., 2016). Also, the extreme phenotype of the Belgian Blue cattle stems from a 11 bp nucleotide deletion, resulting in a non-functional myostatin gene that would normally function to repress excessive muscle growth (Kambadur et al., 1997).

3.7.2 Deletions

Deleting DNA sequences in coding or regulatory regions is an effective way to generate a loss of function variant of the gene or disrupt normal expression of the gene of interest (Britten et al., 2003; Mills et al., 2011; Tian et al., 2008). With CRISPR technology, a DNA segment can now efficiently be deleted by generating two nearby DNA double-strand breaks, whereof the region in between these two sites is removed during the repair. Simultaneous introduction of two targeted breaks can in theory also be designed to delete genomic regions up to several mega-bases in size. This technique can be used to remove an entire genomic element, such as an enhancer region, or restoring the expression of a truncated gene correcting the reading frame of a gene. Deletions can also be used to alter the expression levels of the gene of interest by manipulating the distance between the gene coding region, regulatory elements or other genes.

3.7.3 Insertions

Insertion of an additional genomic sequence is an efficient way to introduce a new (or a few new) phenotypic trait(s) (Britten et al., 2003; Tian et al., 2008). Individuals with insertions are traditionally referred to as transgenics. DNA insertion can be used as a mean to duplicate existing genes to enhance the effect of that gene. Alternatively, DNA can be inserted to disrupt a gene directly or to alter the reading frame of a gene that encodes an undesired enzyme. Insertions can also express gene products that interfere with expression of native gene products, e.g. siRNA silencing. Insertion of a DNA-segment, and especially a foreign designed DNA-construct, is one of the most utilised genome-editing methods applied within agriculture to introduce foreign phenotypic traits, such as resistance to certain pests or pesticides. Such insertions can be achieved through many different techniques. *Agrobacterium* transformation now is the most commonly used mode of transmission in plants.

3.7.4 Inversion

Inversions are DNA rearrangements where the DNA segment between two breakpoints is inverted before the breaks are rejoined. Inversions are balanced rearrangements and do not change the overall amount of the genetic material. Inversions are important contributors to

species divergence in a variety of organisms, including plants and even humans. In animals, inversion rearrangements are often associated with genetic diseases. The widespread occurrence of inversion rearrangements in plants are believed to be associated with adaptation to environmental changes. However, the functions of inversion mutations in plant genomes are poorly understood, due to lack of tools to create targeted inversion mutations prior to introduction of the CRISPR-Cas system. Recently, a research group reported that using a pair of RNA-guided endonucleases of CRISPR/Cas9, they created targeted inversion mutations in Arabidopsis *thaliana* (Zhang et al., 2017a). In the future, this approach may potentially be used to introduce targeted DNA inversions where the intent is crop improvement, or simply to study chromosomal rearrangement and gene function in plants.

3.7.5 Translocation

Translocations are DNA rearrangements whereby a DNA segment is transferred from one genomic location to another (Brunet and Jasin, 2018; Lekomtsev et al., 2016). Translocations are balanced rearrangements and will usually not change the overall amount of the genetic material. Unlike inversion, however, translocations will often result in loss (or gain) of genomic material when passed on to the next generation and are therefore often associated with lethality or severe genetic diseases in animals. The effect of translocations is less severe in plants, as these tend to have multiple copies of the same gene. Translocations can be grouped into chromosomal fusion, chromosomal rearrangements and selection/exchange of whole chromosomes.

3.7.6 Gene replacement

Gene replacement means that a native gene is replaced with an alternative version of the gene (Morton and Hooykaas, 1995; Schaeffer and Nakata, 2015). The alternative version can e.g. be a mutated form of the native gene, lack certain gene/exon regions, or be a homologous gene cloned from another species. Gene replacement is rare in nature but may be produced in the laboratory using either homologous recombination or CRISPR-Cas9 genetic engineering to replace or substitute an endogenous gene (Schaeffer and Nakata, 2015). Replacing a native gene by homologous recombination is a form of genome editing that will result in expression of an alternative gene resulting in an altered phenotype.

3.7.7 Epigenetic alterations

Epigenetics are heritable, reversible alterations in gene expression or phenotype not caused by alterations in the nucleic acid sequence. These heritable changes in gene expression are regulated by different combinations of epigenetic modifications on the genome. The most well described epigenetic modifications include DNA methylation, histone modifications and non-coding RNAs (short and long). In general, promoters of inactive genes exhibit distinct combinations of histone modifications and a high level of DNA methylation, while promoters with active transcription are associated with other unique patterns of modifications, and

hypomethylation. The ability of epigenetic modifications to transmit acquired environmentally adaptive traits to offspring is highly advantageous. The heritability of DNA methylation patterns suggests that epigenetics has played a role in plant domestication and evolution (Stelpflug et al., 2014; Stroud et al., 2013; Zhang et al., 2013). Further, it has been reported in rice that genes of stress-responsive pathways displayed an accumulation of epi-mutations due to drought stress over several successive generations (Zheng et al., 2017b), and the changes in DNA methylation pattern were observed to be stable and heritable.

An understanding of the effect of epigenetic variation such as DNA methylation on plant phenotype may provide an opportunity to further accelerate the crop improvement process. Editing of epi-alleles, which are alleles with identical DNA sequence but different expression due to different epigenetic modifications, would have important implications for plant improvement. Constructs of a fusion of dCas9 with the DNMT3 (DNA methyltransferase 3) in combination with a locus-specific sgRNA can be used to mediate epigenetic silencing of the specific locus (Adli, 2018). Further, the fusion of dCas9 to other epigenetic modulators, such as TET1 demethylase, histone demethylase or the p300 histone acetyltransferase can modulate the expression of endogenous genes. Overall, gRNA-dCas9-fusion variants offer a platform for RNA-guided DNA targeting for stable and efficient modulation of transcription and epigenetic editing (Adli, 2018).

3.7.8 Gene drive

Gene drive is a powerful genetic engineering technology that increases the probability that a specific allele will be transmitted to offspring more efficiently than at the Mendelian ratio (higher than the natural 50% probability). Gene drive can be achieved by different molecular mechanisms that will result in addition, deletion, disruption, or modification of genes. The CRISPR-Cas technology has been developed into one of the most powerful gene drive systems. A gene-drive cassette will express the Cas9 endonuclease, a sgRNA designed to target a desired locus, and most often a third expressed gene product. In addition, the cassette will contain gene segments homologous to the targeted locus to enable the cassette to integrate itself into the same locus. With this strategy, the tools needed to make the genetic alteration will insert itself and disrupt the native locus in all offspring whenever the host is mated with an individual of the same species. With time, the gene-drive cassette will eventually replace the native sequence in most (or all) individuals of a population of a species. When the targeted locus is in a non-coding region, the outcome will be insertion of a gene product. When the targeted locus is a gene coding region, the outcome will be a deletion, disruption, or modification of a gene.

A concern with gene drive, as with any potentially powerful technique, is misuse in a variety of ways or unintended consequences. A proposed use of gene drive technology is its ability to control or eradicate a insect population of a pest species or a disease vector (Nielsen, 2021). By targeting a locus important for reproduction in one gender, the other gender will ensure that the gene-drive cassette is inherited but at the same time minimising the chance of generating fertile offspring of both genders. As a consequence, reproduction of the population as a whole will decline and extinction of the breeding populationcan be the end result. This strategy has been planned to eradicate the Malaria-carrying mosquito but is not in use as all long-term

effects on biodiversity cannot be predicted (Hendrichs et al., 2021; Nielsen, 2021). A concern is that a gene drive intended to affect only a subpopulation of a species might mutate itself and end up spreading across an entire species or to closely related species.

3.8 Overview of what the various genome-editing techniques are used for

Table 3.1: Overview of the various techniques that has been used to improve organisms used in food and feed, and the various types of genomic alterations these are able to generate. The shades of blue colors reflect whether the technique is regarded as 1st, 2nd, or 3rd generation genome-editing techniques.

Outcome / Technique/ delivery method	Point mutation(s	Deletion	Random insertion	Site-specific	Inversion	Translocation	Gene replaceme	Epigenetic alterations	Gene repression	Gene drive
Chemically induced mutagenesis*	X	X	-	-	X	X	-	-	-	-
Radiation induced mutagenesis*	X	X	-	-	X	X	-	-	-	-
Transposon / insertional mutagenesis	-	X	X	-	-	-	-	-	-	-
Gene gun / Biolistics	-	-	X	-	-	-	-	-	-	-
***Agrobacterium* transformation**	-	-	X	[X]	-	-	-	-	-	-
Nuclear transfer	-	-	-	-	-	-	-	-	-	-
Pronuclear injection	-	-	X	[X]	-	-	-	-	-	-
Blastocyst injection	-	-	-	-	-	-	-	-	-	-
Chloroplast or Mitochondrial transfer	-	-	-	-	-	-	-	-	-	-
Oligonucleotide directed mutagenesis	X	X	-	X	-	-	-	-	X	-
Meganucleases	X	X	-	X	-	X	X	-	X	-

Zink Finger Nucleases	X	X	-	X	-	X	X	X	X	-
TALENs	X	X	-	X	-	X	X	X	X	-
CRISPR/Cas9	X	X	-	X	X	X	X	X	X	X
CRISPR/Cpf1	X	X	-	X	X	X	X	X	X	X
CRISPR/Other modified Cas	X	X	-	X	X	X	X	X	X	X
CRISPR/CasX	X	X	-	X	X	X	X	X	X	X
CRISPR/Cas13	-	-	-	-	-	-	-	X	X	-
CRISPR/dCAS	-	-	-	-	-	-	-	X	X	-
Base editors and prime editors	X	X	-	X	-	-	-	X	X	-
RNAi	-	-	-	-	-	-	-	X	X	-
Morpholino	-	-	-	-	-	-	-	X	X	-
Riboswitch	-	-	-	-	-	-	-	X	X	-

4 Use of genome-editing technologies in plant breeding (ToR 2)

4.1 Main applications today

Genome-editing techniques, with transcription activator-like effector nucleases (TALENs) and especially CRISPR-Cas9, have been used already for several years for research purposes in the model plant *Arabidopsis thaliana* (thale cress), and many genes have been targeted to generate mutant plants. Recently, research groups and commercial companies have used the novel technology for faster, cheaper and more precise development of new crop varieties in plant breeding. Studies of more than 20 crop species developed with CRISPR genome editing have been published since 2012 (Ricroch et al., 2017), and several new projects on other crops are underway. Most edited plant species in the pipeline are relevant for Norway, either for import, like soybeans used in feed production, or for agricultural production, like potatoes. Plant products in which CRISPR has been used to knock out genes to improve traits, e.g. stress tolerance and improved nutritional value, are closest to market today, while we can expect knock-in mutants harbouring a gene or part of a gene from a relative or another species to be introduced in the future.

In this chapter, examples of genome-edited plant species that potentially could be applicable in a Norwegian setting are presented. We have focused on a few examples of crops with new traits that are most likely to be found on the international market in the near future and that could be relevant for Norway.

4.1.1 Plant-specific methods of genome editing

Successful genome editing depends on efficient genetic transformation and regeneration of plants from edited plant cells (Altpeter et al., 2016). There are mainly three methods that are used to transfer the components of the genome-editing apparatus to the plant cell nucleus. In Agrobacterium-mediated transformation, the natural gene transfer system in the soil bacterium *Agrobacterium tumefaciens* is used to introduce the gene expression cassettes for the gRNA and for the *cas9* gene into the plant cells. Another frequently used method is particle bombardment or the 'gene gun', whereby the expression constructs are coated on gold or tungsten particles and shot into the cells. For both particle bombardment and Agrobacterium-mediated transfer, plants are regenerated from cells where the gene expression constructs are integrated into the plant cell genome (Altpeter et al., 2016). The expression constructs are subsequently crossed out from the plants regenerated from the edited plant cells to remove any foreign DNA. For plants that reproduce asexually it is impossible to remove the DNA-constructs by outcrossing, thus an alternative method is to regenerate plants from protoplasts transfected with preassembled complexes of Cas9 protein and gRNA to create edited but non-transgenic plants (Woo et al., 2015). Genome editing is even useful in polyploid plants, since it is possible

to knock out or modify several redundant gene copies on all the homologous chromosomes in a single transformation event if the gRNA is targeting a region that is conserved among the alleles (van de Wiel et al., 2017; Wang et al., 2018).

4.2 Genome-edited plants relevant for import to Norway

Many emerging genome-edited crop varieties are not relevant for cultivation in Norway. This is due to growth requirements of the crop itself, e.g. soybean, rice, or maize, none of which are well adapted to the Norwegian climate, and/or they may have redundant traits, e.g. resistance to a disease or pest not found in Norway. Genome-edited plants not suitable for cultivation in Norway may still be relevant for import, however, if the new traits confer other benefits e.g. improved taste or nutritional value.

4.2.1 Soybean (*Glycine max* L.)

Soybeans cannot be grown in Norway, but substantial amounts of soybean and soybean products are imported, mainly for feed, but also for food. The average annual import in the period 2014–2016 was 409,000 tonnes soybean, 58,000 tonnes soybean cake, 7000 tonnes soybean oil and 1700 tonnes soya sauce (FAOSTAT, 2019), in addition to imports of many types of processed foods in which soybean is an ingredient. In comparison, the total average annual production of cereals in Norway during the same period was 1,307,000 tonnes.

The majority of scientific papers documenting genome editing of soybeans focus on method development. In some studies, genes controlling important agricultural traits, such as herbicide tolerance (Li et al., 2015), seed fatty acid composition (Du et al., 2016) and flowering time/photoperiod responses (Cai et al., 2018), have been edited. Genome editing of these genes/traits may therefore soon be exploited in plant breeding for commercial use. The various methods utilising CRISPR/Cas, CRISPR/Cpf1 and TALENs that are established for genome editing of soybean are listed, together with the relevant literature, in Table 4.1.

Table 4.1. Scientific publications documenting genome editing of soybean.

Purpose of the study	Target gene	Trait associated with the gene	Genome-editing technique	Type of genetic alteration	Delivery system	Reference
Method development to mutate the FAD2–2 gene in soybean to improve the seed oil profile	FAD2-1A, a fatty acid desaturase	Fatty acid metabolism	CRISPR/Cas9	Integration	PEG mediated transformation of protoplasts	(al Amin et al., 2019)
Induce targeted mutagenesis of the GmFT2a gene in soya bean to modify time of flowering	FT2a, (Flower Locus T.)	Photoperiod response, late flowering	CRISPR/Cas9 knockout	Deletion	Agrobacterium	(Cai et al., 2018)
Modify seed fatty acid composition	FADs, fatty acid desaturases	Fatty acid composition	TALEN	Integration	Biolistic	(Demorest et al., 2016; Haun et al., 2014)
Method development for induced herbicide resistance	DD20, DD43, acetolactate synthase 1	editing at two endogenous genomic sites	CRISPR/Cas9 Cas9-gRNA	Integration	Biolistic	(Li et al., 2015)

4.2.1.1 Altered fatty acid composition

The company Calyxt (USA) has developed a genome edited soybean variety with an altered seed fatty acid composition (80% oleic acid and a 20 % reduction in saturated fatty acids and no trans fat), making the seed products healthier and less prone to fatty acid oxygenation. Members of the fatty acid desaturase FAD2 gene family are responsible for conversion of oleic acid to linoleic acid, which is further catalyzed to polyunsaturated linolenic acid by FAD3 desaturase in wild type plants. The altered fatty acid composition in the genome edited variety was obtained by using TALEN to knock-out the activity of two fatty acid desaturase genes, introducing a 63bp deletion of the *FAD2-1A* gene and a 23bp deletion of the *FAD2-1B*-gene. In February 2019, Calyxt announced their commercial launch of Calyno high-oleic soybean oil for the food industry, based on soybean production over an area totalling 34,000 acres in USA (Calyxt, 2019). Calyxt has also commercialised a high-oleic soybean meal to be used as a livestock feed ingredient. Calyxt is also working on development of varieties with other fatty acid profiles, altered protein profile, drought tolerance, higher yield and herbicide resistance.

4.2.2 Maize (*Zea mays* L.)

Maize, also called corn, is a minor crop plant in Norway, and there is only a marginal cultivation of maize, ~280 ha (Bioforsk, 2013) Maize is believed to have originated and been domesticated from wild species by Native Americans in central Mexico some 7000 years ago. During the year of 2017, maize production in the world reached 1,134 million tonnes (knoema.com).

At present, gene edited maize is most relevant for import but not for cultivation. However, with a warmer climate, maize can possibly be grown more extensivly in Norway in the future, especially in the southern and eastern regions.

4.2.3 Altered starch content

Maize kernels normally contain two types of starch; 75% amylopectin and 25% amylose. For industrial uses however, the amylose content has to be removed. The high amylopectin corn-starch can be used as an adhesive, food thickener and to improve freeze-thaw properties of food. The company Pioneer is now developing a "waxy-corn" mutant with altered starch content. This mutant was recently declared non-regulated by the USDA and is expected to be the first CRISPR-product to hit the market. Waxy maize kernels contain >97% amylopectin compared to the normal 75% content. This is due to a deletion of a ~4 kb region, constituting almost the entire starch synthase endogenous waxy gene (*wx1*), using CRISPR editing. This leads to disruption of amylose production (Waltz, 2016a). CRISPR has enabled a much faster and efficient knock-out of the *wx1* gene directly in elite inbreeds, which would not have been possible with conventional breeding, where one would also risk losing yield potential in the introgression process.

4.2.3.1 Enhanced drought tolerance

Researchers at Pioneer have developed a drought-tolerant maize variety by constitutively expressing the Auxin Regulated Gene involved in Organ Size (ARGOS) gene, which is a negative regulator of ethylene response, known for its innate ability to promote drought tolerance. To overexpress the ARGOS gene, the native maize GOS2 promoter (Protein translation factor SUI1 homolog), which confers a moderate level of constitutive expression, was introduced into the ARGOS8 5' UAS region and swapped with the ARGOS8 promoter using CRISPR-Cas9 (Shi et al., 2017). The GOS2 promoter was introduced via homology directed DNA repair (HDR) using one gRNA targeting the ARGOS8 5'UAS region for GOS2 promoter insertion, or two specific guide RNAs spanning the native ARGOS-promoter for GOS2 promoter swapping. The CRISPR-DNA constructs and GOS2 template were transformed into maize immature embryos using biolistic-mediated transformation and callus regeneration of plants. Both the modified promoter insertion and promoter swapping variants showed ubiquitous and elevated expression of ARGOS8 in multiple tissues compared to the spatial and non-uniform expression of the endogenous ARGOS8 mRNA in the wild type. When tested in field trials, the new ARGOS8 genome-edited corn variants showed increased grain yield compared to wild type under field drought stress conditions, and no reduction of yield under well-watered conditions. This is an

example of a potentially important commercial variety that differs from most other gene edited plant products on the way to market because it replaces one gene region with another having attributes other than merely performing mutations or deletions to knock out gene function. The variety is currently undergoing additional trials under varying stress conditions to reveal commercial potential (Pioneer).

Table 4.2 Genome editing case studies in maize and rice.

Purpose of the study	Target gene	Trait associated with the gene	Genome-editing technique	Type of genetic alteration	Delivery system	Reference
Improve maize grain yield under field drought stress conditions	ARGOS8	drought tolerance	CRISPR/Cas9	deletion/Insertion	Agrobacterium	(Shi et al., 2017)
Altered starch content in maize kernels	Wx1	Amylose production	CRISPR/Cas9	deletion	*	(Waltz, 2016a)
Improving crop yield in rice	PYLs	abscisic acid (ABA) signal network	CRISPR/Cas9	*	Agrobacterium	(Miao et al., 2018)

(*) Not assessed / No information provided.

4.2.4 Rice

Rice is a crop that is not relevant to grow in Norway, but gene edited rice products can be imported. The most relevant traits developed through genome editing are knock-outs of susceptibility genes conferring resistance against biotic stress like bacterial and fungal pathogens, abiotic stress like drought, and yield.

4.2.4.1 Improved yield

Several research groups have used CRISPR to target genes that improve crop yield. A Chinese group of scientists used CRISPR to introduce mutations in the genes encoding the ABA receptors pyrabactin resistance 1-like 1 (PYL1), PYL4, and PYL6 ((Miao et al., 2018). These genes are members of a subfamily of receptor genes for the key phytohormone abscisic acid (ABA) that control stress responses and growth in plants. Generally ABA production is induced upon abiotic stress stimuli. Binding of ABA to the PYL-receptors triggers physiological and biochemical responses in the plant that adapt it to abiotic stress while also inhibiting growth. However, due to functional redundancy among the genes in the PYL-family, the scientists were able to reduce growth inhibition by introducing mutations in 3 of the receptors, without losing

stress tolerance. In field tests in China, plants from the rice cultivar 'Nipponbare' used for research was edited to include mutations in all 3 PYL-receptors showed 25–31% yield improvement compared to wild type controls without losing stress tolerance. The next step in the development will be to introduce the same mutations in elite varieties that are used for farming.

4.3 Crops relevant to grow in Norway

The majority of GMOs marketed today are varieties of crops that are not relevant for cultivation in Norway. Mainly due to the growth requirements of the crop itself, e.g. soybean, maize, or rice, poorly adapted to the Norwegian climate, and/or they may have redundant traits, e.g. resistance to a pest not found in Norway, or tolerance to a herbicide unsanctioned in Norway. The use of genome-editing techniques has led to the development of new varieties within a much broader range of agricultural crops, including crops commonly cultivated in Norway. Some of these new crops contain traits relevant for Norwegian farmers and consumers.

4.3.1 Potato

Potatoes are produced in most agricultural areas of Norway. During the Second World War and in the post-war years, potatoes were cultivated on more than 500,000 decare (daa), and in 1949 potatoes were grown on 582,000 daa. The area of potato cultivation has declined during the last fifty years of the twentieth century. During the last two decades, the area of domestic potato cultivation has decreased from 150,180 daa in 2000 to 115,810 daa in 2016 (Statistics Norway, 2017; VKM, 2018a) Potato is the third most cultivated food crop in the world after wheat and maize.

Cultivated potato is tetraploid and highly heterozygous with an outcrossing nature, causing great challenges for potato breeders. It is estimated that it takes about 100,000 seedlings to obtain one new cultivar (Eriksson et al., 2016; Lindhout et al., 2011). Further challenges in potato breeding are the genetic complexity surrounding biotic stress mechanisms in higher plants and the genotype by environment (GxE) interactions affecting the performance of potato cultivars. Moreover, potato cultivars have a narrow genetic base, making it very susceptible to emerging pathogen strains. Novel breeding technologies, using genome editing, are now established in many crops (Quetier, 2016), including potato (Nadakuduti et al., 2018), to improve variety development. Based on the literature review, there are 19 articles addressing genome-editing technology in potatoes (Table 4.2). The 19 articles on genome-editied potato can be divided into four categories; (i) starch production; (ii) herbicide tolerance; (iii) quality; and (iv) disease resistance.

Table 4.3. Genome-editing case studies in potato. (Adapted from: (Nadakuduti et al., 2018)).

Purpose of the study	Target gene	Trait associated with the gene	Genome-editing technique	Type of genetic alteration	Delivery system	Reference
Identify key enzyme in the biosynthesis of cholesterol.	Sterol side chain reductase 2 (StSSR2)	Steroidal glycoalkaloids reduction in tuber	TALEN	Deletion	*Agrobacterium*	(Sawai et al., 2014)
Transient expression of TALENs in potato protoplasts for targeted mutagenesis and regeneration	Acetolactate synthase1 (StALS1)	Herbicide tolerance	TALEN	Insertion	Protoplasts	(Nicolia et al., 2015)
Tuber improvement for cold storage	Vacuolar invertase (StVInv)	Tuber improvement for cold storage	TALEN	Deletion	Protoplasts	(Clasen et al., 2016)
Use of TALENs for targeted T-DNA integration	StALS1	Herbicide tolerance	TALEN	Insertion	*Agrobacterium*	(Forsyth et al., 2016)
Rapid testing and effective delivery of TALENs	1,4-alpha-glucan branching enzyme gene (SBE1), StVInv	Degree of starch branching, cold induced sweetening	TALEN	*	Agroinfiltration	(Ma et al., 2017)
Transient expression of CRISPR/Cas9 in potato protoplasts for targeted mutagenesis	Granule-bound starch synthase (StGBSS)	Tuber starch quality	CRISPR/Cas9	Deletion	Protoplasts	(Andersson et al., 2017)
Use of RNPs for genome-editing in potato protoplasts	StGBSS	Tuber starch quality	CRISPR/Cas9 RNPs	Insertion	Protoplasts	(Andersson et al., 2018)
Understand the molecular basis of phosphate stress responses in potato	Transcription factor gene StMYB44	Phosphate transport via roots	CRISPR/Cas9	Deletion	*Agrobacterium*	(Zhou et al., 2017)
Targeted mutagenesis in potato	StALS1	Herbicide resistance	CRISPR/Cas9	Insertion	*Agrobacterium* GVR	(Butler et al., 2015)

Gene targeting via homologous recombination	StALS1	Herbicide resistance	CRISPR/Cas9 TALEN	Insertion	*Agrobacterium* GVR	(Butler et al., 2016)
Targeted mutagenesis using native StU6 promoter driving the sgRNA	StIAA2	Petiole hyponasty and shoot morphogenesis	CRISPR/Cas9	Insertion	*Agrobacterium*	(Wang et al., 2015)
Knock-out of self-incompatibility gene S-RNase in diploid potato	Stylar ribonuclease gene (S-Rnase)	Self incompatibility	CRISPR/Cas9	*	*Agrobacterium*	(Ye et al., 2018)
Development of a Gateway system for rapid assembly of TALENs in a binary vector	StGBSS	Tuber starch quality	TALEN	Insertion	*Agrobacterium*	(Kusano et al., 2016)
α-solanine-free hairy roots	St16DOX	α-solanine-free hairy roots	CRISPR/Cas9	*	*Agrobacterium*	(Nakayasu et al., 2018)

(*) Not assessed / No information provided.

4.3.1.1 Increased starch quality

Potato starch is an important component in various food and industry products (e.g. adhesives, textiles, paper, toys, etc.). Normal potato starch is composed of amylose (20%) and amylopectin (80%). The ratio between these determines the quality of the starch produced. For industrial applications only amylopectin is useful, and the presence of amylose leads to many industrial problems of a technical nature. The chemical process to separate amylose to make the starch suitable for industrial applications is laborious and energy intensive, making it environmentally unfriendly and economically unfavourable. Therefore, it would be beneficial to develop potato varieties that produce starch only in the form of amylopectin.

One single gene was found to be responsible for amylose synthesis: granule-bound starch synthase (GBSS). The gene was originally identified in maize (WAXY gene). GBSS exists in a single locus (*GBSS1*) almost in all plants, and the gene has four alleles in the tetraploid potato. In a recent study, Andersson et al. (2017) efficiently performed a multiallelic mutagenesis of the for GBSS alleles in potato by transient CRISPR-Cas9 expression in protoplast. In this study, most mutations resulted in small indels of 1-10 bp, but in 10% of the analyzed mutants they found inserts of 34-236 bp. The introduced mutations in all GBSS alleles leads to potatoes that produce starch only in the form of amylopectin.

4.3.1.2 Improve cold storage and reduce acrylamide level

Cold storage of potato is a commonly used method to reduce sprout growth and extend postharvest shelf life by reducing moisture losses, diseases and rotting. However, cold temperature causes accumulation of reducing sugars in potato tubers in a process called cold-induced sweetening (CIS). This process is caused by the breakdown of starch to sucrose, which is cleaved to glucose and fructose by vacuolar acid invertase (Brummell et al., 2011). Tubers with an increased amount of reducing sugars become less suitable for consumption and tend to have poor processing qualities; dark-coloured, bitter-tasting French fry and chip products with high amounts of acrylamide (Clasen et al., 2016).

Vacuolar invertase (VInv), is a key determining factor reducing sugar accumulation during CIS. Clasen et al. 2016 used transcription activator-like effector nucleases (TALENs) to knock out VInv from the Ranger Russet commercial potato variety. In this study, 18 plants were identified as containing mutations in at least one VInv allele, and five of these plants had mutations in all VInv alleles. Tubers from complete VInv-knockout plants had significantly lower levels of reducing sugars and acrylamide in heat-processed products.

4.3.2 Rapeseed, canola, oilseed rape (*Brassica napus* L. subsp. *napus* syn. *B. napus* L. var. *oleifera*)

Rapeseed is a common agricultural crop in Norway that is mainly used as feed but is also used for human consumption, both as oil and as a food ingredient. According to FAOSTAT, the average annual production during the period 2014–2016 was 10,500 tonnes on an area of 4,000 hectare, which is conservative compared to cereals (1,307,000 tonnes on 284,000 hectare) (FAOSTAT, 2019). During the same period, we imported 396,600 tonnes rapeseed oil, 161,900 tonnes rapeseed cake and 6,300 tonnes rapeseed per year on average, while exports were insignificant.

A rapeseed variety with tolerance to sulfonylurea and imidazolinone herbicides was developed by the biotech company Cibus (USA) using ODM (Songstad et al., 2017). The variety is on the market in USA and Canada via the seed company Falco. See further description of this case below. Another herbicide-resistant variety developed by Dow AgroScience (USA) using a ZFN-based technology was reported by Ricroch et al. in 2016 to be close to market. The various methods utilising CRISPR/Cas, ODM (RTDS) and ZNF that have been used for genome editing of rapeseed are listed in Table 4.3. Genes controlling important agricultural traits, such as seed fatty acid composition (Okuzaki et al., 2018), resistance to the fungal pathogen *Sclerotinia sclerotiorum* (Sun et al., 2018a), flowering time (Yang et al., 2018) and seed shattering (Braatz et al., 2017), have been edited. Genome editing of these genes/traits may therefore soon be exploited in plant breeding.

Table 4.4. Genome-editing case studies in rapeseed.

Purpose of the study	Target gene	Trait associated with the gene	Genome-editing technique	Type of genetic alteration	Delivery system	Reference
Modify seed fatty acid composition	Fatty acid desaturase 2	Fatty acid composition in seeds	CRISPR/Cas9	Insertion	*Agrobacterium*	(Okuzaki et al., 2018)
Improve disease resistance	BnWRKY11, BnWRKY70 (transcription factors)	Resistance to *Sclerotinia sclerotiorum*	CRISPR/Cas9	*	*Agrobacterium*	(Sun et al., 2018a)
Increased seed yield	CLAVATA1, 2 and 3	Flowering time	CRISPR/Cas9	*	*Agrobacterium*	(Yang et al., 2018)
Reduce seed shattering	ALCATRAZ	Seed Shattering	CRISPR/Cas9	*	*Agrobacterium*	(Braatz et al., 2017)
Herbicide tolerance	AHAS1C	Herbicide tolerance	Rapid Trait Development System (ODM)	*	Protoplast	(Gocal, 2015; Songstad et al., 2017)
Herbicide tolerance	*	Herbicide tolerance	ZNF	*	*	(Ricroch et al., 2017)

(*) Not assessed / No information provided.

4.3.2.1 Herbicide tolerance

The gene encoding acetohydroxyacid synthase (AHAS), also known as acetolactate synthase (ALS), is located on chromosome C1 in rapeseed. This enzyme is inhibited by the binding of sulfonylureas and imidazolinones, which are groups of compounds that are active ingredients in some herbicides. This inhibition blocks the synthesis of the amino acids valine, leucine and isoleucine, leading to rapid cessation of cell division and growth (Devendar and Yang, 2017).

The *AHAS* gene has been edited using ODM, or more specifically the Rapid Trait Development System (RTDS™) developed by the company Cibus (Gocal, 2015). The mutation leads to a spescific replacement of the amino acid serine with the amino acid asparagine, altering the bindig site for the herbicide of the enzyme ALS. This mutation makes rapeseed tolerant to herbicides containing sulfonylureas and imidazolinones. The oligonucleotide used for the mutation was introduced to protoplasts via polyethylene glycol treatment. Mutant plants were then regenerated from the edited protoplasts (Songstad et al., 2017).

4.3.3 Tomato (Solanum lycopersicum L.)

According to data from SSB (2017), there were 78 greenhouses producing tomatoes in Norway in 2015 with a total production area of 466 000 m^2 (46.6 ha). Most of the production area is located in the area around Stavanger, Rogaland county. In the period 2010–2016, 1 339 509 m^2 (134 ha) to 1 541 852 m^2 (154 ha) were grown per year, with a mean tomato production of 86,14 kg per ha. During 2006–2016 Norway imported on average 23.587± 1.438 metric tonnes of fresh tomatoes annually (Statistics Norway, 2017; VKM, 2018a).

4.3.3.1 Disease resistant tomatoes

Tomato powdery mildew is a plant disease caused by the fungal species *Leveillula taurica*, *Oidium lucopersici* or *Oidium neolycopersici*. The fungi, spread by spores, infect plants at temperatures below 30 degrees at medium to high humidity and form powdery white patches on the underside of the leaves that appear as yellow spots and patches on the upper side. The disease has been reported to lead to yield losses exceeding 50% in commercially grown tomatoes in the USA (Jackson and McKenzie, 2016). Plants contain genes for disease resistance as well as genes for disease susceptibility. A well-known strategy to confer disease resistance using genome editing is to introduce mutations in the disease susceptibility genes. The wild type *Mildew resistance locus o* (*Mlo*) gene confer susceptibility to fungi causing the powdery mildew disease in several species, and resistant plants have been successfully developed by introducing mutations in this gene with genome-editing technologies like Talen and CRISPR. In tomato, plants resistant to the powdery mildew fungal pathogen *Oidium neolycopersici* were achieved by creating homozygous loss of function mutants through the introduction of a 48 bp deletion in the *SlMlo1* gene using CRISPR-Cas9 technology with two gRNAs spanning the deletion (Nekrasov et al., 2017). *SlMlo1* is the major contributor to disease susceptibility of the 16 *Mlo*-genes in tomato. Transgene free plants were generated by selfing the primary transformants and screen for plants not harbouring the CRISPR-construct (Nekrasov et al., 2017). The whole process introducing the mutation in an elite cultivar usable for commercial production without any foreign DNA and with no off-target effect took less than a year.

4.3.4 Camelina (*Camelina sativa* (L.) Crantz)

Camelina (*Camelina sativa* L.) also called false flax, linseed dodder, or gold-of-pleasure is an annual or winter annual plant in the Brassiceae, or mustard family. Camelina originated in regions of southern Europe and south-west Asia. Camelina has historically been grown as an oilseed crop for both food and feed, mostly due to its high oil content (30-40%).

Camelina has gained some renewed interest recently due to its stress tolerance and interesting oil profile. Camelina has a strong potential to adapt to a wide range of environments. As an example, in the USA, camelina has been successfully grown from the Pacific North to the Southwest, across the Northern and Central Plains (Aiken et al., 2015; Berti et al., 2015; Gesch, 2014; Hunsaker, 2011; Schillinger et al., 2012).

There is an increasing interest in reviving the production of camelina in Scandinavia as well (Kirkhus et al., 2013).

4.3.4.1 Altered fatty acid composition

Several research groups have used CRISPR/Cas9 and Agrobacterium-mediated transformation to alter the fatty acid composition of camelina seeds by knocking out enzymes in fatty acid metabolism, like FAD2 (fatty acid desaturation 2) (Jiang et al., 2017; Morineau et al., 2017) and fatty acid elongase 1 (FAE1) (Ozseyhan et al., 2018). FAD2-mutated lines had an oleic acid

content of up to 50% of the total fatty acid content (as compared to 8% in the wild type), while FAE1-mutated lines had a reduction of very long-chain fatty acids from 22% to 2%. Camelina is hexaploid with three closely related subgenomes, and Morineau et al. showed that triple mutants where both alleles of all the three homologous FAD2 loci had been knocked out had developmental defects not found in double mutants. In 2018, an approved field experiment was set up in the UK, to test and compare the agricultural performance of a triple and double FAD2 mutant line.

Table 2.5 Genome-editing case studies in tomato and camelina.

(*) Not assessed / No information provided.

Purpose of the study	Target gene	Trait associated with the gene	Genome-editing technique	Type of genetic alteration	Delivery system	Reference
Transgene- free powdery mildew resistant tomatoes	*SlMlo1*	Resistant to powdery mildew fungal pathogen	CRISPR/Cas9	Deletion	*Agrobacterium*	(Nekrasov et al., 2017)
Altered fatty acid composition in camelina	FAD2	Fatty acid metabolism	CRISPR/Cas9	(knock-out)	*Agrobacterium*	(Morineau et al., 2017)
Improving seed oil composition in camelina	FAD2	Fatty acid metabolism	CRISPR/Cas9	(knock-out)	*	(Jiang et al., 2017)
Knocking out FAE1 to obtain optimal fatty acid composition in camelina seed oil.	FAE1 genes	Fatty acid metabolism	CRISPR/Cas9	(knock-out)	*Agrobacterium*	(Ozseyhan et al., 2018)

5 Use of genome-editing technologies in animal breeding (ToR 2)

5.1 Main applications today

Genetic improvements through selective breeding have significantly boosted livestock and aquaculture production. Farmed animals that produce more offspring, are more resistant to diseases, grow faster and with better meat quality have emerged as a result of selective breeding programs over decades. Genetic improvement in animal husbandry traditionally relies on observation and characterisation of given traits in a limited number of elite individuals and their progeny (progeny testing). The process of generating production animals from this elite population is limited by several factors, such as ability to accurately identify high merit individuals for further expansion, selection intensity, generation time of the species, maintaining existing genetic diversity and conversion of genetic variation into genetic gain (Gonen et al., 2017; Lillico, 2019).

Genome editing has a broad range of potential applications in production animals, including making livestock more adapted to farming or environmental conditions, enhancing disease resistance, improving growth, fertility and providing better animal welfare. The advances of genome-editing tools has made re-writing the genetic code faster, cheaper and more precise in farmed animals. Tools, such as ZNFs, TALENs and in particular CRISPR, have been used to alter targeted genes to be either active or inactive, both for research purposes and direct applications. Genome-editing, for example, could be used to correct heritable diseases or substitute alleles of a given gene into more desirable alleles, without the need for repeated backcrossing or outcrossing with an animal carrying the desired allele (Van Eenennaam, 2017).

Delivering nuclease-mediated genetic changes to livestock will likely occur in synergy with conventional breeding programmes. Most of the economically interesting traits in animal breeding are typically polygenic traits, where a high number of low-effect genes together control the trait. The majority of these low-effect genes remain to be identified and are therefore not available for editing. However, some single genes with strong effects on certain traits are known and are typical candidates for modification. Such edits can be delivered by genetically modifying somatic cells which are then used as nuclear donors for somatic cell nuclear transfer (SCNT). Another common delivery system is by injection of genome-editing reagents into the cytoplasm of single cell zygotes (fertilised eggs) (Tan et al., 2016).

In this chapter, examples of animal species for which genome-editing techniques could potentially be applicable in a Norwegian setting are presented.

5.1.1 Atlantic salmon (*Salmo salar*)

From the beginning of commercial-scale salmon farming in the 1960s, farming of Atlantic salmon has been the largest aquaculture industry in Norway with a production of 1.377 million tonnes in 2020 (NDF, 2020). The breeding programmes were initially focused on growth rate and reducing early sexual maturation, but from the 1990s focus was also on disease resistance and fillet characteristics (Gjedrem and Rye, 2018).

Two prominent environmental sustainability issues in Atlantic salmon farming are diseases and genetic introgression resulting from escaped farmed salmon spawning with wild populations (Grefsrud, 2021a; Grefsrud, 2021b) The same two factors are the strongest negative factors acting on the viability and status of wild Atlantic salmon (NSACAS, 2020). Further increase in salmon aquaculture in the sea is currently limited by the high prevalence of the parasite salmon louse (*Lepeophtheirus salmonis*) (Taranger et al., 2015) and regulated through a traffic light system that estimates mortality of wild salmon caused by lice from aquaculture.

Traits, such as growth, which would be relevant to enhance through genome-editing techniques are discussed below (summarised in Table 5.1).) In 2014, the first report on genome editing by CRISPR technology in Atlantic salmon was published (Edvardsen et al., 2014).

Table 5.1. Examples of studies documenting genome editing in Atlantic salmon.

Purpose of the study	Target gene	Trait associated with the gene	Genome-editing technique	Type of genetic alternation	Delivery system	Reference
Production of germ-cell free salmon	Dead end (*dnd*)	Fertility	CRISPR/Cas9	Indels at the *dnd* gene	RNA-injection into fertilised eggs	(Wargelius et al., 2016)
Increase of endogenous synthesis of omega-3 polyunsaturated fatty acids (PUFA)	Elongation of very long chain fatty acid protein 2 (e*lovl2*)	Elongation of omega-3 PUFAs	CRISPR/Cas9	Indels in the coding region of *elovl2*	RNA-injection into fertilised eggs	(Datsomor et al., 2019b)

5.1.1.1 Sterility/sexual maturation

Escaped farmed Atlantic salmon represents one of the major challenges for salmon aquaculture. Escapees enter rivers to spawn and have led to unwanted genetic introgression into the wild salmon populations in multiple rivers along the coast of Norway (Karlsson et al., 2016). Targeting genes for fertility is the first step towards a potential production of genetically sterile fish that cannot interbreed with their wild conspecifics. Producing fish that are sterile also opens up opportunities for genome editing of other traits, such as disease resistance since any change to their genome cannot be transferred to wild fish upon escape. In addition, germ cell-free sterile fish do not show the puberty-associated increase in sex steroid production (Kleppe et al.,

2017), avoiding the drawbacks of reduced growth, lower flesh quality and higher susceptibility to disease (Taranger et al., 2010). Genetic deletion of the *dead end* (*dnd*) gene in salmon by CRISPR/Cas9 technology produced completely sterile fish lacking germ cells (Wargelius et al., 2016).

5.1.1.2 Fatty acids

The health benefits of omega-3 polyunsaturated fatty acids (PUFA) are well documented (Calder and Yaqoob, 2009). Fish, in particular farmed Atlantic salmon, are primary sources of PUFA in human diet in Norway. Atlantic salmon in aquaculture are increasingly fed with vegetable oils, which contain less of these desired fatty acids. The elongase enzyme *elovl-2* (Elongation of very long chain fatty acid) plays an important role in the biosynthesis of very long chain fatty acids. Deletion of genes in the fatty acid elongation pathway using CRISPR alters the ability of salmon to produce these fatty acids themselves and has pinpointed key regulators of endogenous PUFA synthesis in Atlantic salmon (Datsomor et al., 2019a; Datsomor et al., 2019b). Manipulation of such regulatory genes has a potential of increasing the PUFA synthesis.

5.1.1.3 Growth

The genetically modifed Atlantic salmon, AquAdvantage, from AquaBounty has inserted a growth hormone-regulating gene from chinook salmon (*Oncorhynchus tshawytscha)*, with expression controlled by a promoter taken from ocean pout (*Zoarces americanus*). This transgenic line was established through plasmid DNA transgenesis in the beginning of the 1980s and is now approved for sale in several countries including the USA and Canada (Waltz, 2017). Atempts to alter growth performance with CRISPR technology has not been done on salmon, but there are other fish species where this habs been accomplished. In catfish (*Ictalurus punctatus*) the myostatin gene has been edited by CRISPR (Khalil et al., 2017). Also, a gene-edited line of the aquaculture species tilapia (*Oreochromis niloticus*), FLT 01, developed by Intrexon and AquaBounty, is illustrating the potential of genome editing for increasing growth in salmon This modified fish has been exempted from GMO regulation in Argentina (The Fish Site, 2018).

5.1.1.4 Disease

Diseases and parasites are the primary threats to sustainable aquaculture production. Although no genome editing related to diseases has been reported, there is a great research interest in this field. Several quantitative trait loci (QTLs) for resistance have been identified, such as for Infectious Pancreatic Necrosis (IPN) (Houston et al., 2008; Moen et al., 2009) which is implemented in salmon breeding programmes. Based on the success with genome editing in other species, such as the PRRSV resistance in pigs (Burkard et al., 2017), it is likely that efforts on developing disease resistance in Atlantic salmon and other farmed fish will increase. Also, sea lice represent a significant health and welfare issue for the aquaculture industry and

although no strong QTL for resistance has been reported, genome editing may be a potential solution.

5.1.2 Cattle (*Bos taurus*)

Cattle (*Bos taurus*) are among the most utilised farm animals and are an important source of both meat and milk, as well as other dairy products. More than 1000 breeds have been produced through various selection programmes around the world, where the aim has been to enhance the quantity and quality of milk or meat (Eriksson, 2018). Selective breeding has also resulted in cattle that are locally adapted to the different environments. In Norway, the Norwegian Red breeding has focused on animals with good health and high fertility. The majority of the traits being selected for are so called complex traits, controlled by a high number of genes and also being influenced by the environment. However, some traits are known to be controlled by one or a few genes and are the primary targets for genome editing. Some of these are described below and summarised in Table 5.2.

Table 5.2. Examples of studies documenting genome editing in cattle.

Purpose of the study	Target gene	Trait associated with the gene	Genome-editing technique	Type of genetic alternation	Delivery system	Reference
Production of hornless dairy cattle	*P_C POLLED*	Hornlessness	TALENs	Homozygous and heterozygous introgression of *P_C POLLED*	Somatic cell nuclear transfer	(Carlson et al., 2016)
Increased muscle growth	Myostatin (*mstn*) gene	Regulation of muscle mass	ZFN	5-bp indels at the *mstn* gene	Somatic cell nuclear transfer	(Luo et al., 2014)
Knockout of prion protein (PRNP)	Bovine *prnp* gene, encoding the PrP^C glycoprotein	Bovine spongiform encephalopathy caused by accumulation of the misfolded isomer PrP^{BSE} in the brain	CRISPR/Cas9	Indels and large deletions at the *prnp* gene in bovine cell lines and embryos	Somatic cell nuclear transfer and zygote editing	(Bevacqua et al., 2016)
Knockout of bovine beta-lactoglobulin (BLG)	*blg* gene	Hypoallergenic milk lacking the whey protein BLG	ZFN	17- and 16-bp indels leading to frameshift mutations	Somatic cell nuclear transfer	(Sun et al., 2018b)

5.1.2.1 Hornless cattle

One naturally occurring phenotype is hornless cattle. This is widespread among many breeds, but in low frequency, and particularly rare in the most common dairy breed – Holstein. The hornless phenotype results from one of two mutations, either an 80kb or a 212 bp duplication, in the *POLLED* locus of *B. taurus* (Carlson et al., 2016). This is a desirable trait in dairy cattle. Horns will need to be removed from the calves to avoid injury to other cows or to humans, which is both an economic and animal welfare issue. Linebreeding to fix the desired *POLLED* variant would be a possible way to avoid these issues, but linebreeding also results in the accumulation of other unwanted alleles and is also very time consuming. Using a combination of the TALENs genome-editing technique and somatic cell nuclear transfer, Carlson et al. was able to replicate this natural phenotype in two calves. This example of genome editing illuminates the potential for non-transgene modifications to enhance animal welfare for cattle.

5.1.2.2 Increased muscle growth

An example, with regard to meat quantity, is the extreme muscle growth in the Belgian Blue and Piemontese breeds. These breeds have been produced through linebreeding (inbreeding) that has fixed a naturally occurring mutation (11 bp deletion) of the *myostatin* gene (MSTN) (Kambadur et al., 1997). This gene is responsible for normal muscle cell growth, and the frame-shift mutation resulting from the deletion inactivates this gene, resulting in a highly abnormal muscle growth. Due to their excessive size, calves can usually not be born naturally, but are routinely delivered by caesarean section. As this mutation occurs naturally, enhancing this trait through selection and other types of breeding is not regulated. However, the same result has been achieved through genome editing of the cattle genome using both ZNFs in combination with somatic cell nuclear transfer (Luo et al., 2014) and zygote editing (Proudfoot et al., 2015). This mutation of the Myostatin gene illustrates that even though the genomic change is relatively small and does not involve any insertion of DNA, the phenotypic change can be profound.

5.1.2.3 Prion knockout

Genome editing can be used to increase food safety while maintaining animal welfare, by targeting the production of specific proteins that are involved in specific diseases (Richt et al., 2007).

In 2016, Bevacqua et al., reported successful disruption of the bovine prion protein gene (PRNP) in somatic cells using CRISPR/Cas9, which can result in resistance to Bovine Spongiform Encephalopathy (BSE) in cattle (Bevacqua et al., 2016). Norwegian dairy goats lacking prion protein due to a naturally occurring nonsense mutation in the *prnp* gene has been reported earlier (Benestad et al., 2012).

5.1.2.4 Udder health improvements

Many cattle breeds suffer from udder health issues. One of the major goals for genome editing in cattle is thus to target these issues in order to improve udder health. So far, results in this field have been achieved through production of transgenic cows, using transfected fibroblasts from Jersey fetuses that serve as nuclear donor cells (Wall et al., 2005). An example is *Staphylococcus aureus* resistant dairy cattle that do not develop mammary gland infection (mastitis), as these transgenic cows express lysostaphin in the milk (Donovan et al., 2005; Wall et al., 2005).

5.1.2.5 Other milk protein modifications

The improvement of milk quality, in terms of protein and enzyme composition, has been the focus of many genome engineering projects. For example, bovine milk completely free from β-lactoglobulin, a major allergen, has been produced by ZFNs (Sun et al., 2018b).

5.1.3 Domestic pig

Worldwide meat production from pigs was 113.08 million metric tonnes in 2018, compared to 95.5 million metric tonnes for chicken meat, which is the second largest terrestrial meat production in the world (Statista, 2018). In Norway, targeted selection has been nationally organised in a farmer-owned breeding company for decades (Norsvin). Their current breeding goals can be grouped under the categories of production, slaughter quality, meat quality, litter size, reproduction, mothering ability and robustness/health. Today, Norwegian pig genetics is sold to 54 different countries, in close collaboration with the Dutch breeding company Topigs (Topigs Norsvin) (Norsvin). In general, production traits like growth rate, meat and fat content are considered to be efficiently handled by the traditional breeding methods, which is far cheaper than using CRISPR technology. The interest in using genome-editing technologies is therefore aimed at diseases, robustness, sex ratio, pigment spots etc. (see Table 5.3), which are not as easily handled by the breeding programme. In any case, current genome-editing methods will normally be used on a limited number of animals. However, this also means that prioritising certain genetically modified individuals for the future generations may decrease valuable genetic variance in the population.

Table 5.3. Examples of studies documenting genome editing in domestic pig.

Purpose of the study	Target gene	Trait associated with the gene	Genome editing	Type of mutation	Method used	Reference
Resistance to Porcine reproductive and respiratory syndrome (PRRS) virus	*cd163*	A lectin, present on the surface of macrophages, involved in the entry process of PRRS virus	CRISPR/Cas9	Deletion of exon 7 at scavenger receptor cysteine-rich (SRCR) domain 5	Zygote editing	(Burkard et al., 2017)
Cold resistance	Uncoupling protein 1 (*ucp1*)	Heat generation and regulation of energy homeostasis	CRISPR/Cas9	Insertion of exons 3-5	Knock-in of mouse adiponectin-*ucp1* combined with somatic cell nuclear transfer	(Zheng et al., 2017a)
Removal of boar meat taint	Kisspeptin receptor 1 (*kiss1r*)	Trigger of puberty and regulation of Gonadotropin-Releasing Hormone	TALEN	Indels ranging between 1-3 bp	Somatic cell nuclear transfer	(Sonstegard et al., 2016)

5.1.3.1 Disease resistance

Porcine reproductive and respiratory syndrome (PRRS) is the most economically important disease of swine in North America, Europe and Asia. The causative virus (PRRSV) seems to enter the cells via the CD163-receptor (Van Gorp et al., 2008). CRISPR-Cas9 has been used to generate pigs lacking functional CD163 (Burkard et al., 2017). The modified animals appear to be fully resistant to PRRSV infection (Whitworth et al., 2015). To avoid disrupting the biological function of CD163, more precisely engineered receptors have also been made, in which only the virus binding part of the receptor has been deleted. Animals carrying these engineered receptors seem to be fully resistant to PRRSV infection, while the hemoglobin-haptoglobin scavenger function of CD163 is still intact (Burkard et al., 2017; Burkard et al., 2018).

5.1.3.2 Thermoregulation

Uncoupling protein 1 (UCP1) plays a key role in brown adipose tissue-mediated adaptive non-shivering thermogenesis (Wang and Seale, 2016). UCP1 is found in most mammals, but has lost its function in some mammalian lineages, including pigs. Consequently, pigs have poor thermoregulation and piglets are therefore easily lost due to cold stress at birth (in cold regions). Fat deposition in pigs is one way to compensate for the lack of thermoregulation, but has been selected against over decades, making modern pigs more susceptible to cold stress. Restoring UCP1-function in domestic pigs could potentially improve thermoregulation and

reduce fat deposition, beneficial to both pig welfare and production. Using CRISPR/Cas9-mediated site-specific integration of functional UCP1, Zheng et al. showed that these modified pigs had a significantly improved thermoregulation, and that fat deposition was reduced without altering physical activity or daily energy demands (Zheng et al., 2017a).

5.1.3.3 Boar meat taint

Physical castration of male pigs is commonly used to avoid boar taint, the particular odour and flavour from male pig meat. The boar taint is mainly caused by the sexual steroid androstenone and the tryptophan break-down product, skatole. Skatole is metabolised in the liver, but this process is reduced by sexual steroids, resulting in accumulation in fat in males (Poulsen Nautrup et al., 2018). Based on both animal welfare and production costs concerns, physical castration is undesirable. Several researchers have reported QTL with effects on skatole or androsterone (Burkard et al., 2018). Kisspeptin plays a role in the onset of mammalian puberty and reproduction. TALENs-edited pigs that have had their receptor gene, *kiss1r*, knocked out have been shown to lack testicular development, but yet react to gonadotropin treatment (Sonstegard et al., 2016).

5.1.4 Chicken (*Gallus gallus domesticus*)

As the second most consumed animal species in the world, eaten by humans of all ages and religions, chicken (*Gallus gallus domesticus*), as well as eggs, are in high demand. In a society with high consumption and increasing demands for market ready (and cheap) meat, genome-editing techniques targeted at growth rate, production of allergen-free or allergen-reduced eggs or meat, nutrient content and disease resistant traits are likely to be utilised by the food industry soon.

Genetic modification of chickens (summarised in Table 5.4) has lagged far behind that of other organisms because of the difficulty in accessing and manipulating the zygote (Mizushima et al., 2010). Transgenic chickens have, in general, been produced by two different procedures; viral transfection systems or genetically modified embryonic cells transferred directly into the recipient embryo (Bednarczyk et al., 2018). However, viral vector infection of the early stage embryo has often resulted in high embryonic lethality, as well as transient, variable and off-target effects. Primordial germ cells (PGCs) can be cultured and genetically modified *in vitro* and subsequently injected into recipient embryos, generating transgenic chickens. The PGCs can also be transplanted into sterile males to produce transgenic offspring (Trefil et al., 2017). Using newer techniques such as TALEN and, especially, CRISPR/Cas9, the transgenesis is dramatically more efficient and specific (Dimitrov et al., 2016; Oishi et al., 2016; Park et al., 2014; Sid and Schusser, 2018; Taylor et al., 2017) . The CRISPR/Cas9 system has also been utilised to assess efficiency of gene knockouts on avian embryonic somatic cells (Abu-Bonsrah et al., 2016; Gandhi et al., 2017).

Table 5.4. Examples of studies documenting genome editing in chicken.

Purpose of the study	Target gene	Trait associated with the gene	Genome editing	Type of mutation	Method used	Reference
Disruption of egg white genes in chicken	Ovalbumin (*ova*) and ovomucoid (*ovm*)	Allergenicity in eggs	CrispR/Cas9	1-31 bp deletions	Transfection of cultured PGCs, which are transplanted into chicken embryos	(Oishi et al., 2016)
Generation of pathogen-resistant poultry (review)	Genes for specific membrane receptor proteins for cell entry for various pathogenic viral agents	Genetic susceptibility of infectious diseases	*	*	*	(Sid and Schusser, 2018)
Sex determination (review)	*	*	Use of GM techniques that enable early sex determination (by inserting a fluorescent green protein in parents) hatching eggs before incubation	*	*	(Bruijnis et al., 2015)

* Not assessed / No information provided

5.1.4.1 Allergen-free eggs

Hen's eggs are a widely consumed source of protein. In 2016, Oishi et al. showed that the CRISPR/Cas9 system could be used for production of allergen-free or allergen-reduced hen's eggs by disrupting ovalbumin and ovomucoid genes in parent generations. Later, in 2018, the same group demonstrated that transgene insertion at the chicken ovalbumin locus resulted in stable expression of exogenous protein in the egg white, making it an excellent bioreactor for production of pharmaceutical proteins.

5.1.4.2 Disease resistance

The chicken industry faces many animal health, animal welfare and undesirable environmental effects of the production systems, problems that potentially could be improved by genome editing. For instance, as a high output industry, poultry are often kept in unnaturally high densities and numbers. This allows diseases to spread easily and affect many individuals. To prevent diseases, the farming systems in many countries have a record of high antibiotic usage,

which is not only expensive but also can disturb the natural microbiota of the animal and, even more detrimental, may cause resistance development in zoonotic pathogens and disseminate such antibiotic traits in the environment. Genome editing targeted at disease resilience could potentially increase both animal health and welfare, while ecologically reducing the risk of antibiotic resistance. While still in early stages, results are promising towards producing avian virus resistance, with no side-effects of virus-resistant alleles on animal health or reproduction detected so far (Koslova et al., 2018; Sid and Schusser, 2018).

5.1.4.3 Sex determination

Another area suited for genome editing is that of sex determination. As of today, the common industry practice is to kill male chickens one day after hatching (Krautwald-Junghanns et al., 2018). Ensuring only female offspring could eliminate this practice and increase animal welfare at both individual and population level. Using genetically engineered hens, with green fluorescent marked Z chromosomes, sex can be determined before incubation (Bruijnis et al., 2015). However, fluorescent spectroscopy necessitates shell perforation, which is associated with reduced hatching rate (Krautwald-Junghanns et al., 2018). In mice, a recent study provided first evidence of genetically biasing the offspring sex ratio through the CRISPR/Cas9 system (Yosef et al., 2019). The technology is promising, but still in its infancy and not yet tested in poultry.

5.1.5 Sheep

A limited number of genome-editing experiments have so far been carried out in sheep. Typically, genes with well-known phenotypic effects, such as *mstn* (associated with myostatin-related muscle hypertrophy), *asip* (encoding Agouti Signalling Protein, a peptide that determines coat colour) and *bco2* (encoding beta-carotene oxygenase 2, associated with fat tissue colour) has been used in proof of principle experiments (Niu et al., 2017; Wang and Seale, 2016; Zhang et al., 2017b). CRISPR/Cas9 has also been used to introduce loss-of-function mutations in the fibroblast growth factor 5 (*fgf5*) gene, known to regulate hair length in several mammals. The modified sheep showed increased wool staple length, stretched length and fleece weight, and the authors suggest that this could be used for increasing wool length and yield (Hu et al., 2017; Li et al., 2017). In addition, a gene related to fecundity, *bmpr-ib* (bone morphogenetic protein receptor type IB), has been modified by CRISPR/Cas9 in *in vitro*-generated sheep embryos (Zhang et al., 2017b).

5.1.6 Honeybees (*Apis mellifera*)

Genome editing of honeybees (*Apis mellifera*) is slightly on the side of the mandate, as bees themselves are not consumed for food, but their major product, honey, is. Honey is produced inside the honey stomach of the bees by mixing flower nectar (secreted from plants) or honeydew (secreted from other insects) with enzymes and saliva. The resulting honey is then regurgitated into the honeycombs of the hive and can be collected.

Recently, substantial losses in bee colonies have been reported (EFSA, 2021e). There are several scenarios in which genome editing of the honeybee could improve their health and well-being, e.g. host resilience toward parasites, insecticides and bacterial, fungal or viral infections.

The last few years, several genome-editing protocols for the honeybee have been successfully established; i) RNA interference (RNAi) (Amdam et al., 2003; Demares et al., 2014; Hasselmann et al., 2008) ii) the PiggyBac transposon system (Schulte et al., 2014) and iii) the CRISPR/Cas9 method (Hu et al., 2019; Kohno and Kubo, 2018; Kohno et al., 2016; Roth et al., 2019). None of these techniques have yet been used to modify bees that will be used for food production, but as the protocols are quite newly established, it is not unlikely to happen in the future.

6 Use of genome-editing technologies in microorganisms (ToR 2)

6.1 Main applications today

6.1.1 Fungi and bacteria

The use of bacteria and yeasts in fermented foods is common worldwide. Bread, dairy products, fermented meats and fermented beverages, such as beer and wine, have been consumed by cultures for thousands of years. In addition to their traditional uses, bacteria and yeasts are currently utilised by the industry to synthesise diverse value-added compounds that have applications in pharmaceutical, cosmetic, food and feed products. However, the choice of a particular strain or species for a specific industrial application is often based on historical, rather than scientific grounds. In recent years, newly developed tools for genome editing have made it possible to efficiently alter traits of organisms in a specific and targeted way, avoiding random mutagenesis. Furthermore, these technologies have now become available for use in many different species of bacteria and yeasts. This offers opportunities for the industry to generate new strain variants that perform better than the strains used previously.

The most frequently used of the above-listed methods is CRISPR/Cas. CRISPR/Cas-based technologies have already opened new avenues for more rapid development of probiotics and starter strains/cultures for the food and feed industry. An expressed goal is to develop safe, better tasting and health-promoting products for human and livestock consumption. In this regard, the methodological utility of CRISPR/Cas and similar technologies has the potential to become a game changer for the industry.

The larger field of synthetic biology is growing rapidly, especially in model host systems such as *Saccharomyces cerevisiae, Escherichia coli* and *Bacillus subtilis. S. cerevisiae* has served as the major eukaryotic organism in this field, but it lacks the metabolic potential present in many of the more than one thousand yeast species identified to date. Similarly, there is a great unexplored metabolic potential among the plethora of species constituting the prokaryotic domain. The so-called non-conventional yeasts and bacteria have remained difficult to exploit, however, as there have been few genetic tools available to access their underlying metabolic networks. The CRISPR/Cas-based technologies have been shown to be adaptable to an increasing number of non-conventional species, hence their limitations as synthetic biology platforms no longer represent a major obstacle. This will likely enable industrial biotechnology to use non-conventional organisms for the economical production of small molecules and proteins.

The key examples from the articles selected from the literature searches (2013–2018 and 2018–2019) have been summarised here:

The most common microorganisms used for genome editing are yeasts (fungi) and bacteria. Fermented foods are a staple in the modern diet, with milk, meat, grains and cabbage being the most common substrates. Other microorganisms are used as "probiotics". Probiotic microorganisms may also be added as adjuncts in fermented products or other food matrices or supplements (Stout et al., 2017).

Microorganisms, in particular lactic acid bacteria (e.g. *Lactobacillus* spp.) and yeasts (e.g. *Saccharomyces*), used both as start culture for fermentation and "probiotics", have an especially high occurrence of native CRISPR-Cas systems. Such loci appear in about 63% of analysed lactobacilli and 77% of bifidobacteria genomes (Briner et al., 2015; Stout et al., 2017; Sun et al., 2015). A similar system is also found in *Saccharomyces* (Yan and Finnigan, 2018).

- Fungi belonging to the genus *Saccharmyces* and in particular *Saccharomyes cerevisiae,* but also a number of non-conventional yeasts with different evolutionary distance to *S. cerevisiae*, have increasingly attracted attention for production of pure chemical substances, oils and recombinant proteins (Cai et al., 2019). *S. cerevisiae* is a widely used yeast in the biotechnology industry. It exhibits high tolerance against harsh industrial conditions and has therefore been developed as a platform microorganism for metabolic engineering (Lian et al., 2018).

- Lactic acid bacteria, in particular *Lactobacillus* spp. are relevant for use in fermentation processes or as probiotics to elicit health benefits (Barrangou and van Pijkeren, 2016; Selle and Barrangou, 2015b; Stout et al., 2017).

- Use of other bacteria with potential in food and feed production, for example *Bacillus* and *Streptomyces* (Chan et al., 2016; Liu et al., 2018) , constitutes a minor proportion of the published studies.

CRISPER, TALEN, and Zinc finger nucleases are the major genome-editing technologies used in microorganisms . Among these, CRISPR-based techniques are the most commonly used.

To the best of our knowledge, most gene edited microorganisms are intended for contained rather than open use. Current regulation of contained use considers the potential for dissemination of recombinant organisms in the environment.

6.2 Potential use of genome-edited microorganisms in the near future

6.2.1 Improvement of probiotic properties

Teichoic acid (TA) is present in all *Lactobacillus* spp. The presence of TAs in probiotic *Lactobacilli* might stimulate dendritic cells and lead to inflammation in the colon. The role of TAs in immunomodulation is a potential target for improving the probiotic properties of relevant strains and species (Van Pijkeren and Barrangou, 2017; van Pijkeren and Britton, 2014). However, some species such as *L. casei*, *L. rhamnosus, L. fermentum* and *L. reuteri* have been found to lack wall teichoic acid. The CRISPR-Cas system might be used to change the properties of TAs in probiotic strains to reduce their inflammatory properties.

6.2.2 Antimicrobial activity

Native CRISPR-Cas may act as a tool and programmable antimicrobial with the ability to selectively eliminate targeted strains or control mixed start culture population, used in fermentation. For more information see (Stout et al., 2018).

6.2.3 Enhanced native antimicrobial activity

The review article by Van Pijkeren and Barrangou draws attention to antimicrobial compounds such as bacteriocins, which are produced by *Lactobacillus* spp. and can inhibit or kill other bacteria (Van Pijkeren and Barrangou, 2017). They are small, ribosomally synthesised peptides whose antimicrobial properties against pathogens have not been fully explored. Several attempts have been made to construct a tailored probiotic with high *in vivo* killing activity. However, it remains to be seen whether naturally acquired or engineered bacteriocins impact the *in vivo* fitness of the host bacterium.

6.2.4 Vaccination of industrial microorganisms (plasmid based)

Predatory viruses (bacteriophages) constitute a significant threat to starter cultures in the processing plant environment. A phage attack may slow or stop the fermentation process, resulting in reduced product quality (Selle and Barrangou, 2015a; Stout et al., 2017). In fact, a phage attack on a mono-culture population may cause the entire population to crash. Genetic transfer of plasmids containing native phage resistance mechanisms and/or CRISPR can be used to combat phage attacks on the starter strain population (Barrangou and Horvath, 2012).

6.2.5 CRISPR-based genotyping

Due to inherent diversity of microorganisms and their ability to undergo horizontal gene transfer, typing of bacterial strains represents a challenge. CRISPR-array genotyping offers a rapid, affordable and high-resolution typing of bacterial strains carrying such arrays. Today

CRISPR-based genotyping is used for many different bacterial species, including industrial fermentation starter cultures such as *Streptococcus thermophilus* and "probiotics" such as *L. casei* strains (Selle and Barrangou, 2015a). CRISPR/Cas repeat–spacer arrays is a new and effective method that can be added to other techniques, such as pulsed-field gel electrophoreses, repetitive-PCR, and 16S rDNA sequencing. Using microorganisms with CRISPR/Cas in many fermentation microorganisms, the start culture industry is situated in a favorable situation to use CRISPR/Cas in strain typing application (Stout et al., 2017).

A list of gene edited microorganisms with potential to be used in food and feed is shown in Tables 6.1 and 6.2.

Table 6.1. Examples of industrial fungi with potential to be used in food and feed, engineered using CRISPR/Cas systems (modified after Donohoue et al., 2018).

Species	Purpose of study/industrial relevance	Modifications/type of alteration	References
Fungi			
S. cerevisiae	Common production strains	- Donor-mediated gene disruption - Multiplexed donor-mediated gene disruption - Multiplexed recombination - CRISPRa, CRIISPRi *	(David and Siewers, 2015; Jakociunas et al., 2015) (Bao et al., 2015; Biot-Pelletier and Martin, 2016; DiCarlo et al., 2013; Gilbert et al., 2013; Ronda et al., 2015; Ryan et al., 2014; Smith et al., 2016; Zalatan et al., 2015)
Aspergillus oryzae	Industrial-scale production of recombinant proteins and enzymes	Mutagenesis: multiple gene deletions/integrations	(Katayama et al., 2019)
Aspergilus niger	Constitutive production of pectinase. Pectinase produced by *A. niger* are used in food industry. Pectin as polysaccharides in plants is the substrate for *A. niger*.	Mutation in Gaar, the regulator of D-galacturonic acid-responsive genes	(Alazi et al., 2019)

*CRISPRa; CRISPR activation, CRIISPRi; CRISPR interference.

Table 6.2. Examples of industrial bacteria with a potential to be used in food and feed, engineered using CRISPR/Cas systems (modified after (Donohoue et al., 2018)).

Species	Purpose of study/industrial relevance	Modifications/type of alteration	References
Bacteria			
L. reuteri	Probiotic strain and producing of biotherapeutics	Recombination: oligonucleotide-mediated deletion	(Oh and van Pijkeren, 2014)
L. casei	Biotechnological production of acetoin (flavouring compound naturally occurs in wine, honey, milk, coffee, etc.	Using single plasmid genome (system), 4 different genes (*hicD3, pflB, ldh*, and *phdC*), responsible for acetoin biosynthesis	(Xin et al., 2018)
Lactococcus lactis	Nisin-controlled inducible expression. High nisin concentration important for inhibiting pathogenic bacteria in fermented food	Single plasmid system; simultaneous inducible co-expression of multiple recombinant genes	(Berlec et al., 2018)
Streptococcus thermophilus	Probiotic and industrial fermentation strains provided resistance against phages attacks	Engineered immunity because of acquired spacers in phage resistance mutants	(Barrangou et al., 2007)

7 Implications for risk assessment of genome-edited organisms (ToR 3 and 4)

In recent years, techniques have been developed for genome-editing of plants, animals and microorganisms. The potential implications for the established framework of risk assessment need to be evaluated for the new genome-editing techniques. Are current risk assessment methodologies developed for traditional GMOs adequate to evaluate potential risk of organisms developed by genome editing?

Today, VKM's risk assessments of genetically modified organisms are conducted in accordance with the EFSA guidance documents. Therefore, this report aims to ascertain the adequacy of or challenges associated with the guidance documents in risk assessment of genome-edited organisms. The suitability of the following EFSA guidance documents have been evaluated;

1) Guidance for risk assessment of food and feed from genetically modified plants (EFSA, 2011a)
2) Guidance on the environmental risk assessment of genetically modified plants (EFSA, 2010a)
3) Guidance on the risk assessment of food and feed from genetically modified animals and guidance on animal health and welfare aspects (EFSA, 2012a)
4) Guidance on the environmental risk assessment of genetically modified animals (EFSA, 2013)
5) Guidance on the risk assessment of genetically modified microorganisms (EFSA, 2011e)

The potential implications of different genome-editing techniques on the established framework of risk assessment are discussed.

Selected genome-edited plants, animals and microorganism described in Chapters 4, 5 and 6 are used to evaluate the adequacy of the guidance documents. The evaluation of the applicability of the guidance for risk assessment of microorganisms was performed at a general level, given the limited number of examples of such organisms that are expected to reach the food chain in the near future. Microorganisms are rarely relevant as a single defined food or feed source alone in contrast to plants and animals. Microorganisms are, however, important in processing food and feed, e.g. in fermenting and for prodcing food and feed addditives in industrial production facilities.

A more detailed step-by-step approach was taken in the evaluation of the guidance for risk assessment of plants and animals where specific and relevant case examples could be found. Six cases of genome-edited plants and five cases of genome-edited animals are presented in Box 7. These cases were chosen based on the editing techniques used, types of edits

introduced and their potential relevance in Norwegian food production. The types of edits are categorised based on the extent of molecular changes introduced. The different types are described below.

7.1 Site-directed nucleases (SDNs) and oligonucleotide-directed mutagenesis (ODM)

The new techniques for genome editing of plants, animals and microorganisms are based on the use of engineered site-directed nucleases (SDNs) for targeted mutagenesis of genes or targeted insertion of DNA sequences (Friedrichs et al., 2019; Grohmann et al., 2019). Genome-editing techniques can efficiently induce specific changes in the genome of the target organism and include approaches with Meganucleases (MN), Zinc Finger Nuclease (ZFN), Transcription activator-like effector nucleases (TALENs), CRISPR/Cas systems (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein) and Oligonucleotide-directed mutagenesis (ODM), with CRISPR becoming the predominant genome-editing technique. Recently described base- and prime-editing techniques can be employed to edit single nucleotides as well as alter sequences of DNA at specific sites without involving double-strand breaks (DSBs), respectively.

Genome-editing techniques usually use SDNs, which cleave DNA at specific sites and trigger the organism's own DNA repair mechanisms. SDNs are nucleases that are directed to specific DNA sequences through a DNA binding moiety. CRISPR, TALEN and ZNF all use SDNs to induce a DNA break at specific sites in the genome. On the contrary, ODM uses a site-specific oligonucleotide (20 – 100bp) to cause specific changes to one or only a few bases of the DNA without inducing a DNA break. The oligonucleotide is identical to the DNA sequence in the organism, except for the base-pair change(s). The organism will repair this 'mismatch' by incorporating it into its own DNA sequence, resulting in a specific targeted change in the genome. Genome-editing techniques can induce modifications such as insertions/deletions (INDELs) of nucleotides, gene inversions or translocations, changes in the nucleotide sequence, or even deletion or insertion of larger pieces of DNA (see Chapter 3).

All SDN variants target a specific locus in the genome and use enzyme (nuclease) activity to induce DSBs in the DNA. However, they trigger different repair outcomes depending on whether a repair template is introduced or not. SDNs can be divided into three categories; SDN1, SDN2 and SDN3, reflecting the complexity of the genome editing (EFSA, 2012c; Lusser et al., 2012; Podevin et al., 2013) (Figure 9). ODMs, on the other hand, introduce mutations without DSBs (Figure 10). In this report, these categories will be used to evaluate the adequacy of the EFSA guidance documents in risk assessment of genome-edited organisms.

For the SDN1 category, no repair template is added to the cells together with the enzyme (SDN). The DSB is repaired by Non-Homologous End Joining (NHEJ), an error-prone mechanism, in most cases, generating small, site-specific INDELs that can create frameshifts that knock out the gene function. Larger regions can be deleted if two guide RNAs (gRNAs) are used to flank the region to be removed. In the case of insertions (e.g. 1-10 bp), the inserted material is derived from the organism's own genome, i.e. no exogenous material is introduced.

Making edits categorised as SDN1 is an efficient and versatile way of introducing new traits in an organism when the genetic basis and target site is known. Compared to conventional breeding techniques, techniques used to introduce such edits (SDN1) provide more predictable outcomes by specifically editing targeted DNA sequences, thereby reducing the time needed to attain and identify samples (plants) with the desired traits. However, although the site of DSB is specific for the SDN1 cateory, the outcomes of the repair are more or less random and further selection is needed.

To introduce genome edits categorised as SDN2, a repair template (an oligonucleotide) is added to the cells simultaneously with the SDN. The template is identical to the sequences flanking the DSB introduced by the SDN, except for one or a few nucleotides. The DSB is then repaired by Homology Directed Repair (HDR), an error-free mechanism, generating a site-specific desired point mutation (specific nucleotide substitutions of a single or a few nucleotides or small insertions or deletions). The efficiency of introducing edits categorised as SDN2 is lower than for SDN1 because the introduction of the SDN-tool to the cell must be coordinated with the delivery of the DNA repair template.

For genome edits categorised as SDN3, a DNA fragment is introduced at a predefined site (locus) in the genome with a template containing an exogenous DNA fragment or gene cassette (construct) together with flanking DNAs showing homology to the target locus, in combination with a nuclease (SDN) (Podevin et al., 2013) . The construct is then inserted at the site targeted by the gRNA (CRISPR), ZF-finger (ZNF) or TALE-effectors (TALEN) by HDR resulting in the introduction of the genetic material, e.g. the insertion of foreign genes. The result is a transgenic organism if the DNA fragments or genes are derived from other species. The efficiency of introducing edits categorised as SDN3 is lower than for both SDN1 and SDN2. Organisms with transgene inserts categorised as SDN3 differ from organisms developed by other transgene techniques (e.g. transformation by *Agrobacterium tumefaciens*), since the insertion of the transgene is targeted to a predefined region of the genome by the technique used (e.g. by CRISPR/Cas9), as opposed to random insertion (EFSA, 2012c).

ODM, however, does not use a nuclease to induce DSB for introducing site-specific mutations. Instead, a synthetic single-stranded oligonucleotide, which is complementary to the target sequence except for one or a few mismatches, is delivered to the cell where it precisely binds to the DNA target (Mohanta et al., 2017). The cellular mismatch repair mechanism recognises the mismatch and repairs the target DNA with the synthetic oligo as a template, introducing single nucleotide polymorphisms (SNPs) to the target gene (Mohanta et al., 2017).

In recent years, a new technique called base editing (BE) has been developed. Base editing enables a directly targeted and irreversible conversion of a specific DNA base into another at a targeted genomic locus without requiring DSBs. The base editors, either a cytidine deaminase or an adenine deaminase linked to a nCas9 nickase, together trigger the DNA mismatch repair system converting a C:G pair to an A:T pair or an A:T pair to a C:G pair, respectively. There are several examples of base editing for crop improvements developed during the last couple years, especially in rice, and also in wheat, potato, tomato and watermelon (Mishra et al., 2020).

Prime editing (PE) is another new genome-editing tool, which was reported recently by Anzalone et al. (Anzalone et al., 2019). Prime editing is a 'search-and-replace' genome-editing tool that facilitates targeted insertions, deletions and all possible base-to-base conversions, without requiring DSBs or donor DNA templates. The prime-editing method has been used to introduce point mutations, insertions and deletions in rice and wheat. However, the editing efficiency was relatively low (Lin et al., 2020; Tang et al., 2020).

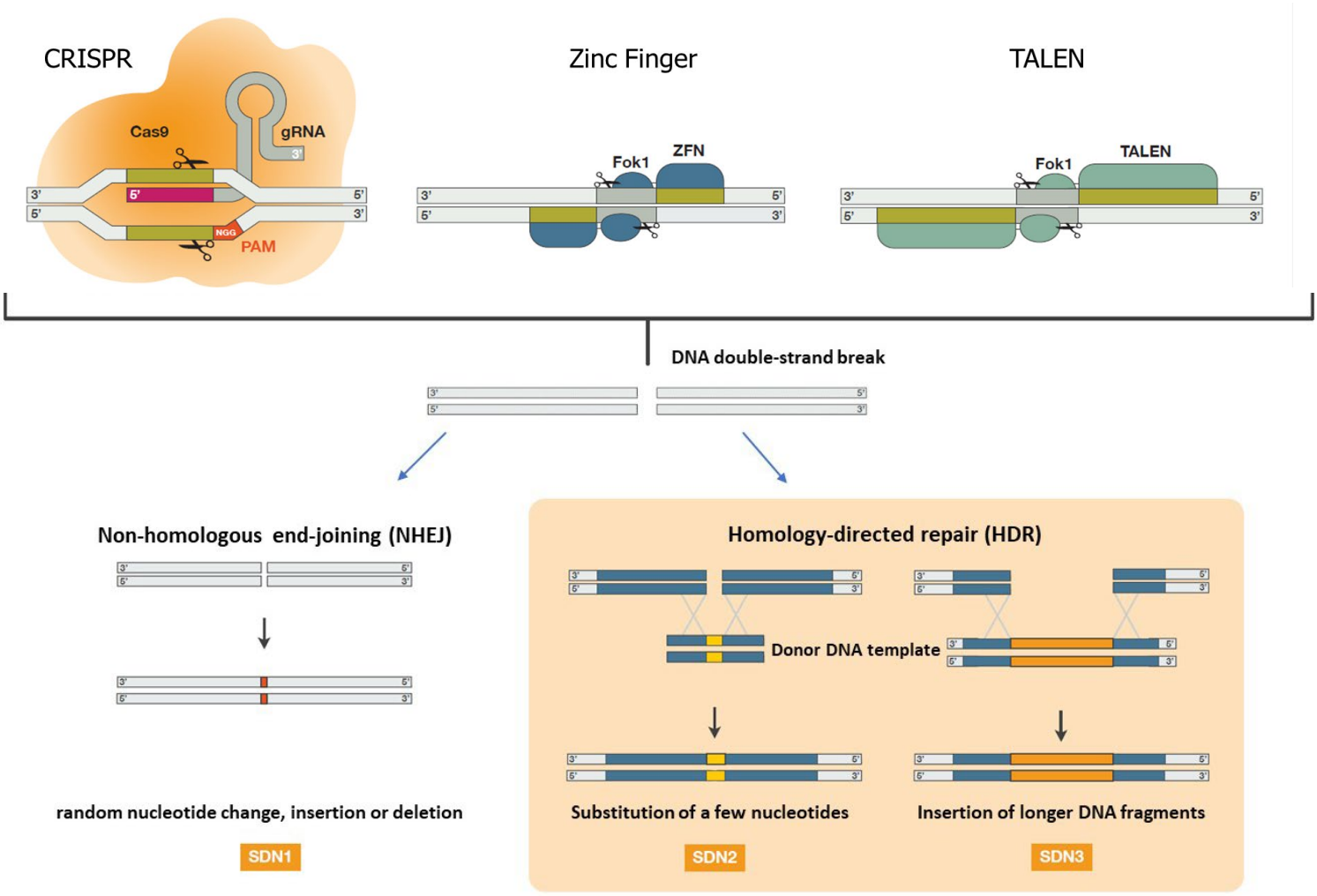

Figure 9. The outcome of genome editing with engineered site-directed nucleases (CRIPSR, ZFNs and TALENs) divided into three categories, SDN1-3. The starting point for each genome editing is that SDN "molecular scissors" cuts DNA at specific sites directed by their DNA-binding moiety, introducing a double-strand break (DSB) which triggers cellular DNA repair mechanisms. If no template (donor DNA) is added, the induced break is repaired by NHEJ (Non-Homologous End Joining) pathway and the outcome is defined as a SDN1 category. If a homologous repair template containing one or several single nucleotide variants is added, the break is repaired by HDR (Homology Directed Repair) pathway and the the outcome is defined as SDN2 category. If the added template contains DNA insertions flanked by sequences homologous to the target DNA site, the construct is inserted by either HDR or NHEJ. This outcome is defined as a SDN3 category. Base editing and prime editing techniques (not shown in the schematic figure) use modified Cas9-protein (nCas9-nickase) and they edit DNA bases without inducing DSBs or without donor DNA templates.

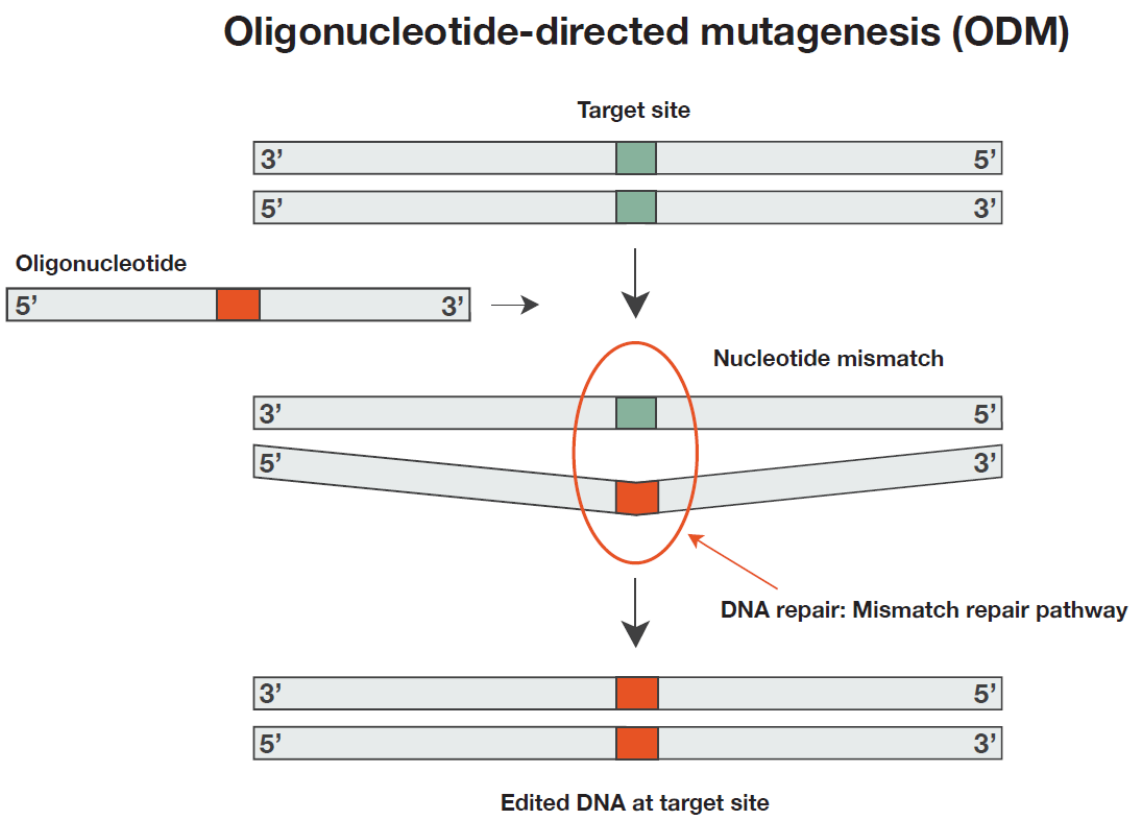

Figure 10. **Outcome of genome editing with oligonucleotide-directed mutagenesis (ODM).** A short DNA fragment (oligonucleotides; < 200 nucleotides) homologous to the target sequence with the exception of a few nucleotides (1-5 nucleotides) is temporarily exposed to the cells. The oligonucleotide containing the desired modification targets binds to the corresponding homologous DNA sequence. Once bound, the cell's natural repair machinery recognises the single base mismatch between its own DNA and that of the repair template. ODM can change, insert or delete one or a few base pairs of DNA.

Box 7.

Genome-edited plants and animals used as case examples

Genome-edited plants

Case 1 is a genome-edited potato (*Solanum tuberosum* L.) developed with the CRISPR/Cas9 technique and categorised as a SDN1 (Andersson et al., 2017). Improved starch quality was achieved after the introduction of a few nucleotide changes (1-10 bp indels) into all four alleles of the potato granule-bound starch synthase (GBSS) gene. The resulting loss of function of the GBSS enzyme elimnates the synthesis of amylose, thereby increasing the amylopectin content of the potato. This gene target and phenotype is the same as for the genetically modified Amflora potato developed by BASF (BASF).

Case 2 is a genome-edited soybean (*Glycine max* (L.) Merr.) developed with the CRISPR/Cas 9 technique and categorised as a SDN2 (Li et al., 2015). Tolerance to the herbicide chlorsulfuron was introduced by editing the acetolactate synthase 1 (ALS1) gene. The edit causes an alteration in the ALS enzyme making it less sensitive to the herbicide. ALS is a key metabolic enzyme in biosynthesis of branched-chain amino acids like valine and isoleucine, targeted by many herbicides. In conventional soybeans, chlorsulfuron would block branched-chain amino acid biosynthesis by disrupting the enzyme, killing the plants.

Case 3 is a genome-edited maize (*Zea mays* L.) developed with the CRISPR/Cas9 technique and categorised as a SDN3 (Shi et al., 2017). The drought-tolerant maize variety was developed by overexpressing the negative ethylene regulator ARGOS8. This was achieved by inserting the constitutively expressed native maize promotor GOS2 in the promotor region of the ARGOS8 gene. Ethylene is a phytohormone known to play an important role in regulating plant response to abiotic stress, including water deficits and high temperature. A higher yield can be achieved by decreasing the sensitivity of maize to ethylene.

Case 4 is a genome-edited oilseed rape (*Brassica napus L.*) developed with the oligonucleotide-directed mutagenesis (ODM) technique (Songstad et al., 2017). Tolerance to sulfonylurea and imidazolinone herbicides was achieved by single nucleotide changes in two genes encoding subunits of the AHAS (acetohydroxyacid synthase, also known as ALS enzyme (as in case 2 and 5)). The changes result in a single amino acid substitution in each protein subunit, which induces conformational alterations in AHAS conferring tolerance to the herbicides. In conventional rapeseeds, sulfonylurea and imidazolinone would block branched-chain amino acid biosynthesis, killing the plants.

Case 5 represents both a genome-edited tomato (*Solanum lycopersicum* L.) and a genome-edited potato (*Solanum tuberosauma* L.*)*) developed with the base editing (BE) techniques (Veillet et al., 2019). Tolerance to the herbicide chlorsulfuron was achieved in both plants with cytidine base editors to direct a C-to-T base conversion, editing the acetolactate synthase (ALS) gene. The edit causes an alteration in the ALS enzyme making it less sensitive to the herbicide. ALS is a key metabolic enzyme in biosynthesis of branched-chain amino acids like valine and isoleucine, targeted by many herbicides. In conventional tomatoes and potatoes, chlorsulfuron would block the enzyme ALS, killing the plants.

Case 6 is a genome-edited apple tree (*Malus* × *domestica* (Suckow) Borkh.) developed with the CRISPR/Cas9 technique and categorised as a SDN1 (Pompili et al., 2020). Reduced susceptibility for fire blight infection was achieved by knockout of the gene *MdDIPM4*. Fire blight is a contagious disease affecting apples and pears. The bacterium *Erwinia amylovora* is the causal agent of fire blight disease in apple. Two susceptibility genes, *HIPM* and *DIPM4,* have been identified as key regulators of establishment and proliferation of *E. amylovora* in apple.

Case 2, case 4 and case 5 *represent genome-edited plants (soybean, oilseed rape, tomato and potato) obtained through the use of the editing CRISPR/Cas9, ODM and base-editing, respectively. Despite differences in the systems, all three cases lead to base-changed variants of the endogenous enzyme ALS conferring tolerance to sulfonylurea herbicides and other related herbicides that target ALS.*

*Potato, rapeseed, tomato and apples (**cases 1, and 4-6**) were chosen based on their relevance for cultivation in Norway, whereas soybean and maize (cases 2-3) were chosen because of their significance as imported food and feed.*

Genome-edited animals

Case 1 represents two examples of genome-edited farmed Atlantic salmon (*Salmo salar* L.) developed with theCRISPR/Cas9 technique and categorised as SDN1 (Datsomor et al., 2019a; Datsomor et al., 2019b). In both cases, genes encoding enzymes involved in the production of polyunsaturated fatty acids (PUFA) were edited, resulting in altered fatty acid composition.

Case 2 is a genome edited farmed Atlantic salmon developed with the CRISPR/Cas9 technique and categorsied as SDN1 (Wargelius et al., 2016). Introduced edits in the *dead end* (*dnd*) gene leading to knockout of this gene resulted in a sterile fish without germ cells. The *dnd* gene is a factor required for germ cell survival in vertebrates.

Case 3 is a genome-edited channel catfish (*Ictalurus punctatus*) developed with CRISPR/Cas9 technique and categorised as SDN1 (Khalil et al., 2017). Knockout of the *MSTN* gene encoding the protein myostatin which normally suppresses muscle growth, enhances growth of the fish.

Case 4* is a genome edited cattle (*Bos taurus*) developed with the TALEN technique and categorised as a SDN3 (Carlson et al., 2016). Insert of a 212 bp duplication (homology-directed) into bovine embryo fibroblasts leads to alteration of the gene responsible for development of horns. The altered gene resembles a gene variant found naturally in cattle of Celtic origin (Polled Celtic, P_C *POLLED*) that does not produce horns.

Case 5 is a genome-edited pig (*Sus scrofa domesticus)* developed with the CRIPSR/Cas9 technique and categorised as SDN1 (Burkard et al., 2017; Burkard et al., 2018). Resistance towards porcine reproductive and respiratory syndrome (PRRS) was achieved by a deletion in the *CD163* gene. The virus causing the disease enters immune cells via the CD163-receptor to establish an infection. Animals carrying the modified CD163 receptors seem to be fully resistant to PRRS virus infection.

Farmed Atlantic salmon, cattle and domestic pig (case 1-2, and 4-5) were chosen based on their relevance for breeding and production in Norway. Channel catfish (case 3) was chosen because the gene edit targets muscular growth, which is relevant for most domesticated animals, and because it is an alien fish species in Norway.

The cases of genome-edited animals listed above represent animals intended for confined or semi-confined conditions. There are currently few examples of genetically modified or genome-edited animals intended for open environmental release. A notable exception of environmental release is the genetically modified male sterile mosquitoes developed by Oxitec (Oxitec), which have been field-released in various parts of the world for the purpose

of population control of disease-carrying mosquito populations. Gene drive traits facilitated by the use of CRISPR technology has been proposed as a tool to further develop measures for insect population control (Targetmalaria.org). Gene drive as case examples of traits enabled by CRISPR are not covered in this report. A recent advisory on the risk assessment of engineered gene drives was published by EFSA (EFSA, 2020a).

It is noted that several of the cases above have been developed through a two-step approach during which, in the initial development step, the organism was genetically transformed through chromosomal insertion of the CRISPR machinery. In the second step, the CRISPR machinery was removed through excision mechanisms or negative segregation. The extent of genome-editing present in the final product constitutes the basis for the SDN class assignments suggested above. The regulatory aspects of negative segregants (EFSA, 2011c) are not considered in further detail in this report.

**This example also illustrates the occurrence of unintended effects of the engineering approach. Independent analyses of sequencing data made available by the developers revealed that vector sequences remained in the final cow genome. FDA discovered a stretch of bacterial Plasmid DNA including several genes conferring antibiotic resistance (Norris et al., 2020).*

8 Application of the EFSA guidance for risk assessment of genetically modified plants in risk assessment of genome-edited plants (ToR 3 and 4)

Compared to classical mutagenesis whereby chemicals or radiation randomly create mutations in many genes at the same time, or traditional genetic modification with random insertion of new genes or gene constructs, genome-editing techniques allow a site-specific alteration of the DNA sequence of one or a few selected genes.

Genome editing in plants typically consists of designing and constructing vectors, delivering vectors to plant cells or protoplasts, generally via Agrobacterium-mediated transformation, particle bombardment (also known as "biolistics", where a gene-gun is used to shoot gene sequences into target cells) or Polyethylene Glycol (PEG)-mediated transformation, plant regeneration and screening for modifications/mutations. With biolistics (short for "biological ballistics") delivery or Agrobacterium-mediated stable transformation, the DNA vectors containing the expression cassettes for the editing components are randomly inserted into the plant genome. In the genome they are expressed and carry out the editing process. As an alternative approach, the vectors can be transiently transformed to protoplasts through PEG-mediated protoplast transfection, where the editing components are transiently expressed to introduce genome editing without genome integration of vector DNA. The editing components can also be translated and/or transcribed *in vitro*, pre-assembled and delivered to the protoplasts by PEG-mediated transfusion in a DNA-free approach to induce site-specific mutations without insertion of foreign DNA into the plant genome. Plants resulting from such an approach may fall outside the GMO regulation. Subsequently, edited cells are prepared for callus induction and plant regeneration, modification screening and analysis and phenotypic characterisation for the desired trait. If the CRISPR constructs are stably integrated into the genome of the recipient plant, the initially produced plant (primary transgenic plant) will contain exogenous DNA that must be removed, for example through subsequent backcrossing and selection, until the locus harbouring the integration is segregated out. Offspring containing the intended modification without the foreign DNA are used for further breeding. The traceability or detection of plants obtained by genome-editing techniques may pose challenges since these plants do not necessarily contain foreign DNA fragments.

A systematic review (Modrzejewski et al., 2019) of market-oriented traits introduced by the new genome-editing in plants identified a total of 1328 studies (literature search from 1996 to 2018). Around 68% of the studies (n = 907) were conducted on agricultural crops, mostly rice, and 32% (n = 421) on model organisms. Further, the number of studies (n = 296) using TALENs, ZFN, ODM, MN and BE were lower than the number of studies (n = 1032) using

CRISPR/Cas system. The distribution of SDN tools used to induce changes in the plant genome of the identified 1328 studies was SDN1 (n = 1154 studies), SDN2 (n = 36 studies), SDN3 (n = 68 studies); point mutations induced with the ODM (n = 27) and BE (n = 42). The ODM- and BE-induced point mutations were generally similar to the point mutations induced using SDN2 (Modrzejewski et al., 2019). From this report, it is concluded that the ediiting by CRISPR/Cas resulting in the SDN1 category is expected to be the most predominant method to introduce market-oriented traits; however, the genome-edited plants expected to reach the market first were developed using the TALEN system. According to Modrzejewski et al., typical traits developed by genome-editing techniques were herbicide tolerance, improved agronomic value, improved food and feed quality and enhanced fitness against biotic and abiotic stress.

8.1 Application of the EFSA guidance for risk assessment of food and feed from genetically modified plants in risk assessment of genome-edited plants

According to the EFSA guidance, risk assessment of food and feed from genetically modified plants should include information on the modification; methods used; potential identity of new proteins in the plant to known toxins or allergens; effects on phenotypic and agronomic traits; effects of processing and storage of plant-derived product; effects on the content of chemical components including nutrients and anti-nutrients; estimation on exposure and, lastly, a monitoring plan on potential effects on human dietary patterns and/or side-effects. The different steps are short listed below:

- Molecular characterisation
- Comparative assessment
- Toxicity and allergenicity
- Nutritional assessment
- Exposure assessment
- Risk characterisation
- Monitoring plan

The following sections (8.1.1 – 8.1.13) contain key elements present in the EFSA guidance on the information requirements to perform a risk assessment of a genetically modified plant intended for use in food and feed (EFSA, 2011a). For each main section, an evaluation of the applicability of the EFSA guidance to genome-edited plants is provided using case examples 1–6 (box 7).

8.1.1 Molecular characterisation

Description of the methods used for the genetic modification

The molecular characterisation should provide an in-depth description of the method(s) used for the genetic modification. The molecular characterisation has to describe whether the modification was achieved by *Agrobacterium* transformation of plant cells, or with the use of a gene-gun (biolistics), or by other techniques. In addition, relevant information on the recipient

plant must be provided, such as the species, subspecies and breeding line, whether it is a common food staple, has a history of safe use, or whether information exists on known toxic or allergenic properties.

Source and characterisation of nucleic acid used for the transformation

Information on the donor organism(s) and on the nucleic acid sequence(s) used for transformation including nucleic acid sequence(s) intended to be inserted should be presented in order to define whether the nature of the donor organism(s) or the nucleic acid sequence(s) may trigger any safety issues.

The information should encompass complete sequences of the intended inserts, including information on any deliberate alteration(s) to the corresponding sequence(s) in the donor organism(s). A history of safe use of the gene product(s) arising from the regions intended for insertion should be provided, as well as data on the possible relationship of the gene products with known toxins, anti-nutrients and allergens. Information regarding each donor organism should comprise its taxonomic classification and its history of use regarding food and feed safety.

Nature and source of vector(s) used, including nucleotide sequences intended for insertion

This information should include a physical map of functional elements and other plasmid/vector components together with relevant information needed for the interpretation of the molecular analyses. A circular map of the plasmid vector indicating its size and components should be described. A detailed description of all genetic elements of the vector should be included, not only the sequence intended for insertion, but also genetic markers and vector backbone sequences.

General description of the introduced trait(s) and characteristics which have been introduced or modified

The molecular characterisation should include a general description of the introduced trait(s) and its mode of action, the resulting changes in the phenotype and the metabolism of the plant and of its intended use, e.g. whether the resulting plant is herbicide-tolerant, insect-resistant or has new nutritional properties.

Information on the sequences actually inserted/deleted or altered

Intended modifications and actual modifications will very often differ at some level. Information should therefore be available describing all detectable inserts in the plant such as size and copy number of the intended sequence(s), as well as unintended insertions/sequence(s), deletions and alterations and their positioning in the genome. There should also be information on whether any insertions have unintentionally interrupted the regulation/expression of existing

genes in the plant. All new open reading frames (ORFs) should also be investigated in and around all insertion sites. New ORFs are sequences that may potentially lead to the expression of unwanted harmful products such as allergens or toxins.

8.1.1.1 Information on the expression of the insert(s)

Information should be available as to whether the inserted/modified sequence(s) results in intended changes at the protein, RNA and/or metabolite levels. Information on the levels of new introduced proteins and/or other products and metabolites should be available.

8.1.1.2 Genetic stability of the inserted/modified sequences and phenotypic stability of the genetically modified plant

Information should be provided that demonstrates that the modification(s) is stably inherited genetically and phenotypically in progeny, i.e. that both genetic and phenotypic traits are transferred to new generations.

8.1.1.3 Application of EFSA guidance in molecular characterisation of genome-edited plants

SDN-based genome editing has emerged as an effective engineering method that allows modification of genetic information by adding, altering or removing DNA sequences at a specific targeted location in the genome. For plants developed using the genome-editing techniques, the editing may give rise to a broad set of new genetic combinations and novel traits. In this section, the applicability of the EFSA guidance for risk assessment of food and feed from genetically modified plants will be evaluated with respect to the molecular characterisation data requirement developed using the genome-editing techniques.

The first requirements of the molecular characterisation in the EFSA guidance can be applicable to plants obtained using genome-editing techniques, since genetic elements encoding the SDN tools will be delivered into the organism using similar genetic engineering techniques as genetically modified plants. Once SDN tools have achieved the intended change, the inserted genes can be removed by segregation using conventional breeding. In case of transgene-free genome editing being applied, preassembled nuclease proteins (e.g. TALEN proteins or Cas9 protein – gRNAs) will be delivered into the plant cells and the nuclease proteins will probably be designed with conventional genetic engineering techniques before they are preassembled. The above-mentioned molecular characterisation requirements can partially be applicable to plants obtained using genome-editing techniques outlined in case studies 1, 2, 4, 5 or 6 and be fully applicable in case study 3.

Specific information on the genome-editing processes must be provided, such as the genetic engineering methods and vectors used, and the modified sequences together with the modified traits or phenotypes. This applies for plants in the SDN3 category. Plants in the SDN1 or SDN2 category, or plants obtained by ODM or BE may require less information since the edited plant

does not contain gene sized DNA inserts coding for novel proteins. Regarding data requirements on the introduced trait(s) and mode of action, resulting changes to the phenotype and the metabolism of the plant, and its intended use, the requirements are applicable for all cases. In terms of trait(s) introduced using genome-editing techniques, it is important for risk assessment to consider not only the modification itself, but the impact of the modification and the novel trait(s) on the physiology and phenotypic effects of the plant.

In molecular characterisation, information is required on the sequences actually inserted, deleted or altered, sequence information for both 5" and 3" flanking regions at each insertion site, and the expression of the sequences, as well as genetic stability of the inserted/modified sequences and phenotypic stability of the genetically modified plant. These requirements are not applicable for plants developed in the SDN1 or SDN2 category, or plants obtained by ODM or BE approaches (Case 1, 2, 4, 5 and 6). The requirement of demonstrating the stability of inserted DNA fragments is not relevant to in the SDN1 or, SDN2 category, or plants obtained by ODM or BE base editing approaches (Case 1, 2, 4, 5 and 6) as long as the final product does not contain any leftovers, e.g. vector backbone sequences used during genome-editing process. However, assessment of genetic stability of the modified nucleotides and introduced trait can be relevant for all cases.

Genetic changes obtained by genome-editing techniques, except when exogenous DNA, changes in restriction sites or deletions are introduced, are impossible to characterise by Southern analysis. However, this method can be used to demonstrate the lack of introduced vector sequences in the plant genome. Next Generation Sequencing (NGS) can potentially offer an advantage compared to Southern analysis in terms of the detection of small sequence modifications. A validated and accepted NGS-based approach might be used to fulfil the data requirements for molecular characterisation of plant in the SDN1 or SDN2 category, or obained by ODM or BE approaches (Case 1, 2, 4, 5 and 6), as well as for plants in the SDN3 category, where exogenous genes are inserted. EFSA has assessed the feasibility of using NGS for the molecular characterisation of genetically modified plants (EFSA, 2018e), and this scientific opinion may be adapted to genome editing in plants in future risk assessments. The feasibility of using NGS-generated data needs to be validated and standardised before they are implemented in risk assessment guidance.

Further, the current approaches to verify whether the inserted/modified sequence results in intended changes at the protein, RNA and/or metabolite levels include Western blot, ELISA, Northern blot and compositional analysis (e.g. gas liquid chromatography), are fully (case 3) and partially (Case 1, 2, 4, 5 and 6) applicable for plants developed with genome-editing techniques. These information requirements can be obtained from all the six cases.

Genome-editing techniques can potentially introduce various edits in the DNA such as nucleotide substitutions resulting in amino acid changes affecting active sites of enzymes or overall protein structure, and these types of changes can be assessed with the guidance. Further, DNA edits, such as nucleotide insertions/deletions (INDELS) resulting in frameshift mutations or mutations in splice sites, resulting in alternative splicing generating non-functional

proteins or mutations in regulatory regions resulting in altered gene expression, introduced by genome-editing techniques, can also be assessed with the guidance. However, when standardised, an implementation of the NGS-based approach combined with bioinformatics tools may offer a higher resolution that could support the molecular characterisation.

For both genetical modification and genome-editing techniques, various omics-based system biology (genomic, transcriptomic, proteomic, metabolomics) approaches can be applied to compare their molecular characteristics, i.e. a comprehensive system biology analysis at all levels (DNA, RNA, protein and metabolite) to demonstrate whether the inserted/deleted/modified sequence results in intended changes at the protein, RNA and/or metabolite levels. The applicability of omics-based data for risk assessment needs to be validated. The EFSA's 24th Scientific Colloquium on "OMICS in risk assessment: state-of-the-art and next steps" took place on April 2018, discussed implementing omics techniques to the EFSA guidance document on risk assessment of genetically modified plant products in food and feed.

SNDs or ODM-based approaches can induce unintended off-target mutations, and these mutations can have either positive, negative or neutral effects. Assessment of potential unintended off-target mutations caused by SDNs and internal DNA repair mechanisms, often indistinguishable from natural genetic variation or radiation/chemical-induced mutations that can occur anywhere in the genome, is difficult. Compared to the conventional mutagenesis, where unintended modifications could be enormous, unintended off-target mutations induced by SDNs are usually much fewer and may be reduced in the breeding process. Unintended off-target effects in SDN- and ODM-based approaches are negligible compared to conventional plant breeding, and additional analyses of potential off-targets would be of very limited value for the risk analysis, according to EFSA (EFSA, 2012c; EFSA, 2020b).

Molecular characterisation of potential unintended off-target mutations caused by SDNs is not straightforward. Therefore, on a case-by-case basis, omics-based system biology approaches may be an option. However, omics techniques and analyses need to be standardised and validated for their purpose before they can be used in risk assessment.

8.1.2 Conclusions regarding the molecular characterisation

VKM concludes that the requirements for the molecular characterisation in the guidance can be fully or partially applied for genome-edited plants, exemplified in the six cases. The requirements for the molecular characterisation are fully applicable for the genome-edited plant in case 3 (SDN3) where an exogenous gene was inserted into the maize genome. The plants presented in case 1 and 6 (SDN1), 2 (SND2), 4 (ODM) and 5 (BE) differ from case 3 because in these plants no exogenous gene is present in the final plant. The part of the requirements linked to the characterisation of the introduced transgenes, i.e., the presence of exogenous DNA in the final product, would not be applicable for plants in case 1, 2, 4, 5 and 6. Therefore, not all aspects of the molecular characterisation requirements in the EFSA guidance for risk assessment of food and feed are applicable to genome-edited plants.

8.1.3 Comparative analysis

According to the EFSA guidance, the comparative analysis is a comparison of the genetically modified plant with its conventional counterpart. The aim is to detect differences in the plant's observable appearance such as height and colour – phenotypic characteristics – as well as its agronomic characteristics, such as yield. Moreover, the analysis includes a comparison of the chemical composition, including nutritional values of the plant, with its conventional counterpart. The underlying assumption of the comparative approach is that traditionally cultivated crops have a history of safe use.

Assessment characteristics are production of material for comparative analysis, criteria for selection of comparators, field trials and statistical considerations, experimental design, agronomic and phenotypic traits, compositional analysis, effects of processing. The comparative compositional, phenotypic and agronomic assessment requires the simultaneous application of two complementary tests: the test of difference and the test of equivalence (EFSA, 2010d).

8.1.3.1 Comparators

The first step in the comparative analysis is to find the conventional counterpart. Requirements are found in Regulation (EC) No 1829/2003, which defines a conventional counterpart as "a similar food or feed produced without the help of genetic modification and for which there is a well-established history of safe use". Details on the criteria for the selection of appropriate comparators, under different scenarios, are found in the EFSA Guidance for the selection of comparators for the risk assessment of genetically modified plants (EFSA, 2011c). However, where applicants can demonstrate that a conventional counterpart for the genetically modified plant cannot be made available, either negative segregant(s), or any set of genetically modified plants that all have been risk assessed on the basis of experimental data collected according to the principles of EFSA, molecular characterisation and food and feed risk assessment could be used as comparator(s). In all cases, the applicant should provide information on the breeding scheme (pedigree) in relation to the genetically modified plant, the conventional counterpart and/or other comparator(s) used in the risk assessment together with a clear justification for their selection.

8.1.3.2 Field trials

The second step: field trials are used for production of material for the comparative assessment and should be performed to assess differences and equivalences between three test materials: the genetically modified plant, its comparator and reference varieties. All test materials should be randomised to plots within a single field at each site, usually in a randomised block design. EFSA guidance on statistical considerations for the safety evaluation of genetically modified organisms contains a detailed guidance of the statistical analysis for the safety evaluation of plants (EFSA, 2010d).

8.1.3.3 Agronomic and phenotypic traits

The third step in the comparative assessment of the genetically modified plant relates to agronomic and phenotypic traits (e.g. yield, plant morphology, flowering time, time to maturity, duration of pollen viability, response to plant pathogens and insect pests, and sensitivity to abiotic stress). There is detailed guidance on the agronomic and phenotypic characterisation of genetically modified plants (EFSA, 2015). Specific recommendations are given on the selection of sites and test materials, the quality and design of field trials, the selection of relevant agronomic and phenotypic endpoints, and data analysis.

8.1.3.4 Compositional analysis

The fourth main step is the analysis of the composition. The material to be used for the comparative assessment should be selected considering the uses of the plant and the nature of the genetic modification. The raw agricultural commodity should generally be selected as this usually represents the main point of entry of the material into the food and feed chain. A specific explanatory note on the selection of forage material suitable for the risk assessment of genetically modified feed of plant origin has been published, providing a crop-specific definition of forage for maize, soybean, sugar beet, rapeseed and cotton, mitigating the lack of forage definition in the regulatory context and supporting the appropriate selection of forage material (EFSA, 2018d). Additional analysis of processed products should be conducted on a case-by-case basis. The compositional analysis should be carried out on an appropriate range of compounds in accordance with and as suggested in the OECD consensus documents on compositional considerations for new plant varieties, which include proximates (including moisture and total ash), key macro- and micro-nutrients (vitamins, minerals, fatty acids, amino acids), anti-nutritional compounds, natural toxins and allergens and other characteristic plant metabolites in the plant species (OECD, 2002a; OECD, 2002b; OECD, 2011; OECD, 2012; OECD, 2019).

8.1.3.5 Effects of processing

Available tests will depend strongly on the genetic alterations introduced. The test should show whether the processing and/or preserving technologies applied are likely to modify the characteristics of the end-products compared with their comparators. A detailed description of the processing technologies used on the plant and focussing on steps which may lead to significant changes in composition should be available, with respect to both quality and quantity. Alterations not resulting in specific new proteins or just changes in levels of endogenous proteins will exclude some of the steps of the comparative analysis suggested by the EFSA guidance document.

8.1.3.6 Application of EFSA guidance in comparative analysis of genome-edited plants

The six mentioned cases will be used to exemplify whether the guidance document for the risk assessment of food and feed from genetically modified plants can be used for the comparative assessment of genome-edited plants.

In case 1, CRISPR/Cas was used to introduce mutations into all four alleles of the potato granule-bound starch synthase (GBSS) gene (SDN1 category). No details from the field trial were given in their study, nor any details on the composition of the potatoes except the starch species. All steps in the EFSA guidance could be used for this potato line. The Consensus Document from OECD on new varieties of potatoes could be used to consider key food and feed nutrients, anti-nutrients and toxicants (OECD, 2002b).

In case 2, CRISPR/Cas was used to obtain a chlorsulfuron-tolerant soybean (SDN2 cateory). All steps in the EFSA guidance document on comparative analysis could be used for this soybean line. The Consensus Document from OECD on new varieties of soybean could be used to assess key food and feed nutrients, anti-nutrients, toxicants and allergens (OECD, 2012). Soybean is one of the main foods that account for IgE-mediated food allergies and the document gives a summary of potential soybean allergens. In the paper by Li et al. (Li et al., 2015), only details from the molecular characterisation is given without information on the field trial, agronomic and phenotypic traits, composition or any processing steps.

In case 3, CRISPR/Cas was used to develop a drought-tolerant maize variety (Shi et al., 2017) with the main goal to decrease the sensitivity of maize to ethylene (SDN3 category). All steps in the EFSA guidance document could be used for this maize line. Some details on the experimental randomised block design was described by Shi et al.. The Consensus Document from OECD on new varieties of maize could be used to consider key food and feed nutrients, anti-nutrients, toxicants and secondary plant metabolites (OECD, 2002a).

Case 4 is a rapeseed (*Brassica napus*) variety with tolerance to sulfonylurea and imidazolinone herbicides developed using ODM-technique. All steps in the guidance document could be used for the ODM technology. The Consensus Document from OECD on new varieties of rapeseed could be used to consider key food and feed nutrients, anti-nutrients and toxicants (OECD, 2011).

There are several examples of base editing (case 5) for crop improvements the last couple of years, especially in rice, but also in wheat, potato, tomato and watermelon (Mishra et al., 2020). The most commonly modified trait is herbicide tolerance. In case 5, potato and tomato plants tolerant to the herbicide chlorsulfuron have been developed by base editing of the acetolactate synthase (ALS) gene using cytidine base editors (Veillet et al., 2019). All steps in the guidance could be used for base-edited crops.

Case 6 is an apple tree with introduced knockout of the gene *MdDIPM4*, one of the key regulator genes responsible for the establishement and proliferation of the pathogen *E.*

amylovora in apple (SDN1 category). The knockout resulted in fire blight disease resisant apple tree (Pompili et al., 2020). All steps in the guidance document could be used for this apple line. The Consensus Document from OECD on new cultivares of apple could be used to consider key food and feed nutrients, toxicants and other metabolites (OECD, 2019).

8.1.4 Conclusions regarding the comparative analysis

VKM concludes that the guidance can be applied also for genome-edited plants in the comparative analysis. Considering the cases described above, all steps of the EFSA guidance on comparative analysis, are important. Standardised and validated high through-put approaches, such as transcriptomics, proteomics and metabolomics, may improve the approach to reveal unintended effects of key food and feed nutrients, toxicants and plant metabolites (OECD guidelines) in the risk assessment of all modified plants.

8.1.5 Toxicological assessment

According to the guidance , the toxicological assessment should demonstrate that the genetic modification(s) introduced do not have adverse effects on human and animal health. The assessment should cover all intended and unintended modifications introduced in the novel plant, e.g. if a new protein or other constituents could be considered a toxin to humans or animals. The outcome of the molecular and comparative assessments sets the foundation for the toxicological testing, identifying the presence of new unintended substances (new constituents) to be further investigated in addition to the new proteins.

8.1.5.1 Toxicological assessment of the newly expressed protein(s)

Data on all new proteins should be available for the assessment. Information on the type of studies required to test for potential toxicity should be determined on a case-by-case basis, depending on the knowledge available for the proteins, e.g. their origin, function and history of use for human and/or animal consumption. If sufficient documentation is provided supporting safe consumption of the proteins and the plant itself, data from toxicity testing may not be required for the assessment.

8.1.5.2 Molecular and biological characterisation of the newly expressed proteins

Information on the physical and biochemical properties of all new proteins should be available, e.g. their amino acid sequence, molecular weight, post-translational modifications, description of function, etc. For enzymes, this includes information on enzyme activities, optimal pH and temperature ranges, substrate specificities and reaction products. Information on potential off-target interactions with other plant constituents and their implications for safety should also be available. If specific tests of the protein(s) are required, experimental data with proteins produced in microorganisms are acceptable, provided there is sufficient equivalence between the protein produced in the microorganism and the protein produced in the plant.

8.1.5.3 Bioinformatic search for homology to proteins known to cause adverse effects

Information should be available from up-to-date homology searches to known harmful proteins, e.g. searches in databases to identify if a novel protein has sequence homology to known toxins. Results may trigger the need for additional information from experimental toxicity studies. A search for homology to non-toxic proteins exerting a normal metabolic or structural function may also be valuable information for the toxicity assessment.

8.1.5.4 Stability of the newly expressed proteins under relevant processing and storage conditions and the expected treatment of the food and feed

Information should be available for relevant conditions affecting the stability of the new proteins during storage and processing, e.g. effects of temperature and changes in pH. Modifications to the proteins such as denaturation, production of stable protein fragments or other modifications, should be characterised.

8.1.5.5 Resistance of the newly expressed proteins to proteolytic enzymes

Information on the properties of new proteins with relation to resistance to degradation by proteolytic enzymes, e.g. gastric pepsin and intestinal enzymes, should be available to risk assessors. Preferably, data should come from appropriate standardised *in vitro* studies simulating gastric and intestinal conditions. Stable breakdown products should be described and further evaluated regarding biological importance and/or risks.

8.1.5.6 Repeated dose 28-day oral toxicity study with the newly expressed proteins in rodents

Data from repeated dose toxicity studies with laboratory animals should be available to risk assessors unless other reliable information demonstrating the safety of the new proteins already exists. In this case, the information should encompass the mode of action of the proteins and demonstrate that the proteins are not structurally and functionally related to proteins with adverse effects to human or animal health. The repeated dose 28-day oral toxicity study in rodents should be performed according to OECD guidance 407 (OECD, 2008). Depending on the outcome of the 28-day toxicity study, further targeted investigations may be required. Given indication of synergistic or antagonistic interactions between two or more new proteins, additional studies with combined administration of these proteins should also be available.

8.1.5.7 Toxicological assessment of new constituents other than proteins

Information on the evaluation of the toxic potency of identified new constituents other than proteins (e.g. RNA or peptides) that also may require toxicological testing should be available.

In cases where there is a documented history of safe use of such constituents, toxicological testing is not required.

8.1.5.8 Toxicological assessment of the whole plant

If the composition of the food and/or feed derived from a genetically modified plant is substantially modified, or if there are any indications of potential occurrence of unintended effects based on the molecular, compositional, phenotypic analyses or animal test, it must be decided on a case-by-case basis whether information from other studies on the whole food and feed derived from the plant should also be available to risk assessors. This information must include a 90-day feeding study in rodents in accordance with EU regulation 503/2013.

8.1.5.9 Design and performance of 90-day feeding study in rodents

The EU regulation 503/2013 introduced in 2013 made it mandatory for all applications for genetically modified plants containing a single genetic modification to include a 90-day whole food feeding study in rodents. The preferred test species is the rat. Therefore, information from 90-day feeding studies should be available to risk assessors for all new applications for genetically modified plants after the implementation of the regulation in 2013.

There are detailed guidance documents from EFSA (EFSA, 2011b; EFSA, 2014) concerning the design of a 90-day whole food feeding study for genetically modified plants.

8.1.5.10 Animal studies with respect to reproductive, developmental or chronic toxicity

Depending on the outcome of the 90-day feeding study and other data, it should be decided case-by-case whether documentation is needed from further toxicity studies, e.g. reproductive/developmental toxic effects and chronic toxicity studies.

8.1.5.11 Other animal studies for examining the safety and the characteristics of genetically modified food and feed

Supplemental information to the 90-day feeding studies, obtained from comparative nutritional studies conducted with young rapidly growing animal species, e.g. broiler chicks and lambs, is often available to risk assessors.

8.1.5.12 Interpretation of relevance of animal studies

Regarding this topic, EFSA points out the following:

"Changes in test parameters must be evaluated with respect to: (i) relationship with the applied doses, (ii) possible correlations with changes in other biologically related parameters (iii) incidental occurrence, (iv) gender specificity, and (v) normal biological variation. When a

difference is noted at only the highest dose applied, other factors should be considered to determine whether there is a relationship with treatment. Information on the background variability in a given parameter may be obtained from data from other animals of the same species/strain tested in the same or other experiments, or from internationally harmonised databases. Attention should be paid to the fact that certain effects may be specific for the test animal, but not for humans, due to interspecies differences."

8.1.5.13 Application of EFSA guidance in toxicological assessment of genome-edited plants

According to EFSA guidance, information from the toxicological assessment should disclose whether modifications in the novel plant have introduced undesirable properties with potential adverse health implications compared to the non-modified variant, the comparator. The extent and type of genetic changes introduced, will determine which types of toxicological tests are to be performed for genome-edited plants.

Depending on the extent of changes introduced, not all aspects in the guidance may be relevant. This applies for plants where genome editing has introduced small, intended nucleotide conversions, insertions or deletions (SDN1, SDN2, ODM or BE) in endogenous genes. In contrast, if genome editing results in insertion of a gene encoding a new protein or other new constituents not naturally occurring in the plant, e.g. a transgene or synthetic/engineered protein (SDN3), a broader set of toxicological tests may be applicable.

The case studies 1-6, described in the molecular characterisation and comparative assessment can also be used below to exemplify the applicability of the EFSA guidance in toxicity assessment of genome-edited plants.

Case 1 is a genome-edited potato, an endogenous enzyme potato granule-bound starch synthase (GBSS) was edited by introducing indels (1-10 bp) in all four alleles of the gene for GBSS resulting in a loss of function of the enzyme. Case 6 is a genome-edited apple, where the *MdDIPM4* gene is knocked-out, inhibiting receptor binding and proliferation of the pathogen responsible for fire blight, resulting in fire blight resistant apple. Both cases belong to the SDN1 category.

Case 2, 4 and 5, represent different genome-edited plants (soybean, rapeseed, tomato and potato) developed by the CRISPR/Cas9, ODM and base-editing (BE) systems, respectively. Despite the differences in the systems used, all three cases lead to mutated variants of the endogenous enzyme acetolactate synthase (ALS) conferring tolerance to sulfonylurea herbicides and some related herbicides that target ALS. Case 2 belongs to the SDN2 category.

Finally, Case 3 represents a maize cultivar developed through genome editing, resulting in a directed gene insertion of a native maize promotor conferring drought-resistance through overexpression of a targeted protein. Case 3 belongs to the SDN3 category.

Searches in databases for sequence homology of the newly expressed protein to known toxins or allergens may not be relevant for the six cases since the genome edits are alterations in endogenous genes or insertion of a native promoter. The molecular characterisation will reveal changes at the insertion site; and hence inform the toxicological assessment. In some cases of genome editing, where edits result in several changes in amino acid sequences, bioinformatic based analyses may, however, be relevant to exclude that the changes in protein coding sequences have unintended effects, or express novel proteins through intended or unintended changes in open reading frames.

All cases except case 3 have mutated or knocked-out variants of a specific protein compared with the plants' native versions. If considered necessary, these proteins could be eligible for protein analysis in accordance with EFSA guidance. In case 3, the expression of a native protein was increased by inserting an extra copy of an endogenous constitutive promoter. Unless new identified open reading frames indicate expression of novel proteins that require further investigation, such analyses would not be relevant.

With concern to testing in animals, it would be possible, in theory and if considered necessary, to test the genome-edited proteins introduced in cases 2, 4 and 5, asuming that the proteins can be practically identified and isolated. Ninety-day whole food feeding studies would be possible for all six cases in accordance with EFSA guidance and in compliance to the EU regulation 503/2013 (EC, 2013a). This test is meant to reveal whether there are potential adverse effects to human or animal health as a result of intended or unintended modifications introduced. According to EFSA, off-target effects in SDN- and ODM-based approaches are negligible compared to conventional plant breeding (EFSA, 2020b).

8.1.6 Conclusions regarding the toxicological assessment

VKM concludes that the guidance can also be applied to genome-edited plants in the toxicological assessment. However, depending on the extent of editing, some types of analyses may not be considered relevant for the risk assessment. This should be considered on a case-by-case basis. If indicated by the risk characterisation, feeding studies can be performed for all genome-edited plants.

8.1.7 Allergenicity assessment

Food allergy is an important public health problem causing an adverse reaction to food in individuals where a combined effect of variations in the environment and genetic predisposition has resulted in allergic sensitisation. This is not addressed in traditional toxicity studies in animals and is therefore assessed separately (EFSA, 2010b; EFSA, 2017).

If the protein has similarities to known allergens, minimal alterations such as point mutations could potentially alter protein sequence and/or stability to degradation, resulting in a decreased or increased allergenic potential. In this case, adhering to EFSA guidance, risk assessment could reveal potential impact on allergenicity.

8.1.7.1 Assessment of allergenicity of the newly expressed protein

According to the EFSA guidance, the allergenic potential of a new protein should be assessed, for example by identifying potential IgE cross-reactivity between the protein and known allergens. If the novel protein is derived from wheat, rye, barley, oats or related cereal grains, a possible role eliciting e.g. gluten-sensitive enteropathy must also be investigated.

In line with the recommendations of EFSA (EFSA, 2010b; EFSA, 2017)) and the Codex ad hoc Intergovernmental Task Force on Foods Derived from Biotechnology (Codex Alimentarius, 2009), an integrated, case-by-case approach, i.e. a so-called weight-of-evidence approach, should be used in the assessment of possible allergenicity of new proteins.

In every case, a search for sequence homologies and/or structural similarities between a new protein and known allergens should be performed to identify potential IgE cross-reactivity between the new protein and known allergens. This includes bioinformatics searches where the sequence of the new protein is compared to known allergens over 80-amino acid stretches (in a sliding window search). If a sequence homology of >35% (threshold value) is found, further analyses of potential allergenic properties of the new protein are triggered (EFSA, 2010b).

8.1.7.2 Analysis of potential binding to serum IgE

Specific serum screening is an important test method to assess the potential of the new proteins to elicit an allergic reaction in individuals already sensitised to cross-reactive proteins. Serum screening is based on *in vitro* tests that measure the capacity of specific IgE from serum of allergic patients to bind the test protein(s).

Specific serum screening should be performed if the source of the introduced gene is considered allergenic or if the new protein may be allergenic, based on sequence homology or structure similarity to known allergens. Adequate methods for evaluating IgE-binding are Radio or Enzyme Allergosorbent Assay (RAST or EAST), Enzyme Linked Immunosorbent Assay (ELISA) and electrophoresis followed by immunoblotting with specific IgE-containing sera.

8.1.7.3 Resistance to enzymatic degradation upon ingestion

Resistance of proteins to pepsin digestion is proposed as an additional criterion to be considered. Pepsin resistance test (EFSA, 2010b; EFSA, 2017) should be assessed for reasons of safety for vulnerable groups, e.g. infants and individuals with impaired digestive functions. Possible interactions between the protein and other components, as well as the effects of the processing, should be considered. Depending on the outcome of the *in vitro* digestibility test, it could also be useful to compare intact, heat-denatured and pepsin-digested proteins for IgE binding, since an altered digestibility may impact the allergenicity of the new protein.

In vitro cell based assays or *in vivo* tests in animal models may be considered useful in some cases to provide additional information, e.g. on the potential of the new protein for *de novo* sensitisation.

8.1.7.4 Non-IgE-mediated adverse immune reactions

Non-IgE-mediated adverse immune reactions to antigenic food, e.g. coeliac disease (CD), food protein-induced enterocolitis (FPIES), as well as eosinophilic diseases of the gastrointestinal tract, cannot be evaluated by specific serum screening. The pathology is only known for CD and at the present time, assessment of new proteins concerning non-IgE-mediated adverse immune reactions should therefore focus only on CD (EFSA, 2017). An integrated, stepwise, case-by-case approach should be used in the assessment of the new protein in relation to its potential to cause CD (Codex Alimentarius, 2009; EFSA, 2017). Such investigations include i) knowledge about the protein related to CD and searches for sequence identity, ii) HLA-DQ peptide modelling for the likelihood of a peptide binding to HLA-DQ2 or HLA-DQ8 and iii) *in vitro* approaches with HLA-DQ peptide binding assay.

Endogenous allergenicity should also be evaluated based on (i) relevant crops for analysis; (ii) relevant allergens to be quantified; (iii) methodology for quantification; and (iv) principles to be followed for data interpretation and risk assessment considerations. At present, only soybean is considered a relevant crop for allergenicity (endogenous properties)(EFSA, 2017). The quantification of soybean allergens/peptides should be performed by either enzyme-linked immunosorbent assay (ELISA) or mass spectrometry (MS).

8.1.7.5 Assessment of the allergenicity of the whole plant

If the recipient of the introduced gene is known to be allergenic (e.g. soybean), any potential change in the allergenicity of the whole food derived from a genetically modified plant should be assessed using an appropriate comparator. This is recommended if the genetic modification has induced an unintended effect, e.g. an over-expression of an endogenous allergen.

A case-by-case approach should be applied depending on the available information on the allergenic potential of the recipient organism. Methodologies such as proteomics in association with the use of allergic human sera as probes may be applied.

The integrated process applies to the assessment of the allergenicity of the edible components and the pollen of genetically modified plants (i.e. covers both food and respiratory allergy risk). Labelling of allergenic content should be in accordance with current regulations (EC, 2013a).

Where available, information should be provided on the prevalence of occupational allergy in workers or in farmers who have significant exposure to the genetically modified plant or to the airborne allergens they may contain.

On a case-by–case, post-market monitoring programmes can also be proposed to confirm the absence of increased allergenic risk in actual conditions of exposure.

8.1.7.6 Adjuvanticity

Adjuvants are substances that may increase the allergic response when co-administered with an antigen by increasing the immune response to the antigen. Glycosylation of a new protein can affect allergen recognition by dendritic cells, and glycosylation sites in the protein should therefore be investigated.

8.1.7.7 Application of EFSA guidance documents in allergenic assessment of genome-edited plants

To exemplify whether the EFSA guidance on allergenicity assessment can be used for genome-edited organisms, cases 1–6 described above were used.

Soybean (case 2) is a known allergen; therefore, allergenicity assessment should be performed for the whole soybean fraction. In case 2, the only intended change is a mutated version of a native enzyme. If unintended effects have caused significant changes in allergenic potential, this would most likely be caused by altered levels in endogenous allergens known to be important in soybean. Such changes should be evident in the compositional analyses of the comparative assessment and would dictate further investigations.

Allergy towards apple (case 6) is one of the most common fruit allergies with about 2% prevalence in European children (Hassan and Venkatesh, 2015). The occurrence is frequently related to birch pollinosis, due to cross-reactivity between allergens in appee and birch. Some, but not all, apple allergens are heat sensitive and will be reduced during cooking or in apple juce processing. If overexpression of endogenous or novel constituents with suspected allergen properties is identified, this could warrant further tests in adherence to the EFSA guidance.

None of the plants in cases 1, and 3–5 (potato, maize, oilseed and tomato) are considered important allergens, and since none of the cases 1-6 involve changes to proteins with known allergenic properties, or introduction of novel proteins, e.g. transgenes, the extent of the allergenicity assessment would be primarily dependent on the outcome of the compositional analyses.

Post-market monitoring in all cases could potentially be used to link development of allergies in consumers related to intake of food derived from the genome-edited plants. In practice, only acute reactions would be possible to link to intake of a specific plant or product.

8.1.8 Conclusions regarding the allergenicity assessment

VKM concludes that the guidance can also be applied for genome-edited plants in the allergenicity assessment. Depending on the extent of editing, some types of analyses may not be relevant for the risk assessment. This should be considered on a case-by-case basis. For genome-edited plants, it is expected that analyses of allergenic potential would mostly apply to plants win the SDN3 caegory, especially if exogenous DNA inserts have been used and there is a lack of history of safe use of the donor organism or recipient plant. Information about IgE

binding, glycosylation sites, HLA-DQ binding, resistance to pepsin degradation, heat and low pH could reveal whether the modification introduces alterations in allergenicity. Post-market monitoring could be used on a case-by-case basis to detect allergies related to the intake of food derived from genome-edited plants.

8.1.9 Nutritional assessment

According to EFSA guidance, a nutritional evaluation should be performed to demonstrate that the food and feed derived from a genetically modified plant is not nutritionally disadvantageous to humans and animals. When the comparative assessment has identified compositional characteristics of the food derived from a genetically modified plant that are different and/or not equivalent to those of its comparator their nutritional relevance should be assessed further, for instance performing specific studies in poultry and/or livestock depending upon the genetically modified crop under assessment. Animals should be selected depending on the genetically modified crop being assessed (e.g. poultry, pigs, fish or ruminants). Results from other tests demonstrating that the nutritionally altered feed fulfils the expected nutritional value should be provided on a case-by-case basis.

The evaluation should include an assessment of the nutritional relevance of new proteins and other new constituents, the changes in the levels of endogenous constituents in the genetically modified plant and derived food and feed as well as the potential alterations in the total diet for the consumers/animals.

8.1.9.1 Nutritional assessment of food derived from genetically modified plants

The nutritional assessment of food derived from genetically modified plants should consider the composition of the food with regard to the levels of nutrients and anti-nutrients as well as the bioavailability and biological efficacy of the nutrients in the food. In addition, the following should be considered: potential influences of transport, storage and expected treatment of the food, the anticipated dietary intake of the food and the resulting nutritional impact.

If an altered bioavailability may raise concern for specific sub-population(s), the level of the nutrient in the food should be determined, taking into account all the different forms of the compound. The methods to test bioavailability should be selected on a case-by-case basis.

8.1.9.2 Nutritional assessment of feed derived from genetically modified plants

In plants modified with traits aiming at enhancing animal performance through increased nutrient density (e.g. increased oil content) or through a higher level of a specific nutrient (e.g. an essential amino acid or a vitamin), an appropriate control diet with similar nutrient profile should be formulated. Such diet should use a control supplemented with the specific nutrient as that present in the genetically modified plant. In the event a food is derived from animals fed a feed with modified nutritional value, it may be necessary to assess the animals' nutritional profile.

8.1.9.3 Application of EFSA guidance in nutritional assessment on genome-edited plants

To exemplify whether EFSA guidance on nutritional assessment can be used for genome-edited plants, cases 1–6 described above will be used.

Case 1 describes potatoes with improved starch quality using CRISPR/Cas (SDN1 category) (Andersson et al., 2017). Genome-edited amylopectin potato has its uses both in food, feed and technical applications. The guidance on nutritional assessment can be applied to case 1. The paper by Anderson et al. describes the molecular procedures related to the genom editing and also characterises starch by light microscopy, but there is no information on other constituents in the gene-edited potato.

Case 2 is a study by Li et al. from DuPont Pioneer Agricultural Biotechnology, which applied Cas9-guide RNA to generate targeted mutagenesis in soybean (*Glycine max*) resulting in herbicide-resistant soybean (SDN2 category) (Li et al., 2015). Li et al. describe the genom editing procedure, but no information is documented related to the nutritional quality. The EFSA guidance on nutritional assessment can be applied to case 2. According to the guidance, genetically modified plants carrying traits such as herbicide tolerance would require appropriate treatment comparisons to evaluate safety.

Case 3 is a drought-tolerant maize variety developed by CRISPR/cas resulting in overexpression of the negative ethylene regulator ARGOS8 (SDN3 category) (Shi et al., 2017), with the main goal to decrease the sensitivity of maize to ethylene. All steps in the guidance could be used to assess this maize line.

Case 4 is a rapeseed variety with herbicide tolerance developed using the ODM technique. The mutations in the rapeseed genome result in conformational changes in the enzyme acetohydroxyacid synthase, involved in the biosynthesis of branched amino acids like leucine, isoleucine and valine. The EFSA guidance on nutritional assessment can be applied to case 4. According to the guidance, genetically modified plants carrying traits such as herbicide tolerance would require appropriate treatment comparisons to evaluate safety (Songstad et al., 2017).

For case 5, using base-editing technique to introduce herbicide tolerance by point mutation in the endogenous enzyme ALS in tomato and potato, the guidance can be used (Veillet et al., 2019). According to the guidance, genetically modified plants carrying traits such as herbicide tolerance would require appropriate treatment comparisons to evaluate safety.

Case 6 is a genomedited apple with a knockout of the *MdDIPM4* gene (SDN1 category). MdDIPM4 is the receptor for the pathogen *E. amylovora* and loss of function of this protein inhibits infection and proliferation of this pathogen and results in fire blight resistance apple (Pompili et al., 2020)

8.1.10 Conclusions regarding the nutritional assessment

VKM concludes that the guidance is also suitable for genome-edited plants in the nutritional assessment. According to the guidance, a nutritional feeding study should be performed on a case-by-case basis on traits pertaining to nutritional quality. Any content of toxins or increased amounts of anti-nutrients due to unintended effects should be documented. This information is identified in the molecular characterisation and compositional analysis. The guidance specifies that genetically modified plants carrying specific traits, e.g. herbicide tolerance and insect resistance, require appropriate treatment comparisons to evaluate safety, and this would also be important for genome-edited plants carrying herbicide tolerance (case 2, 4 and 5) or insect resistance.

8.1.11 Exposure assessment

The EFSA guidance states that an estimate of the expected intake is an essential element in the risk assessment of genetically modified plants and derived food and feed. Information on the intended function, the dietary role and the expected level of consumption of the food and feed should be provided. When the modification targets agronomic traits, the intake of the plant species is not expected to be changed.

According to guidance, the concentrations of new proteins should be determined and other new constituents and endogenous constituents with levels altered because of the genetic modification (e.g. due to changes in metabolic pathways) in those parts of the plant intended for food or feed use. Expected intake of these constituents should be estimated taking into account the influences of processing, storage and expected treatment of the food and feed in question, e.g. potential accumulation or reduction.

In cases in which the genetic modification has resulted in an altered level of an endogenous constituent, or if a new constituent occurs naturally in other food and feed products, the change in total intake of this constituent should be assessed, including both realistic and worst-case intake scenarios. The anticipated average and maximum intake levels of the food and feed based on representative consumption data for products derived from the respective conventional plants should be estimated. Probabilistic methods may be used to determine ranges of plausible values.

The guidance states that groups of the population with anticipated high exposure should be identified and considered within the risk assessment. Any assumptions made in the exposure assessment should be described. Recent developments in methodologies and appropriate consumption data should be used. Data on import and production quantities may provide additional information for the intake assessment. Information on known or anticipated human/animal intake considering all possible routes of exposure is also required.

8.1.12 Conclusions regarding the exposure assessment

VKM concludes that the guidance is suitable also for genome-edited plants in the exposure assessment. However, some steps in the exposure assessment would not be applicable. This applies in the SDN1 or SDN2 category, or developed by ODM or BE without expression of new proteins. The genetic alteration introduced will define which tests are necessary to perform.

8.1.13 Monitoring

A post-market monitoring plan reveals information on changes in the overall dietary intake patterns of the product, possible side-effects and whether the product is used as recommended. Such a monitoring plan is required if the modification of the plant introduces differences in nutritional composition or specific health claims (EFSA, 2011f). Potential effects on the environment are detected in the Post-Market Environmental Monitoring (PMEM) of genetically modified plants and are a part of the environmental risk assessment (section 8.2.11).

8.2 Application of the EFSA guidance for environmental risk assessment of genetically modified plants in risk assessment of genome-edited plants

According to the EFSA guidance, environmental risk assessment of genetically modified plants should include information needed to determine the impact of the genetically modified plants on the receiving environment compared to the non-modified counterpart.

The different aspects to be considered are short-listed below:

- Cross-cutting considerations
- Persistence and invasiveness including plant-to-plant gene flow
- Plant to microorganism gene transfer
- Interactions of the genetically modified plant with target organisms
- Interactions of the genetically modified plant with non-target organisms
- Impacts of the specific cultivation, management and harvesting techniques
- Effects on biogeochemical processes
- Effects on human and animal health
- Post-market environmental monitoring (PMEM)

The following sections (8.2.1–8.2.12) contain key considerations extracted from the EFSA guidance supporting the overall environmental risk assessment of genetically modified plants (EFSA, 2010a). The introduction of each topic of the ERA is a synopsis of EFSA's guidance text and is not intended to be a VKM interpretation. For each main section, a VKM evaluation of the applicability of the EFSA guidance to the risk assessment of genome-edited plants is provided using the plant case examples 1 to 6 in Box 7.

It should be noted that intrinsic properties, such as growth and survivability, of a genetically modified or genome-edited plant are very important for the environmental risk assessment. There are several factors to be considered, e.g. if the plant has relatives already present in the environment where it will be introduced (hybridisation potential), has high or low climate adaptability, or whether is an annual or perennial plant etc. While the health risk assessment is concerned with potential new risks due to altered nutritional composition and to the newly introduced components themselves, e.g. a novel protein, the environmental risk assessment is concerned with potential risks associated with the introduced trait(s), and whether it may have an effect on survivability, fitness, fecundity and potential spread of the organism, with implications for the ecosystem and biodiversity.

In the guidance document for environmental risk assessment, the term 'genetically modified plant' refers to the specific genetically modified event for which approval is requested. However, in practice, commercially available genetically modified varieties are often produced from crosses of this event with other varieties. The applicants should discuss potential risks arising from the genetic background of varieties which might subsequently include the genetically modified event and how these might alter the conclusions of the risk assessment. On a case-by-case basis, depending on the nature of the event and according to the scope of the application, data may be required on the safety of the event when present in different genetic backgrounds.

8.2.1 Cross-cutting considerations

These are fundamental considerations that permeate the individual parts of an environmental risk assessment and constitute key information that risk assessors require to perform a sound risk assessment.

In the case of genetically modified plants, the EFSA guidance highlights the following cross-cutting considerations:

Choice of comparators

- A key scientific concept in risk assessment of genetically modified organisms is the comparative approach. This approach dictates that the genetically modified organism in question should be compared to its nearest conventional non-modified genetic relative - the comparator, throughout all the individual trials and analyses that constitute the risk assessment
- The assumption is that the use of an appropriate comparator will disclose whether there are elevated risks of adverse effects associated with the genetically modified in comparison to conventional varieties, for example regarding impacts on the environment

Receiving environment(s)

- Includes information on the genetically modified plant itself, e.g. species, genetic modification(s) and intended use(s)

- Information on geographical zones, e.g. the climate, altitude, soil, water, flora, fauna, habitats, etc. at site of introduction
- Information regarding management systems, e.g. land use and production systems, other cultivated genetically modified plants, cultivation practices, integrated and other pest management strategies, non-production activities and nature conservation activities

General statistical principles

- Encompassing relevant hypotheses, experimental designs and methodologies by use of appropriate statistics

Long-term effects (including techniques for their assessment)

- Should be assessed for a minimum of ten years after the start of cultivation for annual plants, possibly longer for perennial species, and should in all cases cover the time period over which progeny of the genetically modified plant might persist and appear as a volunteers or feral

Risk assessment of genetically modified plants containing stacked transformation events

- While the concept of stacked events (combination of two or more genetically modified plants by conventional crossing) could also be applied to genome-edited plants, this topic will not be considered in further detail in this report as the starting point in a risk assessment is the single event used to produce the stack (Box 8).

Box 8.

The meaning of the wording events, stacked events and negative segregants

Event

In classic genetic modification processes, a fragment of foreign DNA (a transgene, usually expressing a novel protein) is inserted into the genome of the transformed cell. The outcome of a successful transfer is called a transformation event.

The experimental transformation set up in the laboratory targets a high number of cells and only a proportion of those exposed cells will have a successful transfer. Each successfully transformed cell will carry the transgene(s) in one or several unique (random) insertion site(s). Moreover, there may be minor changes in the genomic DNA flanking the insertions.

Subsequent tissue/plant regeneration, as well as selection will determine which of the many initial events generated has the desired properties for commercialisation. The term event thus refers to a unique transformed cell/genome: with integration event(s) (at one or more sites in the genome), potential minor alterations in the flanking DNA sequences as well as the genetic variation arising in the regeneration process due to some clonal variation. Examples of commercialised events are maize MON810, or Bt11. The event will subsequently be crossed into other varieties depending on local agricultural conditions but retain the unique event name.

Stacked event

If two or more genetically modified plants carrying single events are crossed through traditional breeding, the resulting plant will carry stacked events. Repeated crossing of genetically modified plants each of which carries unique events/traits will result in higher order stacks. Such stacks are now commonly produced and form the majority of applications for genetically modified food import in Europe. The seeds of such stacks may contain any number of sub-combinations of the single events.

The term event in the context of genome editing

The wording "event" evolved to describe the outcome of gene transfer mechanisms with random insertions in the genome. In the case of genome-editing techniques, the insertion site is predefined. The use of the word events to describe such non-random processes may be conceived to be less relevant. On the other hand, off-target effects may also occur in genome editing, and the event descriptor of a modified genome may be useful to keep track of the unique origin of the editing process. As mentioned above for plants, outcrossing and segregation may render the original genetic background less relevant over time. For animals with longer generation time, the term event remains a useful descriptor.

Negative segregants

A genetically modified or genome-edited organism may be outcrossed so that the resulting organism will have lost the transgene/genome edit through segregation. The resulting organisms is called a negative segregant.

Negative segregants are sometimes used in classic production of genetically modified organisms, for instance to remove marker genes that are not genetically linked to the transgene of interest.

Similarly, the use of genome-editing techniques may draw on the production of negative segregants to remove the CRISPR genome editing insert and marker genes (if chromosomally inserted) from the desired genome edit.

In both cases, a plant is produced that is a negative segregant for the genes that have no functioning in the final products, whereas the desired genes/edits are retained in the final plant product.

Box 9.

The concepts conventional counterpart, comparator, comparative approach and intended versus unintended effects in different approaches

Comparator

A key step in the risk assessment of genetically modified organisms is the identification of intended and unintended effects. These will be the differences between the genetically modified organism and its conventional counterpart (comparator), taking into account the range of natural variation. The non-modified organism from which the genetically modified organism is derived is termed the conventional counterpart. The counterpart is expected to be isogenic or near-isogenic.The comparator may be identical to the conventional counterpart or another non-modified relative with a genetic background as close as possible to the genetically modified organism. The comparator usually has an established safe history of use. See (EFSA, 2011c) for a further introduction to the use of comparators.

Comparative approach

The comparative approach is central to and structures the risk assessment; both intended and unintended effects can be identified through such comparisons. The approach may reveal unintended changes that have occurred as an effect of the genetic modification process, the inserted or altered genetic material, as well as expression thereof. Such changes, if they occur, may be revealed through molecular analyses, and through feeding trials and controlled field releases.

Intended versus unintended effects

Guidance documents specify analyses needed to substantiate claims of the intended effects that have been obtained, and to exclude any unintended effects with potential adverse outcomes to health and environment.

This dual purpose of guidance is important to recognise when considering its applicability to genome-edited organisms. The targeted modification of defined genomic loci has removed some uncertainty of the effects of random insertions of transgenes. On the other hand, the site-specific nucleases may not always be 100% specific and may have off-target effects resulting in additional genetic alterations occurring elsewhere in the genome. Moreover, some CRISPR applications rely on the initial (random) chromosomal insertion of the CRISPR transgene, with subsequent removal through negative segregation, to result in a final product with the desired gene edits but without the transgene.

The extent to which site-directed nuclease technology has sufficiently reduced uncertainty of the types and occurrence of unintended effects to warrant different regulation from genetically modified organisms is currently debated. It is a complex discussion that may be facilitated by engineering site-directed nucleases with greater specificity, combined with increased opportunity to perform routine, whole-genome sequencing.

EFSA sconsiderations of the SDN1 versus SDN3

Previous discussions by EFSA stated that the SDN3 category can minimise hazards associated with the disruption of genes and/or regulatory elements in the recipient genome (EFSA, 2012c). Although the SDN3 category can induce off-target changes in the genome of the recipient plant, it was presumed that these would be fewer than those occurring with most mutagenesis techniques and would be of the same types as those produced by conventional breeding techniques. It was concluded that there is a need for flexibility in the data requirements for risk assessments.

The SDN-3 catergory was not found to differ from the other genetic modification techniques currently used and that it can be used to introduce transgenes, intragenes or cisgenes. Another opinion by EFSA (EFSA, 2012b) considered the types of transgenes used in plant breeding and the use of the terms cisgenesis and intragenesis. It was concluded that hazards arising from the use of a plant-derived gene from a breeder's gene pool by cisgenesis are similar to those from conventional plant breeding, as similar traits result from the modification. However, when a similar plant-derived gene is used in intragenesis, new combinations of genetic elements may arise that may present novel traits with specific hazards.

In contrast, genome editing within the SDN-1 and SDN-2 category, and ODM approaches result in plants not contaning any additional inserted genes such as a transgene, intragene or cisgene.

A recent opinion of EFSA (EFSA, 2020b) concluded that considerations which are specifically related to the presence of exogenous DNA/transgene, intragene or cisgene are not relevant for SDN-1, SDN-2 or ODM. Hence, the data requirements in the existing guidance to exclude some unintended effects of the transgene (usually encoding a novel protein) may not be relevant for these categories, emphasising the case-by-case approach.

These opinions are useful for understanding how native genes, including their genomic and gene regulatory context will be risk assessed when used in an risk assessment of an organism categorised as a SDN3.

8.2.1.1 Applicability of EFSA guidance regarding cross-cutting considerations

VKM considers the cross-cutting considerations as a fundamental part of ERA. The six cases described at the start of this chapter exemplify the importance of a case-by-case approach for the cross-cutting considerations and the environmental risk assessment itself. The cross-cutting considerations constitute key information that would also be required to perform appropriate risk assessments of genome-edited plants.

It is noted that for genome-edited plants it may become more common to simultaneously introduce several genome edits to produce the first commercial "event". This is somewhat different from the current use of the term "event" that is used to describe the unique gene transfer events thay may later be combined by traditional breeding to form stacked varieties of genetically modified plants. The terminology used to describe the outcome of multiple genome edits versus sequential gene transfer events may be further clarified (Box 9).

8.2.2 Conclusions regarding cross-cutting considerations

VKM concludes that the guidance regarding cross-cutting considerations is applicable also for genome-edited plants. The case-by-case approach centred on the introduced trait(s) and resulting phenotype should dictate the extent of experimental data required and the specific areas of risk to be addressed in the ERA.

8.2.3 Specific areas of risk to be addressed in the ERA of genetically modified plants

Persistence and invasiveness including plant-to-plant gene flow

As stated by EFSA, environmental concerns about genetically modified plants relate to the potential of spreading caused by persistence or invasiveness of the plant itself, or of its compatible relatives, as a result of vertical gene flow. The potential adverse effects can be

divided into two main types. First, enhanced fitness of the genetically modified plant or of wild relatives (with introgressed transgenes) within production systems may make them more persistent. This could exacerbate weed problems, e.g. weeds that may require more complex control strategies that may be harmful. Second, enhanced fitness of feral transgenic plants, or of wild relatives (with introgressed transgenes) in semi-natural or natural habitats may reduce the diversity of valued flora and fauna.

Plant to microorganism gene transfer

The guidance states that likelihood of gene transfer into microorganisms and its stabilisation, e.g. by integration into their genomes, must be considered. Horizontal gene transfer is defined by EFSA as any process in which an organism incorporates genetic material from another organism without being the offspring of that organism. The evaluation includes analysis of the transfer of transgenes to initially receiving micro-organisms and the potential for subsequent transfer to other organisms (e.g. microorganisms, plants). The potential consequences of such a gene transfer for human and animal health and the environment should be considered.

8.2.3.1 Interactions of the genetically modified plant with target organisms (TOs)

EFSA defines target organisms (TOs) as organisms on which specifically designed characteristics of a genetically modified plant are intended to act. These are usually pests or pathogens of the plant. The target organisms should be defined in the application. According to EFSA, all other organisms should be considered as non-target organisms (NTOs). The likelihood of the target pest to develop resistance should be evaluated and a strategy to prevent or delay resistance should also be designed.

8.2.3.2 Interactions of the genetically modified plant with non-target organisms (NTOs)

The ERA should consider the possible immediate and/or delayed environmental impact resulting from direct and indirect interactions of the genetically modified plant with non-target organisms (NTOs). The ERA should address the potential environmental impact on population levels of herbivores, natural enemies, symbionts (where applicable), parasites, and pathogens that are not the intended or target organisms, but that could be affected by the new trait after interaction with the edited organism. Guidance for assessing potential effects of genetically modified plants on NTOs and rationale for data requirements in order to complete a comprehensive ERA for NTOs is provided in EFSAs scientific opinion of potential impacts of genetically modified plants on NTOs (EFSA, 2010c).

8.2.3.3 Applicability of EFSA guidance regarding specific areas of risk to be addressed in the ERA

Any cultivated plant, irrespective of whether or not it is genome-edited, can constitute an environmental risk. As described in the guidance, this is related to the properties of the

organism, for instance the ability to survive and spread outside production systems, the impact on native biodiversity and ecosystem functions, and the ability to hybridise with native species or facilitate horizontal gene transfer. If a cultivated plant possesses such characteristics, whether naturally or introduced or enhanced by genome editing, it constitutes an environmental risk. These aspects are all well covered in the guidance, and can therefore also be applied to ERA of genome-edited plants.

In terms of persistence and invasiveness, the soybean (case 2) and maize (case 3), are both crops/species poorly adapted to the Norwegian climate and do not exist naturally in Norway (Elven et al., 2018b; Elven et al., 2018e). Maize accounts for less than 3% of Norwegian cultivated crops, and cultivation of maize is limited to specific regions of southern Norway. The small production area combined with cold winters means that the probability of survival of maize seeds and accidental dispersal is low. At present, there is no cultivation of soybean in Norway. However, according to NIBIO (NIBIO, 2018), soybean production is spreading northwards in Europe, and there is limited ongoing testing on edamame (immature soybeans) for cultivation in Norway. Nonetheless, the information required for the ERA of such plant species is expected to be less comprehensive than for species that are adapted to Norwegian climate.

The remaining cases represent cultivated species that are long established in Norwegian agriculture and are well adapted to Norwegian climate. Potatoes (cases 1 and 5) and tomatoes (case 5) are not considered important ecological threats in Norway (Elven et al., 2018c; Elven et al., 2018d). Oilseed rape (case 4) can survive the winter in Norway and has the potential to spread as a feral plant, hybridise with other oilseed cultivars as well as certain wildtype *Brassica* species, in addition to establishing as a weed population in agricultural fields with crop rotations (Elven et al., 2018a). In the case of fire blight resistant apple trees *Malus* × *domestica* (case 6), there is a potential for spread as well as hybridisation and introgression with other species of apple, including the wild apple *Malus sylvestris* (Henriksen and Hilmo, 2015). Introduction of the herbicide tolerant oilseed rape and fire blight resistant apple would thus be expected to require more data to complete the ERA compared to the other cases.

According to the guidance, the likelihood of gene transfer into microorganisms (horizontal gene transfer) and its stabilisation, e.g. by integration into their genomes, must be considered. Although this is not applicable for the cases in this report, it might be relevant to other genome-edited plants (notably plants in the SDN3 category with relevant inserts from microorganisms).

The guidance states that all target organisms (TOs) should be defined, and that all other organisms are non-target (NTOs). The ERA should consider the possible immediate and/or delayed environmental impact resulting from direct and indirect interactions of the genetically modified plant with NTOs. This would not be applicable to any of the cases described in this report since none of them are designed to target pests or pathogens (the fire blight apple tree (case 6) is only less susceptible to infection due to a genetic deletion).

The guidance specifically mentions the need for attention to identified protection goals (EFSA, 2016). Regarding alien species, attention to threatened or key-stone species and threatened-

nature types is an integrated part in the assessments performed by the Norwegian Biodiversity Centre (Sandvik et al., 2017). Although all adverse effects of genome-edited plants on native species or ecosystems are unwanted, effects on important or threatened species or nature types would be especially harmful. For instance, the cultivated apple constitutes a risk to the threatened wild apple *Malus sylvestris* through hybridisation and introgression (Henriksen and Hilmo, 2015), and this could also be the case for a genome-edited apple (case 6).

Information on whether the assessed plant constitutes a risk for threatened or key-stone species and threatened nature types is an important part of the ERA, both for genetically modified organisms and genome-edited organisms, and should be considered on a national level as is the case for alien species. Such risk analysis should be based on a conventional counterpart as a comparator as indicated under the cross-cutting considerations.

8.2.4 Conclusions regarding specific areas of risk to be addressed in the ERA

VKM concludes that the guidance for assessment of specific areas of risk to be addressed in the ERA is applicable also to genome-edited plants. The information required is case-dependent, with more and thematically wider information required when the plant has the potential to spread into natural ecosystems or hybridise with native species. For the cases described in this report, the information required would be expected to differ in each case. For instance, under Norwegian conditions neither maize nor soybean are considered environmental threats, while e.g. oilseed rape and apple have wild relatives and therefore have the potential to both hybridise and spread in the environment. In addition, it is emphasised that information on risk to threatened or key-stone species and threatened-nature types should be considered in a Norwegian biodiversity context.

8.2.5 Impacts of the specific cultivation, management and harvesting techniques

The introduction of genetically modified plants for cultivation may require specific management practices and cultivation techniques, and this may lead to additional changes in management and production systems. Introduction of genetically modified plants and their potential environmental impacts shall therefore be seen in the context of the already existing management and production systems and their environmental impacts. According to the guidance, the ERA shall aim at comparing the range of different systems in the practical management of genetically modified crops with non-modified systems. The ERA shall consider whether the specific management and production systems of the genetically modified plant may lead to greater, similar or lower adverse environmental effects than the current systems.

For instance, introducing genetically modified herbicide-tolerant plants will change the herbicide regime, both type of herbicides and application times. This might also change crop rotation and cultivation and alter weed control systems. Minimum tillage or no-till cultivation techniques might affect soil structure, moisture, greenhouse gasses emission and energy balance. Genetically modified insect resistant plants may change crop rotation in response to altered pest

pressure. In general, adoption of pest, drought, disease and herbicide-tolerant genetically modified plants will alter requirements for Integrated Pest Management as well as the cropping system, potentially affecting biodiversity.

8.2.5.1 Applicability of EFSA guidance regarding impacts of specific cultivation, management and harvesting techniques, in risk assessment of genome-edited plants

As for genetically modified plants, the introduction of genome-edited plants for cultivation may lead to changes in management and production systems. The introduction of genome-edited plants and their potential environmental impacts should, according to the guidance, be seen in the context of the already existing management and production systems and their environmental impacts. A case-by-case approach will therefore determine the extent of information required for an ERA.

Soybean, oilseed rape and tomato (cases 2, 4 and 5) can lead to greater changes in agricultural practices than the potato, maize and apple (cases 1, 3 and 6) because of their herbicide-tolerant traits. Herbicide-tolerant plants will change the herbicide regime, both type of herbicides and application time. Further, cultivation of herbicide-tolerant plants may also enable flexibility in tilling and other soil management practices.

As stated above, adoption of pest-, drought-, disease- and herbicide-tolerant plants will alter opportunities for Integrated Pest Management in general as well as cropping systems, potentially affecting biodiversity. For instance, the use of fire blight resistant apple trees (case 6) would require less pesticides to control the disease, whereas the drought-tolerant maize (case 3) could reduce drought damage to crops.

8.2.6 Conclusions regarding impacts of the specific cultivation, management and harvesting techniques

VKM concludes that the guidance regarding impacts of the specific cultivation, management and harvesting techniques is applicable also for genome-edited plants and that the extent of information required should be based on a case-by-case approach.

8.2.7 Effects on biogeochemical processes

According to the guidance, problem formulation should cover the production site and the wider environment. Both direct and indirect impact due to change of cultivation, management and harvesting techniques should be assessed. The cultivation system used for the genetically modified plant should be compared with current production systems. In the guidance document, it is recommended that greenhouse gas emissions, erosion, soil degradation and potential to pollute water courses be addressed. Potential effects of release of genetically modified metabolites and movement of other compounds from root to soil influencing soil fertility, nutrient transformation and food web must be investigated. Altered plant litter

decomposition due to the presence of specific compounds, and uptake and cycling of plant nutrients like fixation of atmospheric nitrogen (e.g. legumes and soybeans), should also be assessed.

The different steps of the assessment should be weighted case by case and emphasised as needed dependent on type of crop and modification. According to guidance, risk characterisation could initially compare existing data from current production systems (e.g. fertiliser and pesticide applications, frequency and depth of tillage) with the practice expected during the growing of the genetically modified plant. The guidance states that the choice of comparator should be carefully justified, as (a) most methods and materials used in current production (e.g. agricultural) cause losses from and reduced storage capacity of the production system, (b) there may be several types of production system operating in a receiving environment and (c) the systems may change over time (e.g. due to phasing out of pesticides). The characterisation should demonstrate that the genetically modified plant and its management do not have more adverse effects than the comparator. Long-term effects of adverse changes in biogeochemical processes should be considered, as well as possible indirect effects on biogeochemical processes as a consequence of altered production practices related to the genetically modified plant.

8.2.7.1 Applicability of EFSA guidance regarding effects on biogeochemical processes in risk assessment of genome-edited plants

As for genetically modified plants, the introduction of genome-edited plants for cultivation may lead to changes in management and production systems affecting biochemical processes. A case-by-case approach will determine the extent of information required. The traits of the soybean, oilseed rape, maize and tomato (cases 2-5), to a greater extent than potato and apple (cases 1 and 6), may change herbicide regimes, tilling, irrigation and other soil management systems affecting biogeochemical processes. However, these aspects would have to be assessed for all cases to ensure that the altered crop management systems do not have more adverse effects on biochemical processes than the existing system. The existing production systems (e.g. fertiliser and pesticide applications, frequency and depth of tillage) may be compared with the practice expected during the growing of the genome-edited plant.

8.2.8 Conclusions regarding effects on biogeochemical processes

VKM concludes that the guidance regarding effects on biogeochemical processes is applicable also to genome-edited plants, and information required should be based on a case-by-case approach.

8.2.9 Effects on human and animal health

An assessment of potential hazards to human and animal health related to exposure to the plant, e.g. to pollen, dust, or other constituents via handling during processing etc., can be performed according to the EFSA guidance. This assessment is required if the modified

organism is not intended for food or feed uses, and where possible effects on human and animal health are not already extensively analysed.

In accordance with the risk assessment for food and feed products, the following should be provided by the applicant:

- Molecular characterisation
- Comparative assessment
- Toxicity and allergenicity
- Nutritional assessment
- Exposure assessment
- Risk characterisation

For non-protein constituents that are intended to be used in medicinal products, comprehensive pharmacological and toxicological information must be provided (EC, 2004b).

The toxicity of any newly expressed protein as well as the whole plant should be assessed. Toxicity testing of other components than protein or possible changes in the level of naturally occurring constituents that might be altered compared to the non-modified counterpart should be performed. Information on known allergenicity of the plant should be provided.

Nutritional assessment is however not considered relevant for non-food and feed products such as plants for technical purpose (textile/paper) or decoration (e.g. blue variants of the carnation *Dianthus caryophyllus*).

8.2.9.1 Applicability of EFSA guidance regarding effects on human and animal health, in risk assessment of genome-edited plants

Considering the six cases, all can be risk assessed for food or feed uses according to the guidance.

The amount of information required to assess effects on human and animal health must be determined on a case-by-case basis.

For example, in case 1, the genome-edited potato is not expressing any new constituents. The loss of function of the granule-bound starch synthase (GBSS) gene is expected to result in minor compositional differences to the comparator, except for the altered ratio between amylopectin and amylose (Starch components). Less toxicity testing would therefore be expected to be required if it can be concluded in the molecular characterisation and comparative assessment that the modification is not resulting in new proteins or biochemical alterations leading to potential differences in toxicity compared to the conventional counterpart (comparator).

8.2.10 Conclusions regarding effects on human and animal health

VKM concludes that the guidance regarding effects on human and animal health is also applicable to genome-edited plants not intended for food and feed uses and that the amount of information required should be based on a case-by-case approach.

8.2.11 Post-market environmental monitoring (PMEM)

8.2.11.1 General

An environmental monitoring plan is required for placing food/feed on the market if it consists of or contains genetically modified organisms. According to the guidance, the extent of the release to the market should be considered. Thus, the monitoring plan should be targeted rather than considering every possible environmental aspect. Genetically modified plants intended to be used as food/feed or ingredients not intended for cultivation within the EU will thus not normally require a detailed environmental monitoring plan. A more detailed environmental monitoring plan is necessary only if it is not clearly shown that environmental exposure is absent or will be at levels that present no risk to other living organisms or the abiotic environment.

According to the guidance, environmental monitoring of the genetically modified plant shall have two aims: (1) to study any possible adverse effects of the plant identified in the formal risk assessment procedure, and (2) to identify the occurrence of adverse unforeseen effects of the plant or its use that were not anticipated in the ERA. Where there is scientific evidence of a potential adverse effect linked to the genetic modification, then case-specific monitoring should be carried out after placing the product on the market, in order to confirm the assumptions of the ERA. Consequently, case-specific monitoring is not obligatory and is required only to verify the risk assessment, whereas a general surveillance plan must be performed.

8.2.11.2 Case-specific monitoring of genetically modified plants

As stated by EFSA, case-specific monitoring should be targeted at the environmental factors (including non-target organisms) identified in the ERA as most likely to be adversely affected by the genetically modified plant. The monitoring should be designed to detect the expected adverse effects as derived from the ERA. The design of the monitoring program should also reflect varying exposure levels in different geographical regions and other site-specific influences. EFSA has published guidance to clarify the objectives, tasks, tools and requirements for post-market environmental monitoring (PMEM) (EFSA, 2011f)).

8.2.11.3 General surveillance for unanticipated adverse effects

The guidance states that the aim of the general surveillance is to identify the occurrence of unanticipated adverse effects (i.e. effects not anticipated in the ERA) of the genetically modified plant or its use on human health or the environment. The general surveillance applies to

genetically modified plants where no adverse effects have been identified in the ERA but is always required in order to detect unanticipated adverse effects (EC, 2002).

8.2.11.4 Applicability of EFSA guidance regarding post-market environmental monitoring (PMEM)

According to the guidance, the post-market environmental monitoring should include consideration of two parts, a general surveillance for unanticipated effects and (where relevant) a case-specific monitoring aimed at detecting adverse effects identified in the ERA. Ideally, these two monitoring systems will identify any adverse effects of the assessed organism on the environment. The aspects covered in the EFSA guidance is also generally applicable for post-market environmental monitoring of genome-edited plants, as environmental effects should be related to the properties of the species regardless of the type of genome-editing method.

The guidance states that the general surveillance for unanticipated effects should 'when compatible, make use of established routine surveillance practices such as monitoring of agricultural plants, variety/seed registration, plant protection, plant health and soil surveys' or questionnaires. However, such monitoring is not designed to detect unanticipated effects on the environment outside of production systems, such as spread to natural ecosystems or effects on native plant populations. On a case-by-case basis, therefore, there may be a need to develop novel monitoring systems aimed at such detection, as is also recognised in the EFSA guidance (EFSA, 2010a; EFSA, 2011d).

Considering the six cases, the guidance regarding post-market environmental monitoring can be applied to all. However, the extent of the monitoring, and the need for case-specific monitoring, will vary between the cases depending on the outcome of the ERA. For instance, for soybean (case 2), maize (case 3), potatoes (cases 1 and 5) and tomatoes (case 5) the monitoring will probably be limited to the general surveillance for unanticipated effects, whereas for oilseed rape (case 4) and apple (case 6) inclusion of case-specific monitoring in line with the potential environmental effects identified in the ERA may be needed.

It is noted that some categories of environmental impact constituted by genome-edited plants may be technically difficult to monitor. This includes for instance events of hybridisation or introgression with native species, as could be the case for oilseed rape (case 4) and apple (case 6) (Henriksen and Hilmo, 2015). For such species, the extent and type of genome editing will influence detectability. Furthermore, some species' characteristics, regardless of the type of genome-editing method, make dispersal and exposure difficult to monitor, such as plants reproduced by edible animal- or bird-dispersed fruits, or by small, wind-dispersed seeds, which can spread far from the production system. Thus, case-specific monitoring of species with genome-edited traits where risks such as hybridisation or long-distance dispersal have been identified in the ERA need especially carefully designed monitoring programmes.

8.2.12 Conclusions regarding post-market environmental monitoring (PMEM)

VKM concludes that the guidance for post-market environmental monitoring can also be applied to genome-edited plants, and the need and extent of the monitoring required is case-specific. However, technical issues may be present in some monitoring efforts, depending on the scope of the environmental release and the extent and type of genome editing introduced (SDN1-3). These challenges can include the need to distinguish on a molecular level between naturally occurring mutants, conventionally bred, and genome-edited plants.

Box 10.

Rapeseed plants and risk assessment

GMO production utilises about 10% of the world's arable land with the US, Brazil and Argentina as the leading producers. In terms of production area, the most common genetically modified crops are soybeans, maize, cotton and canola, as of 2019. In that year, 48.2% of all soybean plants and 13.5% of all the cotton grown worldwide were genetically modified (Statista, 2018). However, in Norway, there is no cultivation of genetically modified crops. In this report, we use oilseed rape as an example case for possible cultivation of a modified plant in Norway.

Oilseed rape production in Norway is limited to a small area, only about 1% of the total cereal production area and only a small part is used for human food. Out of 10,000 tonnes produced in 2018/19, 1300 tonnes was cold-pressed to yield 300,000 litres of oil for human consumption and the rest was used as concentrate in feed (Svanes et al., 2020). Raw rape meal contains anti-nutrient compounds, has poor digestibility, unpleasant taste and colour, and therefore needs refined processing before it can be used in food/feed.

Genetically modified rapeseed is not cultivated commercially in the European Union, but several lines containing herbicide tolerance have been approved for use as food, mostly as cooking oil and margarine and as animal feed. Feral populations of genetically modified rapeseed have been detected along transporting routes, possibly introducing herbicide resistance in sexually compatible weed populations. Accidental dispersal of unprocessed seeds during transport can lead to settlement of volunteers and spread of the plant as ferals. This may be considered a significant risk in the ERA.

Canola seed is a genetic variation of rapeseed that was developed in the 1960s using traditional plant-breeding methods. The first genetically modified canola was the Roundup Ready canola presented in 1995 with herbicide resistance (towards glyphosate). Approximately 95% of the canola grown in Canada and 93% in the US is from genetically modified seed (FDA, 2020; Statista, 2018). In Australia, about 20% of the canola production is derived from GM canola (OGTR, 2018).

The ODM genome-editing approach has also been used to generate canola with herbicide tolerance to sulfunylurea grown in the US and Canada. By using genome-editing techniques, the omega-3 fatty acid content (n-3-LC PUFA) in rapeseed can be introduced, a trait especially interesting for use as feed in the fish industry as a substitute for plankton in the transmission from maritime farming to freshwater (Sprague et al., 2016). As of today, this is achieved in canola by transgenesis with genes from microalgae and authorised in 2020 in Canada for the use in food and feed.

9 Application of the EFSA guidance for risk assessment of genetically modified animals in risk assessment of genome-edited animals (ToR 3 and 4)

The most commonly used alterations of genome editing in animals have so far been in the SDN1 and SDN3 category, with deletion/insertion of few base pairs or introduction of larger sequences or exogenous DNA, respectively. Genome editing has a broad range of potential applications in production animals, e.g. increased adaptation of livestock to farming or environmental conditions, increased disease resistance, improvements in growth and fertility, and welfare aspects. An unresolved challenge is that many or most traits are multi-genic/quantitative and cannot be addressed by single nucleotide variants.

Since the health status of a food/feed-producing animal is an important indicator of the safety of derived foods/feed, health and physiological parameters including welfare aspects is an integrated and important component in the risk assessment.

In this report, VKM focuses on animals that are used for food or feed products. Insects, such as honeybees, are not considered in this report.

9.1 Application of the EFSA guidance for risk assessment of food and feed from genetically modified animals in risk assessment of genome-edited animals

According to the EFSA guidance, risk assessment of food and feed from genetically modified animals should include information on the modification; methods used; possible effects on the composition; toxicity; allergenicity; nutrient content; and effects on animal welfare. These are listed in short below:

- Molecular characterisation
- Comparative analysis
- Toxicity and allergenicity
- Nutritional characterisation
- Exposure (intake calculations)
- Risk of gene transfer
- Assessment of animal health and welfare
- Post-market monitoring

The following sections (9.1.1–9.1.20) contain key elements present in the EFSA guidance on the information requirements to perform a risk assessment of a genetically modified animal intended for use in food and feed (EFSA, 2012a). For each main section, an evaluation of the applicability of the EFSA guidance to genome-edited animals is provided using the case-examples 1–5 (box 7).

9.1.1 Molecular characterisation

9.1.1.1 Description of the methods and vectors used for the genetic modification

The EFSA guidance requires information to identify the nucleic acid intended for transformation and related vector sequences potentially delivered to the recipient animal. The DNA actually inserted in the animal must be characterised and the expression and stability of the intended trait(s) must be evaluated. According to the guidance document, information must be given on the production method of the vector or fragment used for transformation as well as the methods and criteria used for selection. When relevant, a description must also be included of the technologies used to remove part of the insert, to limit the chance of mobilisation of the insert, or to drive the trait through the population.

Furthermore, the EFSA guidance states that information on the cellular or tissue material to be transformed and the nature and source of vector(s) used for transformation must be described. This information is required to identify each component of the plasmid/vector, including the region intended for insertion, as well as its size, origin and intended function.

A physical map of the functional elements and other plasmid/vector components must be presented together with the relevant information needed for the interpretation of the molecular analyses. The region intended for insertion should be clearly indicated. If helper plasmids are used, a detailed description of the *cis*/*trans* acting system must be included. Information on the purity of the preparation containing the construct prior to introduction into recipient animals or cells must be provided.

9.1.1.2 Source and characterisation of nucleic acid intended to be inserted

According to EFSA guidance, information on the donor organism(s) and the nucleic acid sequence(s) intended for insertion should be given. Deliberate alteration(s) to the corresponding sequence(s) in the donor organism(s) and on the techniques used for producing these changes (site-directed mutagenesis, gene shuffling, and production of synthetic nucleotide sequences) must be described. Information regarding each donor organism should comprise its taxonomic classification and its history of use regarding food and feed safety.

In cases where synthetic nucleotide sequences with no gene counterpart in existing organisms are used, information should be provided on the design and the functional elements of the synthetic nucleotide sequences.

According to guidance, information regarding the DNA region(s) intended for insertion; history of consumption of the gene product(s) arising from the inserted regions; data on the possible relationship of the gene products with known toxins, anti-nutrients, allergens or other compounds with potential adverse health effects, should be provided.

If viral vectors, transposons or known zoonotic organisms have been used, information on their natural hosts, target organs, transmission mode and stability, pathogenicity and potential for recombination with endogenous or exogenous pathogens (e.g. viruses) are required. Finally, information related to the occurrence of transposons or viruses in the recipient animals, related to the genetic construct used, and which might be able to provide transacting transposase activity or act as helper virus, should be available.

9.1.1.3 General description of the trait(s) and characteristics introduced or modified

The introduced trait(s), its mode of action, and the resulting changes in the phenotype of the modified animal must be described.

A description of the generation of the animals to be marketed from the initial animals, including the breeding strategy, should be given. Also, information on whether the initial animals were to be marketed is required.

Data on whether the initial animals are hemizygous or homozygous with regard to the sequence(s) actually inserted, and whether the animals were mosaic, must also be given. Information on the ploidy of the genetically modified animals to be marketed should also be included.

9.1.1.4 Information on the sequences actually inserted/deleted or altered

The size and copy number of the inserts, both complete and partial, must be analysed. The analysis should cover sequences that could be inserted into the host animal, such as any parts of the plasmid/vector. The analysis should span the entire insert locus/loci as well as flanking sequences. Further, the organisation and sequence of the inserted genetic material at each insertion site must be analysed. Size and function of the deleted/modified region(s), in the case of intended deletion/modification(s), must also be analysed.

Sub-cellular location(s) of insert(s) (integrated in the nuclear or mitochondrial genome or maintained in a non-integrated form) and methods for ascertaining those sub-cellular location(s) of the insert(s) must be described.

Sequence information for both 5' and 3' flanking regions at each insertion site must be given, and bioinformatics analysis should be conducted using up-to-date databases with the aim of performing both intraspecies and interspecies homology searches.

An analysis of open reading frames (ORFs) present within the insert and spanning the junctions must be conducted. The ORFs should be analysed between stop codons, not limiting their

lengths. Bioinformatics analyses should be conducted to investigate possible similarities with known toxins or allergens using up-to-date databases.

9.1.1.5 Information on the expression of the inserted/modified sequence

Information on whether the inserted/modified sequence results in the intended change(s) at the protein, RNA and/or metabolite level(s) must be provided. If the intended genetic modification leads to the expression of new protein(s), protein expression data will be the most relevant. In other cases (e.g. silencing approaches or where biochemical pathways have been intentionally modified) the analysis of specific RNA(s) or metabolite(s) may be the most informative.

The EFSA guidance states that the data should be derived from animals bred, fed and reared under representative conditions. Information on tissues of the animal where the inserted/modified sequence is expressed, and tissues where the expressed products are localised, must be given. Data on expression levels from those parts of the animal that are used for food/feed purposes, and relevant to the scope of the application, are considered necessary in all cases. Where tissue-specificity is intended, information on expression and presence of expression products in different tissues, fluids and other compartments should be provided. The requirement of information on developmental expression should be considered on a case-by-case basis, taking into account the promoter used, the intended effect of the modification and the scope of the application.

In cases such as silencing approaches or where the modification is intended to modify the levels of specific proteins or metabolites, the experimental design should include a conventional counterpart (comparator) in order to compare the levels of relevant endogenous RNA(s), protein(s) and/or specific metabolite(s). If the genetic modification results in new protein(s), the comparative approach is not applicable.

Information on the method used for the analysis must be given, and results of the analysis should include mean and range of concentrations of newly produced proteins or levels of endogenous animal proteins, together with the raw datasets.

When justified by the nature of the insert (e.g. gene silencing through RNA interference), information on the expression of targeted gene(s) and on possible effects on related endogenous genes should be provided. Information on the expression of genes situated near the inserted/modified sequence should be given on a case-by-case basis.

9.1.1.6 Inheritance and genetic stability of the inserted/modified sequence and phenotypic stability of the genetically modified animal

According to EFSA guidance, information to demonstrate the inheritance and genetic stability of the locus/loci altered by the genetic modification and the phenotypic stability and inheritance pattern(s) of the introduced/modified trait(s), should be provided.

Data on the inheritance pattern and the stability of the introduced/modified nucleotide sequences and associated phenotypes in the offspring across multiple sexual generations should be provided. The source of the material, the sampling design, the number of animals used for the analysis and the number of generations should be specified and clearly indicated on the breeding diagram.

9.1.1.7 Application of EFSA guidance documents in molecular characterisation of genome-edited animals

In this section, the applicability of the EFSA guidance for risk assessment of food and feed from genetically modified animals will be evaluated with respect to the molecular characterisation data requirement developed using the genome-editing techniques. Particular attention will be paid to animals in the SDN1 and SDN2 category, base and prime editing (BE/PE)), and ODM techniques.

The five example cases; Atlantic salmon (case 1) with edited PUFA synthesis pathway, sterile Atlantic salmon (case 2), catfish (case 3) with increased growth trait, hornless dairy cattle (case 4) and PRRS-virus infection resistance pigs (case 5), will be evaluated. The applicability of the EFSA guidance requirements for the molecular characterisation are discussed using these five cases.

The first requirements of the molecular characterisation in the guidance may also be applicable to genome-edited animals, since genetic elements encoding the SDN tools will be delivered into the organism using similar genetic engineering techniques as genetically modified animals.

The above-mentioned molecular characterisation requirements are partially applicable to animals obtained by the genome-editing techniques outlined in case studies 1, 2, 3 and 5, and fully applicable to animals in the SDN3 category (case 4). The parts relating to the description of the methods and vectors used for the genetic modification are applicable for all five cases, however, the parts about 'source and characterisation of nucleic acid intended to be inserted' are relevant only to case 4, where hornless dairy cattle were obtained by inserting exogenous DNA (SDN3 category).

The information requirements about the introduced trait(s), its mode of action and the resulting changes in the phenotype of the modified animal are applicable to all of the five example cases and can, thereby, be provided following the molecular characterisation guidance.

The data requirements in the EFSA guidance seem adequate to determine whether the initial animals are hemizygous or homozygous. The characterisation of exogenous DNA-fragments actually inserted seems relevant only for animals in the SDN3 category (case 4). For animals in the SDN1 category (example cases 1, 2, 3 and 5) the following requirements are therefore not considered relevant: 'Information on the sequences actually inserted/deleted or altered: the size and copy number of the inserts, sub-cellular location(s) of insert(s) (integrated in the nuclear or mitochondrial genome, or maintained in a non-integrated form) and methods for ascertaining those sub-cellular location(s) of the insert(s)'.

The guidance also seems suitable to establish if the initial animals are mosaic (not all cells of the animal are modified). Moreover, information on the ploidy of the genome-edited animals is possible to provide for all the five example cases following the molecular characterisation guidance.

The requirements on sequence information for both 5' and 3' flanking regions at each insertion site must be given, and bioinformatics analysis should be conducted. The analysis should be used to reveal modification, e.g. new open reading frames (ORFs), at the insertion site(s). Only case 4, representing insertion of exogenous DNA fragments, fulfils the data requirements on the presence of ORFs within the insert and the spanning junctions.

The requirements of bioinformatics analyses to investigate possible similarities with known toxins or allergens using up-to-date databases are only partially applicable to cases 1, 2, 3 and 5, due to the absence of newly created junctions, but they are relevant to animals in the SDN3 category (case 4).

The requirements on information about the expression of the inserted/modified sequence and whether the inserted/modified sequence results in the intended change(s) at the protein, RNA and/or metabolite level', are applicable in all the five cases and they can be obtained using the guidance document. The level of the modified proteins in all the five cases can be measured and assessed.

The guidance requirements on the inheritance and genetic stability of the inserted/modified sequence and phenotypic stability of the genome-modified animal are fully applicable to case 4, where the stability of inserted exogenous DNA can be assessed. However, the requirement of demonstrating the stability of inserted transgenes is not relevant to animals in the SDN1 or SDN2 category (cases 1, 2, 3 and 5). In these cases, assessment of genetic stability of the modified nucleotides and introduced trait is relevant.

9.1.2 Conclusions regarding the molecular characterisation

VKM concludes that, in general, the molecular characterisation requirements in the EFSA guidance can be fully or partially applied to genome-edited animals exemplified in the five cases. However, the information required could be less extensive and should be determined on a case-by-case basis, particularly for in the SDN1 or SDN2 category.

The requirements of the molecular characterisation are relevant for the genome-edited animals in the SDN3 category (case 4). In case 4, an exogenous DNA fragment was inserted to produce hornless dairy cattle.

The animals presented in cases 1, 2, 3 and 5 (SDN1), differ from case 4 because in these cases no exogenous DNA is inserted. The requirements linked to the introduction of transgenes, i.e., the presence of foreign DNA in the final product, would not be applicable for in the SDN1 and SDN2 category as long as the final animals do not contain any leftovers, e.g. vector backbone used during the genome editing process. Some revision of the guidance would be helpful to

guide which analyses are applicable, recognising the need for flexibility given the broad diversity of animal species possibly targeted by genome-editing technologies.

9.1.3 Comparative analysis

The underlying assumption of the comparative approach in food and feed risk assessment is that traditionally bred animals have a history of safe consumption as food and feed for the average consumer or animal to which the animal-derived products are fed. Traditionally bred animals can thus serve as a baseline for the food and feed safety assessment of genetically modified animals or their products and the welfare of the animals. The aims are 1) to identify similarities and differences between the modified animal and its comparator(s) in phenotypic characteristics (both intended and unintended alterations), including data on health status and physiology and 2) to identify similarities and differences in composition between the genetically modified animal-derived food/feed and its comparator(s).

9.1.3.1 Comparators

The first step in the comparative analysis is to select the appropriate comparator. The criteria in Regulation (EC) No 1829/2003 defines a conventional counterpart as 'a similar food or feed produced without the help of genetic modification techniques and for which there is a well-established history of safe use'. The selection of the comparator animals should consider genetic distance and it should be ensured that between-animal variation is representative of the genetic variability present in populations of traditionally bred animals of that species. When certain modifications may result in husbandry conditions that are appropriate for the modified animals but suboptimal for the conventional counterpart (for example cold-tolerant fish), then the conditions for rearing the genetically modified animal and its counterpart should be as closely representative of typical commercial practice as possible. Another example is rapidly growing genetically modified animals that reach maturity or marketable sizes earlier than their counterparts. In such cases, a counterpart with the same size or weight rather than the same age should be selected in order to represent an appropriate comparator.

9.1.3.2 Animal trials

The next step in animal trials for the comparative analysis is experimental design and statistical analysis. The guidance document provides some general recommendations for experimental design and suggests that the principles of experimental design should be followed from the ILAR Journal (ILAR, 2002). It also highlights the importance of keeping animals that are being compared under the same (conventional) conditions. On a case-by-case basis, it should be considered whether to include different husbandry practices in order to assess whether the effects of the genetic modification are influenced by such practices, and to assess the robustness of the genome-edited animals. There are recommended procedures for statistical analysis involving difference and equivalence tests (EFSA, 2010d). Consideration should be given to the possible need to analyse males and females separately, where appropriate.

9.1.3.3 Phenotypic characteristics, including health, physiological and welfare parameters

Given the broad scope of the guidance document, the description of how to assess phenotypic characteristics, including health, physical and welfare parameters is rather general. Phenotypic, unintended effects of the genetically modified animal may be changes in susceptibility to biotic and abiotic stresses, morphological, biochemical, physiological, developmental or reproductive changes or, on a case-by-case basis, through modified responses to husbandry and dietary regimes. Evaluation of the health and welfare status of the genetically modified animals may also give information about possible toxicity and bioactivity of the new substances. An evaluation of health involves the monitoring of an animal over the course of its commercial lifetime. Moreover, the guidance document gives examples of health evaluations that should be included and highlights the importance of comparing the measured parameters and their confidence interval (e.g. immune responses, biochemical and haematology values) with those obtained in conventional populations.

9.1.3.4 Comparative analysis of compositional characteristics

For the comparative analysis of compositional characteristics of food and feed from genetically modified animals, a table is given in the guidance with examples of materials to be used for the comparative analysis. The edible, unprocessed fraction of the animal should be used for analysis. For aquaculture (fish and molluscs), suggested tissues are fillet or residue body if used for fish meal production. Key measures should include macro- and micro-nutrients, as well as bioactive compounds (if identified as important, for example, hormones and growth factors), and key allergens (EFSA, 2010b). In very specific cases, there may also be anti-nutritional or toxic compounds that need to be included in the comparative compositional analysis. The comparative study on the level of common allergens may be performed in connection with specific allergenicity studies. The preparation of the test material and the analyses must be carried out according to appropriate quality standards.

9.1.3.5 Effect of processing

Almost all food/feed produced by animals will require some form of processing before consumption, such as fat from milk will require pasteurisation/sterilisation, or fermentation. Processed products may be assessed together with the unprocessed food or feed or a processed product may be assessed independently. The applicant should describe the processing technologies.

9.1.3.6 Application of EFSA guidance documents in comparative analysis of genome-edited animals

The first case (case 1) contains two examples of farmed Atlantic salmon developed with the SDN1 type approach by CRISPR/Cas9 (Datsomor et al., 2019a; Datsomor et al., 2019b). In both cases genes encoding enzymes involved in the production of polyunsaturated fatty acids (PUFA)

were edited, leading to altered fatty acid composition in both cases. Both papers describe the molecular procedure including the salmon feeding trial. The description in the guidance regarding how to assess phenotypic characteristics, including health, physical and welfare parameters, is general. Despite its general nature, it still provides enough information about the required details. When changing endogenous pathways of essential nutrients in farmed animals, it is important to have enough documentation of the animal welfare aspects. Based on the guidance that sets the criteria for the comparative analysis in the evaluation, changes in endogenous PUFA synthesis in Atlantic salmon using genome editing can be evaluated using EFSA guidance.

Case 2 is an example of sterile Atlantic salmon using CRISPR/Cas9-induced knockout causing a deletion of the gene *dead end* (*dnd*) which resulted in a completely sterile fish without germ cells (Wargelius et al., 2016). This study indicates the potential of production of sterile salmon which would create new lines of fish that cannot interbreed with wild salmon. The guidance that sets the criteria for the comparative analysis in the evaluation can be used to evaluate sterile Atlantic salmon produced by genome editing.

Case 3 is a channel catfish developed with CRISPR/Cas9 (Khalil et al., 2017). Knockout of the myostatin gene (*mstn1*), which normally suppresses muscle growth, enhances growth of the fish. The description in the guidance document regarding how to assess phenotypic characteristics, including health, physical and welfare parameters, gives enough information about the required details. Based on the guidance document that sets the criteria for the comparative analysis in the evaluation, changes in muscle growth in channel catfish using gene editin, can be evaluated using the guidance document.

In case 4, cattle have been genome-edited with the purpose of producing hornless dairy cattle to avoid injuries to other animals or humans as well as painful and costly dehorning procedures. In this study, a TALEN procedure was used to insert a 212 bp duplication into bovine embryo fibroblasts, imitating a polled allele naturally found in cattle of Celtic origin. These cell lines were cloned by somatic cell nuclear transfer and full-term pregnancies were established to produce two calves with the desired trait (Carlson et al., 2016). The guidance can be applied to assess animal health and welfare.

Case 5 is a pig developed by CRIPSR/Cas9 (Burkard et al., 2017; Burkard et al., 2018). Resistance towards porcine reproductive and respiratory syndrome (PRRS) was achieved by a deletion in the *CD163* gene. The virus causing the disease enters immune cells via the CD163-receptor to establish an infection. Animals carrying the modified CD163 receptors seem to be fully resistant to PRRS virus infection. The comparative assessment is applicable to evaluate animal health and welfare.

9.1.4 Conclusions regarding the comparative analysis

VKM considers that the guidance for comparative analysis of genetically modified animals can also be applied to genome-edited animals

The health status of a food/feed-producing animal is an important indicator of the safety of derived foods/feed. Therefore, comparative analysis of the phenotypic characteristics including health and physiological parameters of the modified animal with the traditionally bred animal is considered an important component in the risk assessment. All steps of the comparative analysis are relevant.

It is noted that there is currently no guidance document on compositional considerations for the safety of food and feed derived from genetically modified animals. This is in contrast to those available for the compositional assessment of key crop plants e.g. the OECD Consensus documents on compositional considerations of new varieties of plants, key food and feed nutrients, anti-nutrients, tocicants and allergens (OECD, 2002a; OECD, 2002b; OECD, 2011; OECD, 2012; OECD, 2019).

9.1.5 Toxicological assessment

The purpose of the toxicological assessment is to identify any adverse effects to humans and animals after consumption of the genetically modified animal. The toxicological risk assessment considers both potential effects of the intended as well as unintended modifications, identified in the molecular and comparative assessments.

The health of a food/feed producing animal has traditionally been used as an important indicator of the safety of derived foods. Therefore, the health and welfare status of the genetically modified animal should be carefully observed and compared in detail to the health and welfare status of closely related comparator(s). If the genetic modification has no negative impact on the genetically modified animal, this is a strong indication that consumption of the genetically modified animal-derived products will not have adverse effects on the health of the consumers. The principles of toxicological risk assessment of food and feed from genetically modified animals are described in the following sections. It is emphasised that, in general, toxicological testing of most genome-edited animal-derived food and feed will not be necessary.

9.1.5.1 Standardised guidance for toxicity tests

When toxicity testing is required based on the molecular and comparative assessment, the internationally agreed test methods described by the OECD or by the European Commission (EC, 2002) should be used. The toxicological tests to be performed depend on the type of genetically modified animal-derived food/feed, genetic modification, intended and unintended alterations, intended use and exposure/intake, and the available knowledge. This should be scientifically justified and documented.

9.1.5.2 Phenotypic comparison

Health and welfare of the genetically modified animal may reflect any potential adverse health effects of new proteins, other new constituents and/or changed levels of natural constituents. If there is no negative impact on the health of the genetically modified animal due to the

modification, this is a strong indication that consumption of the genetically modified animal-derived products will not have adverse health effects.

9.1.5.3 Assessment of newly expressed proteins

All new proteins should be assessed for potential toxicity, and the tests should be selected on a case-by-case basis. This depends on the health of the genetically modified animal, the protein source, function/activity and history of human/animal consumption of the protein. Specific toxicity testing may not be required if safe consumption of the genetically modified animal is properly documented. If testing is required, it is essential that the tested protein is derived from the genetically modified animal or with documented equivalence if expressed by microorganisms.

Molecular and biochemical characterisation of the newly expressed protein

Amino acid sequence, molecular weight, studies on post-translational modifications and a description of the function should be described. Temperature and pH range for optimum activity, substrate specificity and possible reaction products should also be provided and is especially relevant if the new protein is an enzyme. Possible interactions between the new protein and other animal constituents should be evaluated with respect to potential risks.

Bioinformatic search for homology to proteins known to cause adverse effects

Information should be available from up-to-date homology searches for known harmful proteins, e.g. searches in databases to identify if a novel protein has sequence homology to known toxins. A search for homology to non-toxic proteins exerting a normal metabolic or structural function may also be valuable information for the toxicity assessment.

Information on the stability of the newly expressed protein during processing and storage

Information should be available for relevant conditions affecting the stability of the new proteins during storage and processing, e.g. effects of temperature and changes in pH. Modifications to the proteins such as denaturation, production of stable protein fragments or other modifications should be characterised.

Resistance of the newly expressed protein to proteolytic enzymes

In vitro investigations on resistance to proteolytic enzymes (e.g. pepsin) of the new proteins should be performed. Stable breakdown products should be characterised and further evaluated with regard to potential biological importance and/or risks.

Repeated-dose 28-day oral toxicity study with the newly expressed proteins in rodents

Data from repeated dose toxicity studies with laboratory animals should be available to risk assessors unless other reliable information demonstrating the safety of the new proteins already exists. In this case, the information should encompass the mode of action of the proteins and demonstrate that the proteins are not structurally and functionally related to proteins with adverse effects to human or animal health. The repeated-dose 28-day oral toxicity study in rodents should be performed according to OECD guideline 407 (OECD, 2008). Depending on the outcome of the 28-day toxicity study, further targeted investigations may be required.

9.1.5.4 Assessment of new constituents other than proteins

Identification of new constituents other than the intended protein(s) should be assessed. These could be compounds with e.g. endocrine, pharmacological or immunological activity. In line with the safety assessment strategy of food and feed, the health status of the genetically modified animal expressing these compounds should be carefully monitored. Toxicological testing may be included on a case-by-case basis, taking into consideration the assessment of their toxic potential and presence in the processed food/feed from the genetically modified animal.

9.1.5.5 Assessment of altered levels of food and feed constituents

Alterations in the natural variation of food and feed constituents in the genetically modified animal-derived food and feed should be assessed. Such constituents could be e.g. macro- and micronutrients, anti-nutrients and bioactive animal metabolites. The outcome of the health status of the genetically modified animal producing these compounds determines if, and to what extent, toxicological tests are required.

9.1.5.6 Assessment of the whole food/feed derived from genetically modified animals

The risk assessment of the genetically modified animal-derived food/feed is based on molecular characterisation, comparative health status, compositional analysis and the toxicological evaluation of the intended and unintended effects. When these analyses indicate differences from the comparator or uncertainties, a toxicity study of the whole genetically modified food/feed can be performed. The 90-day rodent feeding trial with specific tissues and/or organs of the genetically modified animal may be considered to identify whether the genetically modified animal-derived food/feed is as safe as the conventional counterpart.

9.1.5.7 Design and performance of 90-day feeding study in rodents

The animal study should be performed according to the principles of OECD guideline 408. Special attention should be paid to the selection and spacing of doses and the avoidance of

problems of nutritional imbalance. The lowest dose level should be equivalent to the one consumed by humans or animals and the highest dose level should be the maximum achievable without causing nutritional imbalance.

Alternative studies to identify possible unintended effects may be comparative growth studies conducted with young, rapidly growing animal species (broiler chicks as an animal model for non-ruminants, lambs for ruminants or other rapidly growing species).

When the phenotypic and compositional comparison as well as the molecular characterisation have demonstrated no difference between the genetically modified animal-derived food/feed and their comparator, the performance of animal feeding trials is of little additional value and is therefore not recommended.

9.1.5.8 Application of EFSA guidance documents in toxicity assessment on genome-edited animals

Atlantic salmon with changes in endogenous PUFA synthesis (case 1) is edited with the intention to increase nutritional quality. Compositional differences from the comparator is therefore expected and the EFSA guidance for toxicity assessment can be applied. Salmon made sterile (case 2) and channel catfish with modified myostatin gene (case 3), as well as hornless cattle (case 4) and PRRS-resistant domestic pigs (case 5), are examples of genome-edited animals intended for easier breeding and husbandry. The guidance for risk assessment of genetically modified animals used in food and feed can also be applied in these cases with a focus on the health and welfare of the animal. When no new proteins are expressed and potential harmful unintended events or interactions are ruled out after assessment of molecular and compositional characteristics, animal health and welfare is the focus of the toxicological assessment.

9.1.6 Conclusions regarding the toxicological assessment

VKM considers that the guidance for toxicological risk assessment of genetically modified animals can also be applied to genome-edited animals. VKM agrees with the EFSA guidance stating that when no new proteins are expressed and molecular, compositional and nutritional assessment as well as animal health and welfare show no difference to the comparator, animal testing would not be required. This applies to genome-edited animals as well.

9.1.7 Allergenicity assessment

IgE-mediated food allergy has been the focus in the risk assessment of allergenicity of genetically modified animals. An integrated, case by-case approach (i.e. so-called weight-of-evidence approach) shall be used in the assessment of possible allergenicity of new proteins. To assess any possible increase in the risk of *de novo* sensitisation to the genetically modified animal-derived foods, post-market monitoring may be required.

9.1.7.1 Assessment of allergenicity of the newly expressed protein

Amino acid sequence homology comparisons between a new protein and known allergens to identify potential IgE cross-reactivity between the new protein and known allergens should be performed. This includes bioinformatics searches where the sequence of a new protein is compared to known allergens over 80-amino acid stretches (in a sliding window search). If a sequence homology of >35% (threshold value) is found, further analyses of potential allergenic properties of the new protein are triggered.

In vitro serum screening should be performed for IgE cross-reactivity to the new protein in serum from individuals sensitised to known allergens.

Pepsin resistance and *in vitro* digestibility tests should be performed to investigate possible alterations in stability to proteolytic enzymes. Proteins with high resistance to degradation are in general considered to be more allergenic, possibly by higher affinity and binding to IgE.

Additional tests may be included, such as *in vitro* cell-based assays or *in vivo* tests on animal models, to provide additional information on e.g. the potential of the new protein for *de novo* sensitisation. Validation of such tests for regulatory purposes, however, have not yet been performed.

9.1.7.2 Assessment of allergenicity of the tissues, organs and products from the genetically modified animal

Small changes in the amino acid sequence or post-translational modifications may affect the allergenic potential of known allergenic proteins. The allergen profiles, qualitatively and quantitatively, may vary between the breeds, and between individual animals, depending on the age/physiological status and environment. Interactions with other constituents within the animal-derived food product or processing (e.g. cooking) may also alter the allergenicity of the whole food in an unpredictable manner.

It is recommended that the same management measures as implemented for the non-genetically modified animal products be applied when the recipient of the genetic modification is an animal whose products are known to be common food allergens (e.g. milk, eggs, fish etc.). The same management would also be recommended when there are no indications of possible interaction(s) between the metabolic pathways involved in the expression of the recombinant protein compared to biosynthesis of endogenous allergenic proteins naturally present in the conventional animal tissues.

Post-market monitoring may be proposed to reveal any increase of the risk of *de novo* sensitisation to the genetically modified animal-derived foods.

If the purpose of the genetic modification is to reduce allergenicity of the animal products, evidence should be given using actual data obtained from experimental studies, including (human) clinical studies, to substantiate the claim. In addition, information on the prevalence of

occupational allergy (both food and respiratory allergy) in workers or in farmers who have significant exposure to genetically modified animals and derived products should be provided.

9.1.7.3 Adjuvanticity

Adjuvants are substances that may increase the immune response when administered together with an antigen. The adjuvanticity, and thereby the allergic response, may be affected by interactions with other constituents in the food or by the processing with alterations in structure and bioavailability.

9.1.7.4 Application of EFSA guidance in allergenicity assessment on genome-edited animals

VKM considers that the guidance can be applied also for allergenicity assessment in the cases described above.

For case 1, PUFA content is not associated with allergenicity, and animal testing is therefore not warranted if the results from the molecular and compositional assessments rule out other metabolic interactions due to possible harmful unintended effects.

For cases 2–5, where the modified traits are not affecting compositional and nutritional quality and other interactions are ruled out in the molecular and compositional assessments, animal testing of allergenicity is not considered relevant.

Since all cases, except case 5 (PRRS-resistant domestic pigs), include animals associated with food allergy, labelling for allergens of the derived food products should comply with current regulations. Evaluations of altered allergenicity according to the guidance should also be considered, but animal testing would not be indicated.

9.1.8 Conclusions regarding the allergenicity assessment

VKM concludes that the EFSA guidance for assessment of allergenicity can also be applied to genome-edited animals. When the modification is associated with alterations in allergenicity of the whole food, the allergenic potential of the genome-edited food should be further investigated. If the allergenicity and adjuvanticity are not different from the comparator, the same measures should be taken (i.e. labelling). A case-by case approach should be applied for the extent of the allergenicity investigations.

9.1.9 Nutritional assessment

The purpose of the nutritional assessment is to evaluate whether food and feed derived from a genetically modified animal are as nutritious to humans and/or animals as food and feed derived from traditionally bred animals. The nutritional assessment should therefore consider possible effects of the levels of nutrients and anti-nutrients, quality after transport and storage, and total dietary intake of food derived from the modified animal. If the genetically modified animal-derived food and feed have been assessed as not different from the composition of a comparator and the introduced trait(s) has no nutritional impact, the EFSA guidance states that no further studies to demonstrate nutritional equivalence are required.

9.1.9.1 Specific considerations for the nutritional assessment of genetically modified animal-derived food

The necessity to perform nutritional studies should be determined. If the compositional data provide sufficient information on the nutritional characteristics of the genetically modified animal-derived food and its composition has not been significantly altered, it may not be necessary to perform additional nutritional studies in animals. The EFSA guidance suggests that nutritional feeding studies are relevant only if unintended effects are indicated by a 90-day toxicological feeding study in rodents. Nutritional feeding studies should be conducted with young, rapidly growing animal species (broiler chicks as an animal model for non-ruminants, lambs for ruminants, or other rapidly growing species).

If there are questions related to the bioavailability of specific food components as a result of the genetic modification, the level of the nutrient in the food should be determined. The methods to test *in vitro* and/or *in vivo* for digestibility and/or bioavailability should be selected on a case-by-case basis depending on the food constituent.

9.1.9.2 Specific considerations for the nutritional assessment of genetically modified animal-derived feed

The necessity to perform nutritional studies should be determined. In the case of genetically modified animal-derived feed with intentionally or unintentionally altered nutritional characteristics, livestock feeding studies with target animal species should be considered, taking into account the current legal restrictions on the use of all processed animal protein (PAP) (EFSA, 2007a). Today the use of all processed animal protein (PAP) in feeds for farmed animals is banned throughout the EU with some exceptions (e.g. fish meal for non-ruminants) (EFSA, 2007a). In 2013, the use of non-ruminant and insect PAPs in fish feed was re-authorised through Regulation (EU) No 56/2013 (EC, 2013b). The exact experimental design and statistical approaches of feeding experiments in target animals (e.g. fish, chicken, pigs) to test the nutritional value of genetically modified animal-derived feeds will depend on a number of factors, including choice of animal species, type of animal trait(s) studied and the size of the expected effect.

9.1.9.3 Application of EFSA guidance documents in nutritional assessment on genome-edited animals

VKM considers that the EFSA guidance can also be applied for nutritional assessment in the cases described above. Case 1 is performed to induce expression of endogenous long-chain polyunsaturated fatty acids (PUFA) for improved nutritional quality of the fish meat. Analysis of PUFA in the genome-edited salmon in various target organs was performed to describe the desired trait. Case 2 is performed to produce sterile salmon to prevent crossing (genetic introgression) of escaped farmed salmon with wild populations.In case 3 with increased growth, histological analysis of channel catfish muscle was performed to identify the effect of the myostatin mutations, i.e. increased muscle size. The alterations for cases 4 and 5 consider animal health, welfare and husbandry in cattle and pig. None of these selected cases documented nutritional quality of food or feed products from the genome-edited animals.

9.1.10 Conclusions regarding the nutritional assessment

VKM concludes that the EFSA guidance is also applicable for nutritional assessments of genome-edited animals. If the genetically modified animal-derived food and feed have been assessed as not different in composition from a comparator and the introduced trait(s) have no nutritional impact, further studies to demonstrate nutritional equivalence are not indicated. However, if nutritional equivalence has not been established, a nutritional feeding study can be performed on a case-by-case basis. VKM considers that this also applies to genome-edited animals.

9.1.11 Exposure assessment

Information should be provided on the intended function, the dietary role and the expected level of intake of the genetically modified animal-derived food/feed product(s). If there are alterations in protein expression of other than the intended protein/s (due to alterations in metabolic pathways), this should also be determined from the total diet calculations. VKM has not identified properties with genome-edited animals, as exemplified by cases 1–5 that would exclude an exposure assessment.

9.1.12 Conclusions regarding the exposure assessment

VKM concludes that the EFSA guidance for exposure assessment can also be applied to genome-edited animals.

9.1.13 Risk assessment of animals with introduced non-heritable traits

The guidance defines genetically modified animals as animals whose genetic material has been altered in a heritable way through the techniques of genetic modification. In general, this guidance document is not applicable for the safety assessment of food and feed products derived from animals with introduced non-heritable traits, such as immunisation of animals with naked DNA to improve the health characteristics of the product, or animals expressing growth

hormone for productive improvement, although some aspects may also be applicable in these cases. The guidance document specifies that it may be necessary to analyse (many) more animals in the case of animals with new, non-heritable traits compared with animals with stably integrated heritable traits, in order to better assess the dynamic range of intended effects and possible unintended effects. In the case of animals with introduced non-heritable traits, the confirmation of the non-heritability in subsequent generations is important. None of the cases 1–5 have non-heritable traits such as genome-editing of only somatic cells.

9.1.14 Conclusion regarding risk assessment of animals with introduced non-heritable traits

VKM concludes that the EFSA guidance for assessment of non-heritable traits can also be applied to genome-edited animals.

9.1.15 Assessment of the potential risk associated with horizontal gene transfer

According to the guidance, any potential risk associated with horizontal gene transfer from the genetically modified animal and its products to humans, animals and microorganisms should be assessed. Furthermore, it should be ascertained whether the modification would be beneficial for the recipient (i.e. increased fitness), since such a trait would be associated with greater risk of gene transfer and potential spread. VKM considers that the limited modification introduced in the SDN1 and 2 category does not suggest a horizontal transfer potential beyond native genes. For the SDN3 category, a case-by-case assessment is needed, as exemplified by case 4.

9.1.16 Conclusions regarding the potential risk associated with horizontal gene transfer

VKM concludes that the EFSA guidance for assessment of horizontal gene transfer can also be applied to genome-edited animals.

The potential risk associated with horizontal gene transfer is considered on a case-by-case basis. The transfer potential will depend on the genetic modification, usage levels and factors determining host range and fitness effects in the new host.

9.1.17 Assessment of animal health and welfare

The EFSA guidance outlines relevant questions regarding the extent to which introduced genetic modifications affect an animal's health and welfare and whether changes might lead to other requirements for housing, nutrition or management. The health and welfare of genetically modified animals should be tested under different farming conditions, since they naturally will be exposed to a range of different, and sometimes novel, stimuli and stressors relating to climatic conditions, housing, husbandry, nutrition and other management conditions, as well as

infectious agents. Testing is conducted through a tiered approach going from the laboratory level to the field stage.

9.1.17.1 Application of EFSA guidance documents in assessment of animal health and welfare of genome-edited animals

The EFSA guidance addresses the scientific requirements for the assessment of health and welfare of genetically modified animals bred for food and feed use. The assessment is made in terms of the effective functioning of their body systems in a given environment, and all parts of the guidance are considered relevant for genome-edited organisms as well.

Genome editing has a broad range of potential applications in animals, including making livestock better adapted to farming or environmental conditions, increasing disease resistance, improving growth, fertility and enhancing animal welfare. Through the cases considered, animal welfare may be affected, e.g. dehorned cattle (case 4), or growth-improved catfish (case 3).

9.1.18 Conclusions regarding the assessment of animal welfare

VKM concludes that the EFSA guidance on animal health and welfare is also applicable to animals altered with genome-editing techniques, as exemplified by cases 1–5. Evaluation of animal health and welfare is equally important regardless of whether the animal is genetically modified or genome-edited.

9.1.19 Post-market monitoring

Post-market monitoring includes general health and welfare surveillance of genetically modified animals, as well as changes of the overall human dietary intake patterns of the modified product, or other possible side effects. Monitoring of genetically modified animals can reveal subtle effects on animal health and welfare in a longer perspective (e.g. reproductivity). If the initial risk assessment identifies areas of uncertainty, post-market monitoring could be useful to detect potential toxic effects or food allergies. VKM considers that the same approach can be applied for genome-edited animals and determined on a case-by-case basis. Post-market monitoring is dependent on the traceability of the product.

9.1.20 Conclusions regarding post-market monitoring

VKM concludes that the EFSA guidance on post-market monitoring is also applicable to genome-edited animals.

9.2 Application of the EFSA guidance for environmental risk assessment of genetically modified animals in risk assessment of genome-edited animals

According to the EFSA guidance, environmental risk assessment (ERA) of genetically modified animals should include information on potential effects on the receiving environment. The ERA should describe:

- Cross-cutting considerations
- Experimental design and statistics
- Long-term effects
- Aspects of genetically modified animal health and welfare
- Post-market environmental monitoring

Specific areas of risk for the ERA of genetically modified fish

- Persistence and invasiveness of genetically modified fish and vertical gene transfer to wild and feral relatives
- Horizontal gene transfer
- Impacts of genetically modified fish on biotic components and processes in the ecosystem
- Fish pathogens, infections and diseases
- Interactions of genetically modified fish with the abiotic environment
- Environmental impacts of the specific techniques used for the management of genetically modified fish
- Impacts of genetically modified fish on human health

Specific areas of risk for the ERA of genetically modified mammals and birds

- Persistence and invasiveness of genetically modified mammals and birds and vertical gene transfer to wild and feral relatives
- Horizontal gene transfer
- Pathogens, infections and diseases
- Interactions of genetically modified mammals and birds with target organisms (TO)
- Interactions of genetically modified mammals and birds with non-target organisms (NTOs)
- Interactions of genetically modified mammals and birds with the abiotic environment
- Environmental impacts of the specific techniques used for the management of genetically modified mammals and birds
- Impacts of genetically modified mammals and birds on non-genetically modified animal's health and welfare
- Impacts of genetically modified mammals and birds on human health

The following sections (9.2.1–9.2.8) contain key considerations from the EFSA guidance supporting overall environmental risk assessment of genetically modified animals (EFSA, 2013). The introduction of each topic of the ERA is a synopsis of EFSAs guidance text, not a VKM opinion as such. For each main section, a VKM evaluation of the applicability of the EFSA guidance to the risk assessment of genome-edited animals is provided using the animal case examples 1 to 5 in Box 7.

It should be noted that intrinsic properties, such as growth, survivability, and speed of reproduction of a genetically modified or genome-edited animal are very important for the environmental risk assessment. There are several factors to be considered, e.g. if the animal has relatives already present in the environment where it will be introduced (cross-breeding potential), or has high or low climate adaptability. While the health risk assessment is concerned with new potential risks due to altered nutritional composition, newly introduced components themselves, e.g. a novel protein, and animal welfare, the environmental risk assessment is concerned with potential risks associated with the introduced trait(s) and whether it may have an effect on survivability, fitness, fecundity and potential spread of the animal, with implications for the ecosystem and biodiversity.

9.2.1 Cross-cutting considerations

These are fundamental considerations that permeate the individual parts of an environmental risk assessment and constitute key information that risk assessors require in order to perform a sound risk assessment.

In the case of genetically modified animals, the EFSA guidance highlights the following cross-cutting considerations:

9.2.1.1 Receiving environment(s)

The guidance document provides details on how to perform the ERA and information relating to the conditions of placement or release of a modified organism on the market, the receiving environments and the interactions between a modified organism and the environment. Commission Decision 2002/623/EC (EC, 2002) provides further details related to potential receiving environments.

9.2.1.2 Experimental environment

ERAs of animals are generally more complex than for plants. Animals exhibit more complex behaviour than plants; the mobility of an animal will generally exceed that of a plant, and animals represent higher trophic levels.

ERA questions related to invasiveness and persistence may draw on the theoretical framework regarding alien (non-modified) species, in addition to historical uses of conventional counterparts and data from mandatory field studies.

9.2.1.3 Choice of comparators

The non-modified organism from which the genetically modified animal is derived is termed the 'conventional counterpart'. Interactions between the genetically modified animal and the environment, and changes in management can be estimated in relation to the conventional counterpart. The selection of appropriate comparator may be aided by considering genetic distance between the genetically modified and non-modified animal.

9.2.1.4 Choice of comparators for ERA of genetically modified fish

The ERA should compare the genetically modified fish to (1) its non-modified source progenitor line, (2) populations of wild fish of the same species from locations into which the modified fish will be released, (3) populations of wild fish species exploiting a similar ecological niche as the genetically modified fish in accessible ecosystems and (4) aquaculture lines of the genetically modified fish species.

9.2.1.5 The use of non-genetically modified surrogates

Alternative methods may be used to collect data informative for the ERA, particularly in cases where risk assessment discourages release of genetically modified animals to the natural environment. One solution is to gather data from experiments on genetically modified animals performed in confined and controlled conditions. But this approach is limited by how closely natural conditions can be mimicked. The complexity of factors interacting with the animal in the environment may therefore be better informed by experiments on non-modified surrogates.

9.2.1.6 Experimental design and statistics

General principles

Effect studies rely on data collected from experiments in which hypotheses are tested to ascertain whether there are adverse environmental effects due to the genetically modified animal when compared with its comparator(s) and to measure their magnitude.

9.2.1.7 Long-term effects

The objective of the ERA is to identify and evaluate, on a case-by-case basis, potential adverse effects of the genetically modified animal and its offspring (including their waste products) on human and animal health and the environment. Effects may be direct or indirect, immediate or delayed, including cumulative long-term effects, and may also include those associated with the interactions with other genetically modified organisms.

An important part of the ERA is predicting and assessing (adverse) long-term effects. This requires information about the genetically modified animal, its intended uses and the receiving environments, in terms of both the baseline conditions in the receiving environments and

temporal changes in these conditions, independently of the genetically modified animal, and following introduction of the genetically modified animal. Long-term effects of the genetically modified animal should not be considered in isolation but compared with the long-term effects of its conventional counterpart or another appropriate comparator if present in the receiving environments. If no appropriate comparator is present in the receiving environments, long-term effects should be compared between the presence and absence of the genetically modified animal.

Categories of long-term effects

Long-term effects result from a diversity of primary causes and secondary interactions. Long-term effects may differ from a genetically modified animal's effects, before its placement on the market, for several reasons, which may be classified into two categories (EFSA, 2013):

Category I: Long-term exposure to a genetically modified animal or management practice may result in a delayed response by organisms. An example of such a long-term effect is the development of resistance in the pest-target organism.

Category II: Long-term effects may also occur due to increases in spatial and temporal complexity. Before placement on the market, limited spatial and temporal scales can be empirically tested, whereas the complexity of interactions of genetically modified animals with other species increases with spatial scale.

9.2.1.8 Further guidance on modelling

The guidance advises that a number of parameters and considerations should be addressed to make predictions based on mathematical modelling in an ERA, i.e., when experimental data is used for extrapolation to real world conditions. These include parameter estimation, i.e. the importance of assessing intrinsic variations in wild or conventionally bred populations when comparing these to the genetically modified variant. The guidance also emphasises the need for verification of models and algorithms used, validation of models with real data, sensitivity analysis to account for known uncertainty and variability in all parameter estimates, and identification and evaluation of unquantified uncertainties and their potential impact on the assessment.

9.2.1.9 Uncertainty analysis

The guidance defines several types of uncertainties, their potential impacts on the outcome of an assessment and provides advice on how these can be identified, categorised, quantified and addressed in order to reduce their effects..

9.2.1.10 Aspects of genetically modified animal health and welfare

Applicants shall ensure that minimum health and welfare requirements are met during the different stages of placement on the market of genetically modified animals, i.e. production, transport and release into the environment, as detailed in the guidance. Comparison with the non-modified line is proposed when assessing if the genetically modified animals will have altered management requirements (pertaining to resources such as e.g. space, nutrition or temperature). If no appropriate comparator exists, the health and welfare of the genetically modified animal itself need to be considered.

Health and welfare aspects of genetically modified mammals and birds

A comparison with the non-genetically modified line has been proposed for animals reared for food or feed uses (EFSA, 2012a). For animals with increased growth rate, the demands for nutrients, water and space may be altered, requiring changes in nutritional and spatial management practices. Overcrowding will enhance the risk of disease transmission. The specific management requirements of the genetically modified animal should be considered, or at least not inadvertently jeopardised, with regard to health and welfare.

Health and welfare aspects of genetically modified fish

The health and welfare aspects of genetically modified fish released into the environment range from confined aquaculture facilities to a confined or un-confined aquatic environment (e.g. stream, river, ocean). The same principles as laid down in the EFSA guidance on the risk assessment of food and feed from genetically modified animals including animal health and welfare aspects (EFSA, 2012a) also apply for the assessment of health and welfare of genetically modified fish released into the environment during different developmental stages, different production stages and for different receiving environments.

9.2.1.11 Applicability of the EFSA guidance regarding cross-cutting considerations, in risk assessment of genome-edited animals

VKM considers the guidance applicable for cross-cutting considerations in the ERA of genome-edited animals. The extent of information required is dependent of the trait(s) introduced, and to which species/environment. As underlined by the guidance, the ERA of an animal may be complex. In the ERA of animals, the choice of comparator may be especially challenging.

Cases 1 and 2 represent genetic changes in the SDN1 category, leading to altered fatty acid composition and sterility, respectively. In the fish farming environment, a conventional comparator would be a closely related farmed salmon without the genome edit.

For case 1, the altered fatty acid composition is intended as a health benefit for the consumer. However, lack of these enzymes may impact the health of the fish. More information on long-term health and welfare effects would therefore be required. The environmental effects to be

considered upon escape include both ecological effects (i.e. competition, predation or disease transmission) and genetic effects upon introgression into wild populations of Atlantic salmon (see Figure 11) as well as interspecific hybridisation with brown trout, *Salmo trutta*. Interspecific hybridisation between Atlantic salmon and brown trout is a rare phenomenon in most populations (at less than 1%; (Hindar and Balstad, 1994)), but may be high in rivers with reduced populations (by diseases, habitat modification) and escaped farmed salmon.

For case 2, the intended use is to protect the wild populations from genetic introgression of farmed Atlantic salmon. Atlantic salmon are cultivated in large numbers in open sea cages. Escape accidents are common, and escapees enter rivers where they interact with wild Atlantic salmon during spawning (Box 10). The use of genetic sterility can potentially mitigate this problem. In case 2, the lack of germ cells may require information on health and welfare aspects (Kleppe et al., 2017). Environmental effects would be limited to ecological effects for 100% sterile fish upon escape into the wild (Figure 11). Risks of introgression in wild Atlantic salmon and interspecific hybridisation with brown trout must be considered if sterility is not 100%.

Case 3 is an exotic species for Norway, the channel catfish *Ictalurus punctatus*, which is edited for improved muscle growth in the myostatin gene. In this case, the farmed species (if already in use locally) is a comparator for a large part of the ERA. In case of accidental release (escaped fish) in Norway, this fish would represent a novel species to the environment and long-term effects on the ecosystem would need to be considered if feral populations were established (Figure 11). The species has no known wild relatives in Norway.

Increased growth is a desired trait in animal production. Enhanced growth would make the genome-edited fish an 'ecological novelty' (Tiedje et al., 1989) with potential for altered fitness and competitive ability, as well as a potential for changes at the ecosystem level (Cucherousset et al., 2021). The increased muscle growth would also require surveillance regarding health and welfare.

Case 4 is hornless cattle developed by TALEN (SDN3 category). Potential issues with hornless cattle would be mostly related to health and welfare of the animals. Hornlessness occurs naturally in some breeds of cattle. The animals would be semi-contained and have no wild relatives in Norway. The environmental risk would be similar to that of non-edited cattle.

Case 5 is a virus-resistant pig developed by the CRISPR/Cas (SDN1 category). This case would require long-term assessment regarding health effects, animal management and possible pathogen resistance. If escapes occur, the pigs may also interbreed with wild boars that are growing in population size in Norway (VKM, 2018c). Appraising environmental effects of the introduced trait when considering interactions and introgression between the two subspecies may therefore be relevant for the ERA.

VKM considers that the guidance can be applied for assessing long-term effects of genome-edited animals.

The guidance emphasises the importance of having enough documentation of the animal's health and welfare aspects. The same will apply for genome-edited animals and the phenotypic changes can be evaluated on a case-by-case basis.

9.2.2 Conclusions regarding cross-cutting considerations

VKM concludes that the guidance can also be applied for cross-cutting considerations in the risk assessment of genome-edited animals. The risk assessment may use a staged approach, which suggests that the different end points of the ERA may be the target population: wild-type relatives of the target organism, related species or the entire ecosystem. The staged (step-by-step) procedure as well as a case-by-case approach will define the specific types of information and considerations needed for the ERA.

9.2.3 Specific areas of risk to be addressed in the environmental risk assessment (ERA) of genetically modified fish

Genetically modified fish to be placed on the market may be for food/feed production or non-food/feed uses (i.e. 'ornamental' fish). The ERA must also consider accidental release of the genetically modified fish into the environment and their environmental impacts.

9.2.3.1 Persistence and invasiveness of genetically modified fish, including vertical gene transfer

The fitness consequences of a genetically modified fish breeding in the wild are of two main types:

- Enhanced fitness of the reproducing genetically modified fish may create feral modified populations, or hybrid or backcrossed populations in different habitats, which may change the diversity and/or abundance of flora and fauna. For instance, native fish species may be displaced by genetically modified fish, which in turn might affect food chains and ecosystem processes.
- Decreased fitness of hybrid or backcrossed descendants may cause decline or local extinction of wild fish populations. This includes both intraspecific and interspecific hybridisation.

9.2.3.2 Horizontal gene transfer

Horizontal gene transfer (HGT) is defined as the process in which an organism incorporates genetic material from another organism into its genome without being the offspring of that organism. The evaluation of the impact of HGT from genetically modified fish includes analysis of the potential for exposure and transfer of transgenes and further horizontal dissemination to other organisms.

9.2.3.3 Impacts of genetically modified fish on biotic components and processes

A main issue with genetically modified fish is to determine whether they have different biotic interactions in the environment, compared with appropriate comparators. Biotic interactions include target and non-target impacts. A target organism (TO) is one with which the genetically modified fish is designed to interact. Examples are organisms which are intended to be displaced or consumed by the genetically modified fish (e.g. for control of aquatic weeds). All other organisms that might interact with and be affected by the genetically modified fish would be considered as non-target organisms (NTOs).

9.2.3.4 Fish pathogens, infections and diseases

Infectious diseases are among the major obstacles in aquaculture, causing losses in productivity or mortality and poor animal welfare. The high stocking densities at which fish are normally kept in the production facilities enhance transmission of infections. Specific infectious diseases can have considerable environmental and economic consequences such as reduction of wild populations, loss of production, impact on public health or trade restrictions. Resistance or tolerance to disease is therefore a desired trait in the development of genetically modified fish.

Fish can be genetically modified with the primary goal of making them disease resistant or tolerant (direct effects), either to a specific disease or to many diseases . Fish may also be genetically modified to express other traits which may change their susceptibility to infectious diseases more indirectly.

9.2.3.5 Interactions of genetically modified fish with the abiotic environment

Two aspects of abiotic interactions are relevant for the ERA of genetically modified fish:

- The genetically modified fish may have altered tolerance to abiotic factors. This can be either the desired consequence of the genetic modification or a pleiotropic consequence of it.
- The genetically modified fish may affect the abiotic environment in a different way from non-modified fish, for example by altering the digging behavior of females. This aspect can be divided into direct effects of the genetically modified fish (for example, reduced digging behavior of females) and indirect effects cascading from the direct effects (for example, overgrowth), which may act either on abiotic factors or on biotic components.

9.2.3.6 Environmental impacts of the specific techniques used for the management of genetically modified fish

Genetically modified fish must be adapted to changes in the production systems used for their management, rearing and production. According to the guidance, the environmental impacts of the specific management practices associated with the genetically modified fish compared with non-modified fish should be assessed. The characteristics of the genetically modified fish may

differ from those of the non-modified comparator, and the management of the confinement measures, welfare, health and feeding regimes of the genetically modified fish may be altered and/or adapted to particular locations. If genetically modified fish are adapted to different environmental conditions (e.g. lower temperature), production units (e.g. confined aquaculture facilities) could be located in novel locations and have different impacts. An important aspect of the management of the confined aquaculture facilities is to prevent the accidental escape of the genetically modified fish and so the impacts of changes to confinement measures of the facilities should be considered, including the breeding, rearing, production and any transport between them.

9.2.3.7 Impacts of genetically modified fish on human health

The guidance considers primarily effects of genetically modified fish on human health through routes of exposure other than ingestion or intake; these include ocular and nasal as well as exposure through dermal contact and inhalation. However, the likelihood of oral exposure of humans to genetically modified animals or their products which are not intended for food or feed uses should be assessed. If such exposure is likely and ingestion or intake will occur at levels which could potentially place humans at risk, the assessment procedures described in the EFSA Guidance Document on the risk assessment of food and feed from genetically modified animals and on animal health and welfare aspects (EFSA, 2012a) should be implemented. Examples of new hazards that should be considered are human infections caused by pathogens transmitted from fish or the aquatic environment, changes in phenotype (longer spines, sharper teeth) that can increase hazards to human health and changes in specific management practices for genetically modified fish (e.g. increased use of antibiotics driving the development of potential resistance in human pathogenic bacteria).

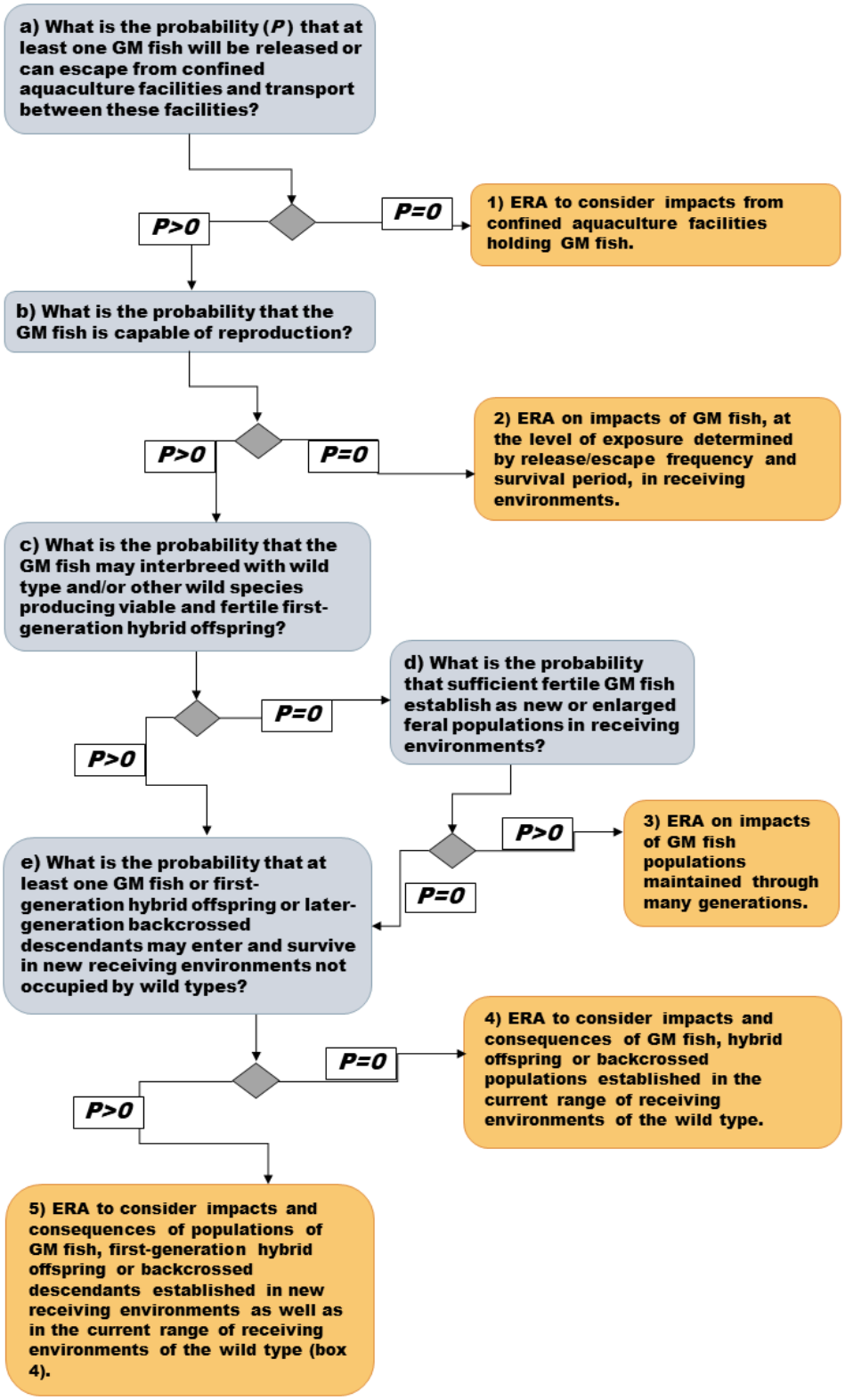

Figure 11. Staged approach for risk assessment of genetically modified fish (adopted from (EFSA, 2013).

9.2.3.8 Applicability of the EFSA guidance regarding the specific areas of risk for the ERA of genetically modified fish in risk assessment of genome-edited fish

VKM considers the guidance to be adequate for the assessments of genome-edited animals such as those listed in cases 1-3, particularly because extensive literature exists on risk assessment of fishes, including invasive fishes (Moyle, 1999), an OECD Consensus Document on the Biology of Atlantic salmon (OECD, 2017), an ICES working group report (ICES, 2016), farmed escaped Atlantic salmon (Glover et al., 2020), marine aquaculture (Waples et al., 2016) and genetically modified fishes (Devlin et al., 2015; Devlin et al., 2006; Kapuscinski et al., 2007).This literature is often but not always salmon-oriented and considers other fishes targeted for genome editing such as channel catfish, tilapia (*Oreochromis niloticus*) and European sea bass (*Dicentrarchus labrax*).

Vertical gene transfer (VGT) by sexual reproduction is the normal route for gene transfer in fishes, and gene transfer must be considered both within a species and among related species such as between Atlantic salmon and brown trout *Salmo trutta* (Hindar and Balstad, 1994). Horizontal gene transfer is a sparsely documented phenomenon. VKM regards the guidance as adequate and appropriate coverage of this topic and recommends that it be considered on a case-by-case basis.

VKM considers interactions between the modified animal and the biotic components and processes in its environment to be the most complex and least studied type of interactions that should be contemplated in the ERA. This is also an area of ecological and ecosystemic inquiry at a more advanced level today than what was the case when the guidance document was developed. It has long been known that fish can be controlling food webs in rivers (Power, 1990) and lakes (Carpenter et al., 1993), but the fact that intraspecific variation within fish species may have consequences for ecosystem dynamics and processes has just begun to be explored (Des Roches et al., 2018; Raffard et al., 2019). The guidance document encourages this to be studied, but the recent focus on biodiversity and ecosystem services, for example by the IPBES report (IPBES, 2019), suggests that the ERA should be encouraged to include a more complex variety of outcomes, including modified fish species. For example, in a meta-analysis of releases of salmonid species and populations, Buoro et al. found stronger ecological effects from releases of native species (but non-native at the population level) than from releases of non-native species (Buoro et al., 2016). Experiments with Atlantic salmon have found that enhanced growth capacity of juvenile salmon may lead to changes in stream ecosystem functioning (Cucherousset et al., 2021).

Knowledge about disease agents in cultured fishes is rapidly increasing, whereas it is still limited for wild fishes. Aquaculture production in freshwater tanks and seawater net pens makes transmission of disease agents between farmed and wild populations an area of central importance for risk assessment. This relates to both exotic disease agents accompanying transport of fish, like *Gyrodactylus salaris* in Norway, and for endemic pathogens and parasites, like sea louse *Lepeophtheirus salmonis* (ICES, 2016). VKM considers the guidance document to be applicable for ERA of fish pathogens, infections and diseases.

VKM considers that the guidance is adequate with respect to assessing aspects of abiotic environmental interactions that are relevant for the ERA of genome-edited fish. The fish taxa considered in cases 1–3 are among the best studied fish species, and environmental tolerance is relatively well substantiated for several abiotic variables, e.g. rainbow trout is among the "lab" species for which LD_{50} is commonly studied for chemical substances in aquatic environments (Rand and Petrocelli, 1985).

Atlantic salmon and channel catfish are among the fish species that have been introduced to many countries outside their range (MacCrimmon and Gots, 1979). The channel catfish is probably the more invasive of the two. Atlantic salmon are considered less invasive than two relatives, the brown trout (*Salmo trutta*) and the rainbow trout (*Oncorhynchus mykiss*), which are both on the list of the World's 100 Worst Invasives (Lowe et al., 2000). The management techniques associated with both conventional aquaculture and genetically modified fish have been subject to considerable debate. This is reflected in the EFSA guidance that considers a variety of topics and procedures.

As net-pen based aquaculture is associated with spread of disease agents and escapes of farmed fish, several techniques have been contemplated for making production confined. These techniques include physical containment on land (with control of in- and outflowing water), introducing sterility, or other traits that unable the fish to survive outside the culture environment. Triploidy as a sterilisation technique is being used on a large scale in aquaculture and requires additional considerations related to feed, rearing protocols and fish health (Stien et al., 2019). The triploidization itself is technically simple even on a large scale. Other types of sterility as case 2 described here may require other management techniques for application on a large scale.

VKM considers that the guidance can be fully or partly applied to the assessment of human health in a case-by-case approach. Cases 1–3 all represent loss-of-function edits, and therefore, some of the studies/data suggested in the guidance would not be relevant to complete the assessment of human health in these cases.

9.2.4 Conclusions regarding the specific areas of risk for the ERA of genetically modified fish in risk assessment of genome-edited fish

VKM concludes that the EFSA guidance can be applied to the ERA of genome-edited fish. The extent of studies and data provided in the application needs to be determined on a case-by-case basis.

9.2.5 Specific areas of risk to be addressed in the ERA of mammals and birds

9.2.5.1 Persistence and invasiveness of genetically modified mammals and birds, and VGT (vertical gene transfer) to wild and feral relatives

Confined, semi-confined and non-confined genetically modified mammals and birds are associated with differences in environmental effects. Confined animals include domesticated animals kept indoors or in fenced-in areas. Semi-confined animals are those that are intended to be under human control, but that sometimes browse freely (e.g. cattle). Non-confined animals are released directly into specific environments (e.g. sterile rabbits released to control wild rabbit populations in Australia).

The ERA differs for these three groups of organisms, as they will be found in different receiving environments. Confined animals will primarily impact their confined environment but can have effects in the wild if they escape. Semi-confined animals will impact both their confined environment and the wild.

Both confined and semi-confined animals will have effects on organisms that are able to move in and out of the area where the genetically modified animal is being held. The escape risk into the wild will differ among species and thus require different risk management strategies. They can also affect the abiotic environment.

The ERA should therefore consider the location in which the species is housed and also all proximal environments (both undisturbed and disturbed) to which the species could escape and potentially relocate. It should also consider all aspects of housing, transport, storage, handling and processing.

An evaluation of the genetically modified organisms' persistence, invasiveness and potential for hybridisation with wild relatives should be performed.

If the genetically modified organism can hybridise with reproductively compatible relatives to produce viable and fertile offspring, potential changes (e.g. in fitness, diet and/or habitat use) caused by the genetic modification need to be assessed.

The main sources of data are expected to be literature sources, modelling where applicable, and any experiments conducted during the development of the genetically modified animal. All comparative data must be accompanied with suitable quality assurance and a full explanation of the methodology used. EFSA provides detailed guidelines for quantifying natural variability in the non-modified organisms that should be considered when compared with genetically modified organisms(EFSA, 2010d). Even though experimental field trials will be mostly relevant for plants, some of the principles proposed for statistical comparison of multiple variables could also be applicable for ERAs of animals.

If data are collected from outside the EU, it should be justified why these data are relevant for the range of potential receiving environments in the EU.

9.2.5.2 Vertical and horizontal gene transfer

In mammals and birds, the most common form of vertical gene transfer (VGT) is sexual reproduction. VGT from inbred lines could possibly lead to loss in genetic diversity, and according to EFSA, the ERA should assess the full range of outcomes due to the introduction of a genetically modified mammal or bird into the environment. The exposure assessment should focus attention on a worst-case scenario.

Horizontal gene transfer (HGT) from genetically modified mammals and birds is expected to be rare but may have consequences for human and animal health and the environment and is therefore considered in the ERA on a case-by-case basis. Both multicellular eukaryotes and microorganisms should be considered as potential recipients.

9.2.5.3 Pathogens, infections and diseases

According to the EFSA guidance, genetically modified mammals and birds may pose a potential risk to the environment if they are either infection/disease-resistant or have changed susceptibility to infection due to altered traits pertaining to growth, productivity or reproduction. The ERA should compare the genetically modified animal with its conventional counterpart, where possible, or with other non-genetically modified comparators, under all receiving environmental conditions.

Two hazards to consider are that disease-tolerant, genetically modified animals might be silent carriers of pathogens and transmit disease to non-genetically modified animals. Moreover, resistance in genetically modified animals may cause selection for pathogen strains with increased virulence.

9.2.5.4 Interactions of genetically modified mammals and birds with target organisms (TO)

Animals may be modified to enable them to increase resistance or tolerance to pathogens or pests. These are then the TOs, or target organisms; (in the case of genome-edited sterile rabbits, the TOs are wild rabbits). The assessment of the environmental effects of management of the target organism by the genetically modified animal should always include the indirect effects of such management on non-target organisms (NTOs). For disease resistant genetically modified animals, the potential loss of efficacy of the characteristics of the animal in its interactions with the TO is a concern. The risk under the worst-case scenario for adverse environmental effects should be evaluated.

9.2.5.5 Interactions of genetically modified mammals and birds with non-target organisms NTOs

Non-target organisms (NTOs) are all species, except the target organism, that are directly and/or indirectly exposed to the genetically modified animal. The ERA should consider potential

immediate and/or delayed environmental impact of the direct and indirect interactions between the animal and NTOs, including the impacts on populations of competitors, prey, hosts, symbionts, predators, parasites and pathogens.

The potential hazards will differ between receiving environments depending on the presence of a conventional counterpart or an ecologically similar comparator species to the genetically modified mammal or bird.

The environmental impact will also differ between confined, semi-confined and non-confined genetically modified animals.

Knowledge gaps and scientific uncertainties are especially relevant for the ERA pertaining to NTOs since identifying all possible direct and indirect interactions between genetically modified animals and NTOs will not be achievable. Furthermore, applicants should discuss uncertainties and propose appropriate risk management strategies for each risk identified.

9.2.5.6 Interactions of genetically modified mammals and birds with the abiotic environment

Assessment of whether genetically modified animals and their associated management practices have potential adverse effects on the abiotic aspects of the environment should be compared with the effects of the non-genetically modified comparator and its current management systems. In all cases, the choice of comparator needs to be considered carefully and justified explicitly. Both the immediate receiving environments and the wider environment, comprising land, water and air should be considered.

9.2.5.7 Environmental impacts of the specific techniques used for the management of genetically modified mammals and birds

According to EFSA, ERA is required when existing breeding, rearing and production systems are changed for genetically modified animals with traits that adapt them to particular environments (e.g. altered dietary range). It should be evaluated whether the changes will lead to greater, similar or lower adverse environmental effects (including disposal of the animal, products derived from the animal and waste products from the production sites).

9.2.5.8 Impacts of genetically modified mammals and birds on non-genetically modified animal health and welfare

EFSA requires a case-by-case assessment of the overall risk of non-genetically modified animal health and welfare. EFSA describes various approaches to compare the health and welfare of genetically modified animals with their respective comparators (EFSA, 2012a).

9.2.5.9 Impacts of genetically modified mammals and birds on human health

It should be considered whether the modified mammal or bird presents a new hazard for human health than their non-genetically modified comparators. Zoonoses can be naturally transmissible from vertebrate animals to humans, and vice versa, and may cause emerging diseases. Thus, both immediate and delayed effects on human health resulting from potential direct and indirect interactions with genetically modified mammals and birds should be evaluated. Potential pathogenic and non-pathogenic impacts on human health through the deliberate or accidental release of genetically modified mammals and birds need to be considered.

9.2.5.10 Applicability of the EFSA guidance regarding the specific areas of risk for the ERA of genetically modified mammals and birds in risk assessment of genome-edited mammals and birds

The guidance provides a detailed approach on how to directly address the potential persistence or invasiveness of genetically modified mammals and birds in the receiving environment and their potential to hybridise with non-modified relatives. It is described how animals with different confinement levels will require different considerations in the ERA. The steps suggested for evaluation of the environmental impact of genetically modified animals will be adequate also for genome-edited mammals and birds.

Vertical gene transfer by sexual reproduction is the normal route for gene transfer in mammals and birds. Case 4, hornless cattle, would be an example of a semi-confined animal, but as cattle have no wild close relatives in Norway there is no hybridisation risk to consider. In North America, chromosomal segments from cattle are found in bison *(Bison bison*) populations (Halbert et al., 2005), but this is mainly resulting from past human mediated cross-breeding for meat production. The European bison (*Bison bonasus*) and cattle have also been intentionally interbred; however, no births of hybrids have been documented in the Białowieża Forest in Poland where the two species co-occur (Krasińska and Krasiński, 2013).

Case 5, PRRS-resistant pigs are more likely to be confined, but escape risk cannot be excluded. Domestic pig can hybridise and produce viable offspring with wild boar (*Sus scrofa*), a phenomenon occurring throughout Europe, also in Norway (Iacolina et al., 2018). In Norway, the wild boar is considered an invasive species and is rapidly increasing in numbers and distribution range (VKM, 2018c). Free-ranging domestic pigs of various breeds are also found in many locations in Norway, even though the contribution to the annual pork production is currently below 1% (Directorate of Agriculture, 2020). Nevertheless, the risk of encounters between escaped pigs and wild or another domestic boar can therefore not be excluded. The heritability of the edited trait (deletion) should be assessed.

The ERA approach for vertical and horizontal gene transfer suggested by EFSA for genetically modified animals will also be applicable for genome-edited mammals and birds.

Regarding pathogens, infections and diseases, the guidance for exposing the risk of disease transmission from genetically modified mammals and birds will also cover gene-edited mammals and birds. The genome-edited pigs in Case 5 have a deletion in the virus binding part of the CD163-receptor making them resistant to porcine reproductive and respiratory syndrome (PRRS). It should be determined whether these pigs can be silent carriers of the PRRS-virus and contribute to selection for pathogen strains with increased virulence.

Concerning the interactions between genetically modified mammals and birds and their target organisms, the guidance will also be applicable for gene-edited mammals and birds. For case 5, the target organism is the virus causing the porcine reproductive and respiratory syndrome. The potential for loss of efficacy of the interactions with its TO (the virus) needs to be assessed.

The EFSA procedure for ERA pertaining to the interactions between genetically modified mammals and birds and non-target organisms covers many aspects. Nevertheless, complete assessment will not be achievable due to the inherent complexity of ecosystems. Thus, as emphasised by the guidance, attention should be given to describing data gaps, uncertainties and mitigation measures.

As cattle (Case 4) are likely to be semi-confined they will interact with a range of non-target organisms. It is unlikely, however, that the modification (hornlessness) will change their interactions with the environment compared to regular cattle. The PRRS-resistant pigs (Case 5), on the other hand, are more likely to be contained, but have the potential of interbreeding with other domestic pigs and wild boar, and thereby spreading the PRRS resistance.

When it comes to interactions of genetically modified mammals and birds with the abiotic environment, the guidance for ERA of interactions of genetically modified mammals and birds with the abiotic environment will be adequate for genome-edited mammals or birds as well. For cases 4 and 5, the non-modified lineages of cattle and pigs could be used as comparators.

The guidance for assessing the environmental impacts of genetically modified animals for which the management practices are changed, will also apply for genome-edited mammals and birds.

As for genetically modified animals, a case-by-case conclusion for the overall risk regarding animal health and welfare should also be required for genome-edited mammals and birds.

Finally, the guidance to evaluate whether the modified mammal or bird presents a new hazard for human health compared to their non-modified comparators will also suffice for genome-edited mammals or birds.

9.2.6 Conclusions regarding the specific areas of risk for the ERA of genetically modified mammals and birds

VKM concludes that the guidance for assessment of specific areas of risk for the ERA of genetically modified mammals and birds can also be applied to genome-edited animals and birds. The information required for the ERA needs to be considered on a case-by-case basis.

9.2.7 Post-market environmental monitoring

An objective of Directive 2001/18/EC (EC, 2001) is to protect the environment, including natural resources and ecosystem services. The EFSA GMO Panel recognises that all human activities can have environmental impacts; thus, there is a general need to consider the impacts of any new product, development or process on environmental protection goals. Directive 2004/35/EC (EC, 2004a) defines environmental damage as a measurable adverse change in a natural resource or measurable impairment of a natural resource service. This directive expects environmental damage to be prevented or remedied.

9.2.7.1 Case-specific monitoring

Case-specific monitoring should be performed to confirm that any assumptions regarding the occurrence and impact of potential adverse effects of the GMO or its use in the ERA are correct.

9.2.7.2 General surveillance

General surveillance should be performed to detect the occurrence of adverse effects, unanticipated in the ERA, from the genetically modified organism or its use on human health or the environment.

9.2.7.3 Applicability of the EFSA guidance regarding post-market environmental monitoring of genetically modified animals in risk assessment of genome-edited animals

VKM considers the principles laid down in the guidance as adequate for the monitoring of potential adverse effects of genome-edited animals. The effects may have been anticipated in the ERA, in which case they should be detected in the case-specific monitoring (CSM), or they may not have been anticipated in the ERA, in which case they should be detected by the general surveillance (GS) according to the guidance document. Whereas the need for CSM depends on the conclusions of the ERA, GS is mandatory for any placement on the market of a genetically modified animal. The same principle is adequate for genome-edited animals.

Following the placement on the market of a genetically modified animal, applicants have a legal obligation to ensure that monitoring and reporting are carried out in accordance with the conditions specified in the consent. Through this monitoring, applicants can also relate to national monitoring programmes that may have a different focus and may be particularly useful in some of the cases considered here.

The five cases are quite different with respect to monitoring of environmental effects. Cases 1 and 2 represent Atlantic salmon, which is a valuable species for Norway, both as a domesticated farmed fish and as a native, wild species in coastal waters and rivers.

Genome editing of salmon would likely take as its starting point one of the commercial strains of farmed salmon (as in cases 1 and 2), in which case the opportunity for environmental

monitoring of the genome-edited type vs. the conventional farmed strain depends on the molecular and phenotypic difference between them.

Case 3, channel catfish, is a North American freshwater fish in the order Siluriformes, and is an exotic species for Norway with one relative (brown bullhead, *Ameiurus nebulosus*, "dvergmalle" in Norwegian) which is located in a few lakes in SE Norway after being imported from North America *c*. 1890 (Hesthagen and Brabrand, 2018). Exotic species are mentioned in the guidance documents with respect to providing potential information on ecological effects (of other exotic species). For the channel catfish, it is anticipated that the additional risk involved by this species not being native to Norwegian waters, should be part of the ERA. The case-specific monitoring, however, should be easier for channel catfish in Norway than for endemic species targeted for genome editing (like Atlantic salmon).

For all cases, any data gaps and uncertainties concerning the environmental impact (including risks for human and animal health) compared to non-edited conspecifics should be surveyed. Data and monitoring plans following the guidelines for case-specific monitoring and general surveillance should be provided.

9.2.8 Conclusions regarding post-market environmental monitoring

VKM concludes that the guidance is also applicable for genome-edited animals with the extent of information required determined on a case-by-case approach.

Box 11.

Atlantic salmon and risk assessment

Norway is the world's largest producer of farmed Atlantic salmon, generating more than 100 billion NOK annually in export value from *c.* 1.4 million tonnes produced. Atlantic salmon is also the most valuable species in aquaculture, even though other fishes are produced in much higher volumes in other countries (FAO, 2020).

Farming of Atlantic salmon gained momentum in the 1960s when it was shown that salmon could be raised in sea water netpens and fenced-off sounds. In sea water, Atlantic salmon showed a higher growth rate than any other salmonid and much higher growth than in freshwater tanks. Today, based on technological development and increased size of the netpens, more than 400 million smolts are released into netpens along the Norwegian coast each year.

Salmon farming has led to reduced fishing pressure on wild salmon. However, salmon farming has also presented new threats to wild salmon through interbreeding between escaped farmed salmon and wild salmon, and increased levels of disease transmission between farmed and wild populations.

On average 344,000 farmed salmon have escaped from fish farms every year during 1993 to 2019 in Norway, according to numbers being reported to the Norwegian Fisheries Directorate. The actual number of escapes is likely 2–4 times as high (Skilbrei et al., 2015). These are high numbers considering that the number of wild Atlantic salmon approaching the Norwegian coast each year for spawning in rivers currently number fewer than 500,000 per year – having been reduced by 50% since the mid-1980s.

The high number of escaped farmed salmon and the reduction in wild salmon numbers inspired the development of sterile salmon. Induction of triploidy from a heat or pressure shock for a few minutes on the newly fertilised egg, is a simple way of producing large numbers of sterile salmon with near 100% triploidy and only 2-3% increased mortality during the egg stage. The technique was known from the 1980s. Triploid salmon show similar growth and survival to diploid farmed salmon, but show problems related to bone formation and cataract, as well as other aspects of fish health (Stien et al., 2019). Triploid salmon show less propensity to migrate into rivers than diploid farmed salmon (Glover et al., 2016). Triploid females do not develop gonads whereas males show gonad development and may participate in spawning but are functionally sterile (Fjelldal et al., 2014). Triploid fillets contain less fat than diploid fillets, but triploid fillets have significantly higher relative levels of important omega-3 long chain polyunsaturated fatty acids than diploid salmon fed a similar diet (Murray et al., 2018).

Another development in the 1980s was the advent of genetic modification aimed at improving production and quality traits in cultured plants and animals. Insertion of a gene construct overexpressing growth hormone into the eggs of Pacific salmonid species showed that extraordinary growth rate (up to 17x weight gain in 14 months) could be achieved in a single generation (Devlin et al., 2001; Devlin et al., 1994). Similar experiments were

conducted in Norway during the late 1980s but received little governmental support and were terminated in 1992 (Nerland, 1996).

As long as traditional farming of Atlantic salmon is uncontained, legislation on genetically modified organisms makes it virtually impossible to conduct an ERA on a scale that is ecologically realistic. When the US Food and Drug Administration approved growth-hormone-genetically modified Atlantic salmon for marketing in the US in 2015, it was based on fish that were also sterile (by triploidy) and reared in tanks on land in locations south of and warmer than the natural distribution area of salmon. Later approval obtained in Prince Edward Island, Canada, uses large indoor tanks on land and also sterility by triploidisation.

In salmonid fishes, a non-modified surrogate and functional comparator for growth-hormone genetic modification has been made by implanting slow-release growth hormone in the body cavity of juvenile fish, creating a fast-growing fish that can mimic a fast-growing genetically modified (or genome-edited) fish for several weeks (Johnsson et al., 1999; Sundt-Hansen et al., 2012). This makes it possible to perform risk assessment experiments in environments that do not meet laboratory standards for containment of genetically modified organisms.

In Canada, contained experiments have been performed on growth hormone-genetically modified salmonids in freshwater tanks/runways both in British Columbia and in Newfoundland (Moreau et al., 2011; Oke et al., 2013; Sundstrom et al., 2007; Sundt-Hansen et al., 2007). No ecologically realistic experiment has been undertaken in the ocean.

Genome editing of Atlantic salmon and other fish species targets some of the same traits as discussed here for farmed salmon and genetically modified salmon. Case 1 uses genome editing for changing fatty acid composition in Atlantic salmon. Case 2 uses genome editing to induce sterility in diploid Atlantic salmon. Case 3 uses genome editing for increased muscular growth in channel catfish. The techniques used are different to traditional breeding and genetic modification, but the ERA may draw on lessons from assessment of the phenotypic changes and management issues induced by other methods.

Monitoring of escaped farmed salmon in Norway was developed in 1989 (Diserud et al., 2019) and is now in its second generation with more than 200 rivers being monitored annually (Glover et al., 2019). Moreover, genetic SNP-based methods have been developed to distinguish farmed salmon from wild salmon. These methods are quite precise for assessing introgression of farmed escaped salmon into wild salmon at the population level (Karlsson et al., 2016). The information goes into a risk assessment programme of aquaculture and the environment in Norway (Glover et al., 2020).

The methods can also be used to assess genetic introgression at the individual level, where it has been used to study effects of introgression of farmed to wild salmon on life-history traits and growth (Bolstad et al., 2017; Hagen et al., 2019). The SNP-based methodology is also used to check all potential wild broodstock for proportion of wild or farmed ancestry before the (wild) fish is spawned.

10 Application of the EFSA guidance for risk assessment of genetically modified microorganisms in risk assessment of genome-edited microorganisms (ToR 3)

The use of bacteria and yeasts in foods such as bread, dairy products, fermented meats and fermented beverages such as beer and wine are common worldwide. In addition, they are currently utilised by the industry to synthesise compounds that are present in pharmaceutical, cosmetic, food and feed products. The choice of a particular strain or species for a specific industrial application is often based on historical usages and most species used have some history of safe use. The most commonly used species are within the genera *Bacillus, Bifidobacterium, Enterococcus, Lactobacillus, Lactococcus, Propionibacterium, Staphylococcus, Streptomyces, Aspergillus, Candida, Fusarium, Penicillium,* and *Saccharomyces.* In recent years, genome-editing techniques have become available for use in many of these species of bacteria and yeasts. See Chapter 6 and Tables 6.1 and 6.2 for a comprehensive overview and examples of major usage areas and species used.

Some of these species are also modified through traditional genetic modification for a number of purposes, ranging from the production of single substances (e.g., vitamins), to live microbes being present in the end product. Genetically modified microorganisms grown under fully contained conditions are also common in the batch production of pharmaceuticals. These are regulated through Directive 2009/41 (EC, 2009) and national legislation and not considered further here. Modified microorganisms not intended for food and feed purposes, e.g., plant promoting agents, do not fall under Regulation 1829/2003 (EC, 2003a) but under Directive EC 2001/18 (EC, 2001).

10.1 Application of the EFSA guidance for risk assessment of food and feed from genetically modified microorganisms in risk assessment of genome-edited microorganisms

Genetically modified microorganisms are used in the production of food and feed. The EFSA guidance on risk assessment of genetically modified microorganisms and their products intended for food and feed use specifies the principles followed in the assessment and the scientific information required in applications (Box 12).

The products of gene technology used on microorganisms intended for food and feed for placing on the market have been categorised into four categories by EFSA (EFSA, 2011e):

Category 1: Chemically defined purified compounds and their mixtures in which both genetically modified microorganisms and newly introduced genes have been removed (e.g., amino acids, vitamins)

Category 2: Complex products in which both genetically modified microorganisms and newly introduced genes are no longer present (e.g., cell extracts, most enzyme preparations)

Category 3: Products derived from genetically modified microorganisms s in which genetically modified microorganisms s capable of multiplication or of transferring genes are not present, but in which newly introduced genes are still present (e.g., heat-inactivated starter cultures)

Category 4: Products consisting of or containing genetically modified microorganisms s capable of multiplication or of transferring genes (e.g., live starter cultures for fermented foods and feed)

The categories above clearly illustrate the broad range of products that are of microbial origin and on which genetic modification processes can be used. The guidance specifies the information needed for the risk assessment of products in each category. The information requirements and the extent of information needed will vary with the category of the product. Thus, both the categorisation and the case-by-case approach will determine the information requirement needed. In general, the assessment focuses on the characterisation of the genetically modified microorganisms including the recipient/parental organism, the donor(s) of the genetic material, the genetic modification itself, and the final genetically modified microorganisms and its phenotype. On a category/case basis, data on composition, toxicity, allergenicity, nutritional value and environmental impact may be needed to complete the assessment.

The comparative approach drawing on comparators with a history of safe use is used as a baseline to support the assessment. The identification of differences between the genetically modified microorganisms and conventional counterpart enables characterisation of these with a focus on adverse effects to health and environment. For the food and feed assessment, the comparator can be a similar food or feed without genetic modification and a history of safe use.

For microorganisms intended for the food and feed chain, familiarity and historical uses supporting the concept of 'history of safe use' have been important. The EU terminology used 'Qualified Presumption of Safety" (QPS) concept is used to support assessments if sufficient knowledge exists on its apparent harmlessness to humans, animals and environment. If the comparator has a QPS status (list maintained by EFSA), the risk assessment can focus on the identification of intended and any unintended changes introduced rather than on the organism itself (EFSA, 2007b).

The key areas of information required in applications for genetically modified microorganisms and their products are listed below (EFSA, 2011e):

- Information relating to the genetically modified organism and the characteristics of the recipient or (when appropriate) parental organism, including the origin of the inserted sequences [donor organism(s)], the genetic modification and the genetically modified microorganisms , and comparison of the genetically modified microorganisms with an appropriate comparator
- Information relating to the product, including information relating to the production process, the product preparation process, description of the product and considerations of the genetically modified product for human health
- Exposure assessment/characterisation related to food and feed consumption
- Potential environmental impacts of genetically modified microorganisms s and their products
- Post-market environmental monitoring, including considerations of case-specific monitoring and general surveillance.

The risk analysis and assessment process follow the well-established four steps: hazard identification, hazard characterisation, exposure assessment and risk characterisation, taking into account uncertainties.

The information requirements bullet-listed above also seem relevant and can be applied to genome-edited organisms. For instance, information related to the genetically modified organism, the genetic modification and the genetically modified microorganisms with an appropriate comparator seems needed to establish a history of safe use and meets the qualified presumption of safety (QPS) for the organism used. On the other hand, the section on information on the origin and characteristics of inserted sequences seems obsolete.

Box 12.

The regulatory basis for genetically modified microorganisms

The EFSA guidance on risk assessment of genetically modified microorganisms and their products intended for food and feed use covers genetically modified microorganisms s and their products for placing on the market under Regulation (EC) No 1829/2003 (EC, 2003a) on genetically modified food and feed , as well as genetically modified microorganisms products under Regulations 1332/2008 (food enzymes) (EC, 2008a), 1333/2008 (food additives) (EC, 2008b), 1334/2008 (food flavourings) (EC, 2008c) and 1831/2003 (feed additives)(EC, 2003c). Additional guidance may also be developed by the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) and the EFSA Panels FAF (the Panel on Food Additives and Flavourings) and CEP (the Panel on Food Contact Materials, Enzymes and Processing Aids). Microorganisms covered in the guidance document (EFSA, 2011e) include archaea, bacteria and eukarya, such as filamentous fungi, yeasts, protozoa and microalgae but not cells of plants or animals or viruses or viroids.

Microorganisms have a long history of use in food and feed production, and there is broad diversity in their uses, in terms of both species and products used. Examples are alive or dead organisms, crude products with enzymatic activity and purified substances. Common to most is a history of strain selection over time. In contained use, there is a history of genetic modification. Often several traits are targeted, and many strains have substantial deviations from their wild counterparts. In this context, genome editing through the use of site-directed nucleases may add further opportunities. The genetically modified microorganisms guidance allows categorisation of products into four different categories. For genom editing, categories 2-4 seem most relevant.

The information requirements on the product, including information relating to the production process, the product preparation process, the product and considerations of the genetically modified product for human health may equally apply to products of genom editing and should be determined on a case-by-case basis. Both novel products and more efficient production of products already existing in the market can be expected. Thus, it is not possible to draw generic conclusions on their applicability beyond the case-by-case approach regarding the data requirements needed.

The same case-by-case rationale will apply to the exposure assessment/characterisation related to food and feed consumption and the environmental impacts and monitoring of genetically modified microorganisms s and their products.

10.1.1 Conclusion on the applicability of the EFSA guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use to genome-edited microorganisms

VKM concludes that the EFSA guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use is also applicable to genome-edited microorganisms. Due to the heterogenous uses of microorganisms/products their regulatory landscape can be considered complex, falling under both a directive, different EU regulations and various guidance documents developed by several of the EFSA panels. The product categorisation presented in the guidance allows for differentiation in the amount of data needed for the assessment. In contrast to animals and plants, the core concept of qualified presumption of safety (QPS) provides a clear baseline for the comparative approach. This combined with a case-by-case approach provides both structure and flexibility to the risk assessment process. The same flexibility is offered to genome-edited organisms within this regulatory framework.

11 Conclusions

The Norwegian Scientific Committee for Food and Environment (VKM) initiated this report to examine the extent to which organisms developed by genome-editing technologies pose new challenges in terms of risk assessment. In 2018, the European Court of Justice decided to include genome-edited organisms in the GMO definition and hence organisms developed by new genome editing techniques for the production of food and feed, are also subject to the obligations laid down by the EU legal framework. In the EU, all new GMO products for food, feed and cultivation are assessed by the European Food Safety Authority (EFSA). This report considers whether the risk assessment guidance on genetically modified organisms, developed by EFSA, is applicable for genome-edited organisms.

- The inherent flexibility of the EFSA guidance makes it suitable to cover health and environmental risk assessments of a wide range of organisms with various traits and intended uses. Combined with the embedded case-by-case approach including the initial hazard identification step, that determines the type and extent of information needed for the assessment, the guidance is applicable to genome-edited organisms. VKM's evaluation has not identified new hazards specific to genome-edited organisms that fall outside the areas of concern established in the guidance.

- The evaluation of the guidance demonstrates that the parts of the health and environmental risk assessment concerned with novel traits (i.e. the phenotype of the organism) may be fully applied to all categories of genome-edited organisms. The guidance on environmental risk assessment is largely concerned with novel traits and assessment of potential effects on biodiversity (e.g. in Norway) stemming from the spread and establishment of genome-edited organisms is fully applicable.

- The evaluation of the guidance demonstrates that the parts of the health and environmental risk assessment concerned with the genetic modification (i.e. the genotype of the organism) may be fully applied to genome-edited organisms with inserted genes or long fragments of DNA, i.e. edits categorised as Site-Directed Nuclease type 3 (SDN3).

  However, these parts are not fully applicable for genome-edited organisms with minor insertions, deletions or single mutations, i.e. edits categorised as Site-Directed Nuclease type 1-2 (SDN1-2), edits obtained by oligonucleotide directed mutagenesis (ODM) or base editing (BE).

In summary, VKM finds that the EFSA guidance on risk assessment of genetically modified organisms provides a functional framework for risk assessment of genome-edited organisms. However, inclusion of specific considerations in the guidance regarding different properties of genome-edited organisms would be beneficial to ensure a common understanding between product developers and risk assessors regarding the type and extent of data needed to perform a risk assessment.

12 Further considerations

New genome editing techniques provide a continuum of organisms ranging from those containing very minor genetic changes, to those currently generated through genetic modification. It will be challenging to fit such a heterogenous set of outcomes from genome editing techniques into the regulatory system developed for genetically modified organisms. Moreover, many of the definitions, terminology and concepts used in the EFSA guidance documents were developed at a time point when genetically modified organisms were near synonymous with the use of species foreign transgenes inserted at random locations into the recipient genome. The applicability of such descriptors may or may not be valid for organisms developed through genome editing techniques.

In the process of addressing the mandate and answering the Terms of Reference, VKM has identified several topics that would benefit from further attention. These topics are not clearly formulated research questions but rather represent areas in need of for instance clarification, consensusbuilding efforts, develeopment of updated precise terminology and harmonized understanding. Several are areas under current development. These topics are presented briefly below.

12.1 Topics for further consideration

12.1.1 Dynamic nature of EFSA guidance

EFSA is continually refining and updating its guidance on risk assessment of genetically modified organisms. The continually evolving nature of EFSA guidance is important to acknowledge, although the main guidance documents were developed several years ago. Rather than reworking the main guidance documents, the EFSA GMO Panel, through various mechanisms including public consultations, wishes to amend these with updated technical notes and opinions. Today there are more than 20 applicable documents. One example of this work is the recent opinion 'Applicability of the EFSA Opinion on site-directed nucleases type 3 for the safety assessment of plants developed using site-directed nucleases type 1 and 2 and oligonucleotide-directed mutagenesis' (EFSA, 2020b). Thus, collectively, the guidance supplemented with technical notes covers new technological developments such as the potential use of omics and next generation sequencing technologies, as well as new genome-editing approaches. VKM emphasises that the overall relevance and suitability of the guidance is based on its dynamic nature. An assessment of the suitability of guidance should therefore not be limited to a narrow interpretation of the suitability of single documents. This report builds on the core principles of the five main guidance documents, as these structure the assessment, illustrate the stepwise procedure and list the key areas of concern. These main documents are further supported by relevant technical notes and opinions related to food/feed and the environment. It is expected that EFSA will continue to amend guidance as new techniques and experience emerge. The processes and timelines behind the dynamic development of new

guidance and opinions are probably not fully understood by all interested parties and could benefit from clarification.

12.1.2 The importance of the case-by-case approach

As mentioned above, the guidance documents are developed to cover a broad set of organisms, environments and intended uses. Clearly, single product developers will not find all areas of concern considered in the guidance relevant to their organism/product. Hence, there will always be a case-by-case approach, whereby developers will consider the aspects in the guidance that should be required to complete and conversely, the aspects that will not be relevant, and noted as N/A. This is commonplace in today´s consideration of genetically modified organisms and will not differ for genome-edited organisms in the same regulatory framework. As stated in EU Regulation 2019/1381 (EC, 2019), pre-submission advice can be provided: '…*It is thus appropriate that, where the Authority may be requested to provide a scientific output, it should provide advice to a potential applicant or notifier upon request, before an application or notification is formally submitted. Such pre-submission advice should relate to the rules applicable to, and the content required for, an application or notification and should not address the design of the studies to be submitted, as that remains the applicant's responsibility'.*

12.1.3 The development of organisms with no species-foreign DNA insertions

The use of genome-editing techniques has shifted focus from adding foreign DNA (transgenes) to alterations of existing nucleotide sequences in the genome. The step away from crossing species barriers and possible different regulatory pathways for various product categories was proposed already in 2003 (Nielsen, 2003), and the concepts of preserving species integrity was considered in the 2012 Opinion from EFSA on Cisgenics and Intragenics (EFSA, 2012b). The EFSA opinion on safety assessment of plants developed through cisgenesis and intragenesis concluded that the EFSA guidance on risk assessment of genetically modified organisms are applicable for the evaluation of food and feed products derived from cisgenic and intragenic plants and for performing an environmental risk assessment. It was emphasised, however, that lesser amounts of event-specific data are needed for the risk assessment of cisgenic plants as compared to transgenic plants, but it must still be determined through a case-by-case approach. The EFSA GMO Panel concluded that similar hazards can be associated with cisgenic and conventionally bred plants, while there might be novel hazards associated with intragenic and transgenic plants. All of these breeding methods can produce variable frequencies and severities of unintended effects and should therefore be evaluated case by case. The considerations made in that opinion, may have relevance to understanding the risk assessment of genome-edited plants, depending on the extent of editing done.

12.1.4 The changing genetic basis for new traits

The transgene-enabled production of a novel protein/trait has until now been a key aspect of the risk assessed organism/product. This engineering paradigm relying on introducing recombined and species-foreign DNA fragments at random places in the genome, is now being

somewhat fused with or replaced by approaches allowed by new genome-editing techniques. Genome editing is often used to confer targeted knock-out mutations/deletions or alter/edit existing nucleotide patterns encoding endogenous proteins at defined place(s) in the genome. This approach to obtaining desired changes by alterations within genomes rather than by introducing external DNA may alter the understanding of the words 'new' or 'novel' or 'newly expressed in relation to the altered trait/protein. Until now, a 'new protein' or 'newly expressed trait' is typically understood as a protein derived from a newly inserted gene obtained by transgene-based engineering. Today, however, in some cases, the same 'new' trait could be obtained through either expression of transgenes, cisgenes, or intragenes, or through genome editing.

The meaning of 'new' would have different nuances depending on the methods used. Moreover, genome editing allows minor nucleotide changes to be introduced at desired places in the genome, for example in regions of the genome controlling expression of a gene of interest, in regulatory sequences and at multiple sites in the same or different genes. These different approaches will certainly create phenotypes with varying degrees of perceived 'novelty'. The shift from a transgene-based paradigm to targeted editing within genomes will benefit from developments of precise definitions and harmonised use of terminology.

12.1.5 The focus on unintended effects

It is noted that a key consideration behind EU regulations and EFSA guidance is focused on unintended effects and how uncertainty can be reduced. This extends beyond the producer´s data needed to document the intended effects of the introduced change(s). The risk assessment serves both to understand the intended effects and to exclude and reduce concerns of unintended effects.

To exclude unintended effects resulting from introduced changes, phenotypically based nutritional, whole food toxicological testing and allergenicity considerations have usually been expected in applications for food and feed use. These aspects extend beyond reporting on the genotype and the molecular characterisation of the intended genetic change. It is important to have the broader scope of risk assessment in mind, when considering its applicability to genome-edited organisms, i.e. the rationale behind risk assessment extend beyond the description of the inserted DNA fragment(s) or nucleotide change(s).

In general, fewer unintended effects may be expected in a genome-edited organism as the changes induced by genome editing are targeted to a predetermined region in the genome. Older technologies were based on random integration that is expected to produce a broader set of unintended effects. Genome editing, on the other hand, may produce other types of unintended effects caused by off-target effects of the nucleases used in the genome, and possible unintended insertions of the genome editing vectors.

12.1.6 Opportunities for whole genome sequencing and other omics-based approaches

The availability of whole genome data of edited organisms and their conventional counterparts is becoming increasingly available at a reasonable cost. Whole genome sequencing data could reduce uncertainty of off-target effects and contribute to the risk assessment process. However, problems may arise in identifying off-target effects in the genome of organisms in the SDN1 cateory, as such breaks can also occur randomly in the genome. The use of a comparator and bioinformatics-based prediction of near identical target sites may help to discriminate between random mutations occurring in single cells versus genomic effects of editing. Discrimination between random processes and genome-editing effects may be easier as longer DNA fragments are introduced in organisms in the SDN2-3 category. Larger insertions, including vector sequences and random integration may be more easily discovered with sequencing technology.

It is noted, however, that whole genome analyses of higher organisms and plants in particular may not be straightforward in terms of bioinformatics and quality assurance at the resolution needed. The same applies for other omics-based technologies, and the uncertainty lies in how to resolve any uncertainty arising from the detection of unexpected nucleotide variants or extra or missing signals in proteomics profiles etc. See for instance EFSA publications on DNA sequencing and on the use of omics as well as public comments related to the development of these (Box 6).

12.1.7 The naturalness of SDN1 edits

The concept of naturalness has been brought up in the debate on the future regulatory status of genome-edited organisms in the SDN1 category. The argument being made is that the same edit or deletion could occur naturally through mutations, and thereby it would not be possible to distinguish genome-edited organisms from their wild or conventional counterpart. Spontaneous mutations occur in all genomes in low frequency. Thus, some genome edits falling in the SDN 1-2 category will not be distinguishable from naturally formed point mutations. On the other hand, case examples of single gene edit mimicking mutations represent only a subset of gene edits possible in the SDN-1 category. A much broader range of genetic opportunities are possible also in the SDN1 category than those resembling single point mutations. Further sub-categorisation may be needed to define edits that also can occur in germlines at relevant frequencies and with a replicative potential.

Fixation of a new mutation by genome editing differs fundamentally from how mutations arise and spread in the natural populations. The spread of spontaneous mutations is determined by population genetic processes such as genetic drift (i.e. chance events) and selection. Strong positive selection over many generations is needed for mutations in a large natural population to resemble the introduction of targeted nucleotide edits.

Therefore, the resemblance between CRISPR-facilitated single nucleotide edits and randomly occurring point mutations may apply to some case examples of SDN1 but not to others. The

comparison to the natural occurrence of spontaneous point mutations is not valid if a organism is defined as belonging to the SDN1 category following genome editing of many genes in the genome simultaneously, as multiple specific combinations of mutations are not expected to occur by random mutagenesis over relevant time scales (Van der Meer et al., 2021).

12.1.8 Lack of consistency in the SDN1-2 category

The SDN1-2 categories are named outcomes of some uses of genome-editing techniques. In many example cases, the approach has targeted one or a few protein-coding genes with the aim to alter the phenotype based on changed protein characteristics/expression patterns, including loss of function. Such examples with well characterised phenotypes may show some consistency. Some, but not all, could be obtainable through mutation-based breeding as well. However, another example of edits in the SDN1 category, e.g. targeting three genes simultaneously, is unlikely to occur through classic breeding in a relevant time frame (Sanchez-Leon et al., 2018). Moreover, introducing a few single nucleotide changes in regulatory genes can cause large changes in the phenotype, proteome and nutritional profile etc. Thus, a minor edit in a genome (compared to nucleotide alterations of previous transgene-based insertions) may not translate linearly to a minor edit in the phenotype. Thus, the risk assessment of phenotypes in the SDN1 category may be vastly different. This aspect of the broad opportunities inherent in the SDN1-2 categories must be considered when categorisation and alternatives to the current case-by-case approach is considered.

12.1.9 Absence of vectors and use of negative segregants

Negative (null) segregants arise when genetically modified organisms lose the transgene insertion through segregation/outcrossing. For instance, when the CRISPR machinery encoding locus/vector is removed from a plant genome after having obtained the desired gene edit elsewhere in the same genome. Null segregants are considered transgenic and regulated as such in the EU, and hence, cannot be used as (sole) comparators even though they are isogenic or near isogenic. The future regulatory status of negative segregants from genome-editing processes may not be fully clear, including the extent of documentation needed to evidence such status. It is noted that many of the organisms produced by genome-editing techniques will, at an early developmental stage, contain DNA-based CRISPR vectors in the cytoplasm or have the CRISPR locus inserted as a transgene into the genome of the edited organism. At that stage, the organism resembles a genetically modified organism carrying a novel trait/transgene. However, subsequent breeding is done to ensure segregation of traits and that the final product will not be carrying the transgene, but only the intended genome edit.

The practice of genomic integration of the SDN encoding locus early in the developmental phase of genome-edited organisms may vary with producer because genome editing may also be done in many cases without the use of introduced DNA. The choice of engineering approach may depend on the organism, type of edit as well as patent rights and other considerations. This heterogeneity in the early developmental stages introducing genome edits is likely to have implications for risk assessment and may complicate the regulatory approaches.

12.1.10 Detection of genome-edited organisms

EU regulation specifies the need for validated detection protocols for genetically modified organisms placed on the market to ensure clear labelling and traceability. This requirement can be met by genetically modified organisms because all current commercialised ones contain DNA sequences inserted stably at unique genomic sites. For some uses of genome editing (e.g. SDN1-2, ODM and base editing) it is not fully clear whether validated detection protocols can be achieved. Such protocols will also be difficult to apply for organisms containing several edits that are not genetically linked.

The individual edits in multi-edited genomes may have separate segregation pathways obscuring both the detection of the initial pattern of nucleotide edits and the establishment of pattern owner/developer. Detection processes will soon become increasingly complicated if various gene edits/traits are further stacked through conventional breeding. Stacking of multiple traits is now commonplace in the current development and breeding of genetically modified plants. The ability to monitor could be upheld by the insertion of a tag at nearby locations of the edit and that have no insertional effects. The potential dilution of edits through random segregation and outcrossing patterns does not facilitate the opportunity to develop clear analyses of long-term effects based on the genetic patterns initially introduced.

VKM notes that the opportunity to complete a risk assessment of a genome-edited organism based on EFSA guidance is usually not dependent on validated detection protocols. This is because the assessment is of the product and the process of production. Detection protocols are validated independently by the EU JRC in Italy.

12.1.11 The concept of uncertainty - EFSA guidance on uncertainty analysis

EFSA has produced general guidance on uncertainty analysis in risk assessment (EFSA, 2018a). There is increased focus on how various forms of uncertainty are handled and communicated in the risk assessment and management processes. It is not yet fully clear how this comprehensive guidance will be used to inform future risk assessment processes, and in what ways the perceived reduced uncertainty of unintended effects suggested by some uses of genome editing will draw support from the guidance on approaches to uncertainty.

12.1.12 Heterogeneity of the regulatory landscape

Organisms and products of various genome-editing techniques have reached various developmental stages. Several field trails have been conducted under national legislation, and some products have reached a commercialised stage (Menz et al., 2020). National authorities have taken different approaches to assessment and regulation in the absence of harmonised international regulatory framework and consensus of their regulatory status (Schmidt et al., 2020). This heterogenic landscape has created uncertainty for product developers, in terms of both regulatory status and opportunities for export/international markets. This also applies to the European market, where national authorities have approached applications for field releases

differently. SDN-based products are yet not authorised for the EU market. There is therefore limited experience available for risk assessors, applicants and other stakeholders.

12.1.13 Increased focus on transparency and sustainability

A new Regulation 2019/1381 focuses on increasing transparency and sustainability of the EU risk assessments in the food chain (EC, 2019). The Regulation articulates conditions for a transparent, continuous and inclusive risk communication to strengthen citizens trust and contribute to greater accountability and legitimacy of the Authority. The Regulation also considers dependency on industry data and that all data and information supporting requests for authorisations should be made proactively available to the public, taking into account intellectual property rights. The conditions provided for in this regulation may be particularly important in the context of genome-edited products and the opportunities offered to their developers in building public trust in the food and feed chain through proactive data sharing and easy public access to disclosed data and information.

12.1.14 Breeding limited by knowledge of genetic variability – not editing tools

It is important to keep in mind that the past and current genome modification and editing techniques allow targeted interventions in the genome. A key limitation on past and current efforts is the lack of biological knowledge of what to modify and how. Commercially interesting new phenotypes can only be technically produced if knowledge exists about the link between genotype and phenotype. GMOs were developed mainly through the addition of species-foreign DNA. Genome-editing techniques continue this single gene-single effect paradigm. Substantial new advances in breeding will depend on in-depth knowledge of the genome being edited. Such knowledge is clearly not at hand for all traits and organisms now amendable to genome editing. Thus, biological knowledge may remain a limitation to technological developments for some time to come, particularly as most commercially interesting traits in higher organisms are known to be multigenic in nature.

12.1.15 Mathematical models, ecosystem modelling, and data-driven approaches

The capability of experiments and theory to predict the ecological consequences of genome-edited organisms in biological communities and ecosystems is limited. Mathematical models of biological processes may be used to inform risk assessment at several levels of biological organisation. At the population level, the fate of a new genetic variant is well known from population genetic theory, given knowledge about population size, relative fitness, and reproductive system. The spread of genetic variants from one population to other populations is also well known and can be modelled based on patterns of gene flow and the geographical population structure of the species.

Ecological effects at the level of biological communities and ecosystems are much harder to model. Here, mathematical models have their greatest strength in making qualitatively informative scenarios and identifying knowledge gaps. Detailed risk assessments, however, at the level of biological communities and ecosystems, must rely on expert judgements that make use of as much knowledge as possible about the interactions between the genome-edited organism and other species, and their environment.

The build-up of systemic approaches, strong computing capacity and analyses of vast amounts of data are likely to be increasingly valuable in environmental risk assessment. An example of this is the recent EFSA report on ERA of bees that discusses modelling and advocates a more holistic environmental approach to risk assessment (EFSA, 2021e).

Box 13

Related recent reports and ongoing activities

Norway

The Norwegian Biotechnology Advisory Board

In December 2018, The Norwegian Biotechnology Advisory Board presented its final recommendations for how genetically modified organisms (GMO) should be regulated. The Norwegian Biotechnology Advisory Board developed a proposal for frameworks that can pave the way for harnessing the potential of gene technology, while at the same time safeguarding health and environment, and promoting societal benefit, sustainability and ethics. The board proposed that requirements for risk assessment and approval should be differentiated in a tiered system based on the genetic change that has been made.

…Relevant criteria can be whether or not the change is permanent and heritable, whether or not the change can also be made using conventional breeding techniques, and whether or not the change crosses species boundaries. At the lowest level, a notification to the authorities (confirmation required before the organism can be released) may be sufficient. At higher levels, organisms would require approval before release is authorised, but may be subject to differentiated risk assessment and approval requirements. In cases where a trait or other case-specific factors warrant a more thorough assessment, the application can be transferred to a higher level. … such a system might be appropriate to reflect the different risk levels that can reasonably be expected for various types of genetic changes, and at the same time allowing for even more suitable and nuanced assessment of sustainability, societal benefits and ethics (Bioteknologirådet, 2018a).

The Advisory Board has on several occasions worked towards operationalising the assessment criteria societal benefit, sustainability and ethics in the Gene Technology Act. In October 2018, the Board published an update of the criteria for evaluating societal benefit of genetically modified organisms. Norwegian Environment Agency and the Board will use this report to determine the societal benefit of a GMO during the approval process. Important factors in the assessment of societal benefit are to identify the effects, both advantageous and disadvantageous, that the authorisation of a GMO will have compared to its rejection and to the groups of society to which this applies. General societal effects, e.g. how Norwegian food production might be affected, are also included in the assessment (Bioteknologirådet, 2018b).

The Norwegian Government

The Ministry of Climate and Environment has tasked a committee with preparing a report describing the technologies and possible risks and benefits of genome modification applied in environmental and health technology, industry and in particular, the food industry. Ethical aspects will be assessed and possible adjustments of the Norwegian legislation on regulation of genetically modified organisms will be evaluated in relation to general benefit for the society novel gene technologies without risk to health and environment are applied (Government.no, 2020).

EU

The European Food Safety Authority (EFSA)

EFSA has published several opinions to address questions regarding risk assessments of genome-edited organisms and the use of genome-editing techniques.

EFSA asked the Dutch National Institute for Public Health and the Environment (RIVM) for an external scientific report, presenting an overview of the 16 scientific opinions from EFSA to be used for risk assessment of plants developed through new genomic techniques (Van der Vlugt 2021). This report concluded that SDN technology and the base-editing technique, but not synthetic genomics, was discussed in the opinions by EFSA (EFSA, 2021d).

The European Council requested EFSA to prepare a similar report with an overview of EFSA and European national authorities' scientific opinions on the risk assessment of plants developed through new genomic techniques (EFSA, 2021c).

In the 2020 opinion from EFSA on Applicability of the EFSA Opinion on site-directed nucleases type 3 for the safety assessment of plants developed using site-directed nucleases types 1 and 2 and oligonucleotide-directed mutagenesis. EFSA concluded that the guidance for genetically modified organisms can also be applied in the risk assessment of organisms in the SDN3, SDN2 and SDN1 cateory (EFSA, 2020b).

In 2020, EFSA published Evaluation of existing guidelines for their adequacy for the molecular characterisation and environmental risk assessment of genetically modified plants obtained through synthetic biology (box 14), and Evaluation of existing guidelines for their adequacy for the microbial characterisation and environmental risk assessment of microorganisms obtained through synthetic biology. EFSA concluded that the guidance on molecular characterisation and ERA of genetically modified organisms can also be applied when risk assessing plants and microorganisms produced through synthetic biology (EFSA, 2021a).

In 2020, EFSA published Adequacy and sufficiency evaluation of existing EFSA guidelines for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of genetically modified insects containing engineered gene drives (EFSA, 2020a). The guidance was found applicable when assessing risks of genetically modified insects containing engineered gene drives.

EFSA has also published technical notes and reports on technology and quality requirements regarding risk assessment of genetically modified organisms.

In 2017, EFSA published a Supporting Publication on baseline information for the environmental risk assessment of RNAi-based genetically modified plants . EFSA discussed here the available information on (1) the uptake and systematic spread of RNAi activity, (2) the mechanisms of gene silencing and the different factors involved in RNAi efficiency, (3) routes of exposure and (4) the environmental fate of dsRNA, siRNA and miRNA; and (5) the various factors that may limit non-target effects (EFSA, 2018b).

EFSA has published a technical note on the use of available techniques for DNA sequencing and quality requirements in the molecular risk assessment of GMOs and launched a webinar to pinpoint the requirements for DNA sequencing quality when using next generation sequencing (NGS) in GMO applications (EFSA, 2018c; EFSA, 2019).

An Event Report or Colloquium was published by EFSA in 2018 describing the use of 'omics' technologies, representing genomics, transcriptomics, proteomics and metabolomics technologies. Discussions focused on genomics in microbial strain characterisation, metabolomics for the comparative assessment of genetically modifed plants and the use of omics for toxicological and environmental risk assessment. It was concluded that omics technologies are a valuable addition in risk assessment of genetically modifed food and feed products (EFSA et al., 2018).

European Commission

The European Commission published a study regarding the status of New Genomic Techniques under Union law, on 29 April 2021:

'The Council of the European Union asked for this study, regarding the status of new genomic techniques under Union Law (Directive 2001/18/EC, Regulation (EC) 1829/2003, Directive 2009/41/EC and Regulation (EC) 1830/2003), in light of the Court of Justice's judgment in Case C-528/16.

The study examined the status of New Genomic Techniques (NGTs), taking into account the state-of-the-art knowledge and the views of the EU countries and stakeholders' (EC, 2021a).

The Council of the European Union requested the Commission (Council Decision (EU) 2019/1904) to submit, by 30 April 2021, a study on the status of novel genomic techniques and implementation of the GMO legislation in the application of plants and animals produced by novel genomic techniques.

This is a continuation of the explanatory note from 2017, published by the Scientific Advice Mechanism (SAM) an independent scientific advice for policy making, describing the difference between conventional breeding techniques, established techniques of genetic modification and the new breeding techniques used in genome editing.

European Network of GMO Laboratories (ENGL)

European Network of GMO Laboratories (ENGL) has reviewed the possibilities and challenges for the detection of food and feed plant products obtained by new directed mutagenesis techniques leading to genome editing. The focus of this report is on products of genome editing that do not contain any inserted recombinant DNA in the final plant. The conclusion was that accurate quantification may be challenging if only changes of just one or a few base pairs are introduced. A targeted (PCR-based) detection method can be applied if the edit is known and not present as natural variants (ENGL, 2019).

Other international activities

Harmonised regulatory approaches, terminology and risk typology are essential parts in the foundation for an international market.

The Convention on Biological Diversity

The Cartagena Protocol on Biosafety to the Convention on Biological Diversity, under the United Nations Environmental Program, is an international agreement which aims to ensure the safe handling, transport and use of living modified organisms (LMOs) resulting from modern biotechnology that may have adverse effects on biological diversity, taking also into account risks to human health. It was adopted on 29 January 2000 and entered into force on 11 September 2003 (CBD, 2000; CBD, 2021).

Recent and current activities within the Conference of the Parties (COP) that adhere to the Cartagena Protocol have been on how to apply Annex I of decision CP 9/13 to living modified fish, and also how to apply the same Annex to living modified organisms containing engineered gene drives (CBD, 2020).

Organisation for Economic Co-operation and Development (OECD)

The OECD genome-editing hub is maintained as a result of the gene-editing project of the OECD Working Party on Biotechnology, Nanotechnology and Converging Technologies (BNCT) (OECD, 2018).

The International Union for Conservation of Nature (IUCN)

In 2019, the International Union for Conservation of Nature (IUCN) issued the report *Genetic frontiers for conservation* which is an assessment of synthetic biology in the light of biodiversity conservation. The technical assessment goes through novel genetic and genomic techniques that could create opportunities for new kinds of biodiversity conservation, but also raise questions and challenges; for example, cases involving the use of genome-editing techniques to combat invasive species or major insect-borne diseases (IUCN, 2019).

Box 14.

Genom editing approaches and synthetic biology

Genome edits can be made systematically throughout a genome of an organism to engineer new functions, biochemical pathways and multigenic traits. This approach is termed synthetic biology and has been defined as 'the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in living organisms' (EFSA, 2021a). The synthetic biology approach is today mostly applied to single-celled organisms intended for contained use in industrial biotechnology, e.g. production of fine chemicals in batch cultures. The EFSA GMO panel recently considered the use of synthetic biology in microorganisms and provided a number of recommendations (EFSA, 2020c). These include a call for wider understanding of community function in the receiving environment and development and deployment of systems approaches.

Although not yet common, notable exceptions can also be found for higher plants (EFSA, 2021c). The boundary between 'simple' SDN-based genome editing and assignment of SDN categories versus the concept of synthetic biology seems not yet fully developed. Future developments in synthetic biology approaches may also challenge both the suitability of EFSA guidance as well as the regulatory framework for genome-editing approaches.

12.2 Future landscape for genome-edited organisms

The opportunity to introduce targeted and minor nucleotide changes in a genome, some of which could arise naturally, has fuelled a debate on whether genome-edited organisms should be regulated and how. As considered and concluded in previous chapters, EFSA guidance on genetically modified organisms can also be applied to genome-edited organisms. This is the case because no new types of biological risks have been identified, and often fewer considerations need to be made, for instance due to the absence of inserted DNA fragments.

The overall diversity of engineering techniques now available to breeders provides endless opportunities for developing products. It is now possible, for example, for a single plant variety to simultaneously contain stacked transgenes from different species, placed both randomly and at targeted sites in the genome, targeted single nucleotide changes at various locations in the genome, and additional deletions and base edits. Such increasingly complex combinations of traits may perform differently when outcrossed into different genetic backgrounds and when grown in different environments. Moreover, non-uniform regulatory authorisations and intellectual property rights (IPR) associated with the various engineered traits will add to complexity.

Important present areas of uncertainty include: what will be the defining point for the regulatory pathway(s) of different product categories, and what types of (de)regulation will emerge in an international context, taking into account the need for harmonisation of approaches to ensure international trade, product recognition and consumer trust. The development of a uniform typology and terminology seems an essential first step for establishing a common baseline.

The legislation has to keep up with the speed of new technological opportunities. For developers and applied users of genome-editing techniques it is essential that the regulatory system as well as the IPR system are clarified and predictable. The regulatory and IPR landscapes are heterogeneous, with no clear and immediate resolution. This ongoing evolution of the regulatory landscape cannot be expected to be a linear outcome of technological opportunity and mainstream science alone. Instead, regulation represents the values and interests of a large set of stakeholders and is negotiated between these, both locally and internationally. The field of responsible research and innovation will also have a role in addressing the broader context of new technological opportunities and developments. In all cases, the emergence of genome-edited organisms has shifted the focus from process-triggered regulation (drawing on a bimodal GMO/conventional distinction) to a product-based focus, where the observed phenotypic difference will be emphasised to an even greater extent.

A new range of products are currently emerging, ranging from products with very minor genetic changes similar to those selected for in conventional breeding to innovative products generated through genetically modified organisms. Understanding how the regulatory landscape will take shape under these broad technological opportunities seems essential, but vulnerable to a plethora of uncertainties.

On this background, it seems pertinent that the development of novel, genome-edited organisms that are intended to meet human needs should be met with the appropriate level and depth of risk assessments before products are placed on the market. For some genome-edited organisms with reproductive potential, the impact of releases into the community and ecosystem of genome-edited organisms may be difficult to predict and hard to ascertain. The comprehensive and evolving EFSA guidance will aid in distinguishing between cases of various degrees of risk.

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Appendix I

Search strategy in order to answer ToR 2

Search title: Genome editing in Plants
Contact: Martin Malmstrøm
Librarian: Marita Heinz and Nataliya Byelyey
Comments: Searches restricted to 2013-2018. An updated search with identical criteria was performed for 2018-2019. The first search resulted in a total of 2498 records in Endnote, which was then corrected to 1131 following automated duplicate removal. The second updated search resulted in 1704 records, which was corrected to 826 after automated duplicate removal.

Data base: Ovid MEDLINE(R) Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily, Ovid MEDLINE and Versions(R) 1946 to June 27th, 2018.
Search date: July 2nd 2018
Articles found: 582

1	(Argonaut* or "Cas 9" or Cas9 or Cpf1 or CRISPR* or dCAS* or ((Gene or genetic or genome) adj (drive* or edit*)) or Meganuclease* or talen or TALENs or "Zinc Finger Nuclease*").tw,kf.	17554
2	Lycopersicon esculentum/ or Malus/ or Solanum tuberosum/ or Brassica rapa/ or Soybeans/ or Zea mays/ or Oryza/ or Brassica/ or Triticum/ or Hordeum/ or Avena/ or Secale/ or Triticale/ or Fragaria/ or Poaceae/	145497
3	("Lycopersicon esculentum" or "Lycopersicum esculentum" or "Solanum lycopersicum" or "Solanum esculentum" or Tomatoes or Tomato or Apple or Apples or Malus or "pome fruit" or "Solanum tuberosum" or Potatoes or Potato or "Brassica rapas" or "Brassica napus" or Rapeseed or Rapeseeds or canola or colza or "oilseed rape" or Soybean or Soybeans or "Soy Beans" or "Soy Bean" or soya or "Glycine max" or Zea or Corn or Maize or Teosinte or Oryza or Rice or Rices or Brassica or Broccoli or "Brussel Sprout" or Kale or Cauliflower or "Collard Green" or "Collard Greens" or Cabbage or Cabbages or Triticum or Wheat or Wheats or "graminis leaf" or Hordeum or Barley or Avena or Avenas or Oat or Oats or Secale or rye or ryes or Triticale or Triticosecale or "Triticum x Secale" or "Triticum aestivum x" or Fragaria or Strawberry or Strawberries or ananassa or Poaceae or Grasses or	281809

	Graminaceae or Gramineae or Graminae or Grass or Arundo or Imperata or Alopecurus or Camelina or "false flax" or "gold of pleasure" or goldofpleasure).tw,kf.	
4	1 and (2 or 3)	759
5	limit 4 to yr="2013 -Current"	582

Data base: Ovid MEDLINE(R) Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily, Ovid MEDLINE and Versions(R) 1946 to June 14th, 2019

Search date: June 17th 2019

Articles found: 477

1	(Argonaut* or "Cas 9" or Cas9 or Cpf1 or CRISPR* or dCAS* or ((Gene or genetic or genome) adj (drive* or edit*)) or Meganuclease* or talen or TALENs or "Zinc Finger Nuclease*").tw,kf.	23120
2	Lycopersicon esculentum/ or Malus/ or Solanum tuberosum/ or Brassica rapa/ or Soybeans/ or Zea mays/ or Oryza/ or Brassica/ or Triticum/ or Hordeum/ or Avena/ or Secale/ or Triticale/ or Fragaria/ or Poaceae/	156265
3	("Lycopersicon esculentum" or "Lycopersicum esculentum" or "Solanum lycopersicum" or "Solanum esculentum" or Tomatoes or Tomato or Apple or Apples or Malus or "pome fruit" or "Solanum tuberosum" or Potatoes or Potato or "Brassica rapas" or "Brassica napus" or Rapeseed or Rapeseeds or canola or colza or "oilseed rape" or Soybean or Soybeans or "Soy Beans" or "Soy Bean" or soya or "Glycine max" or Zea or Corn or Maize or Teosinte or Oryza or Rice or Rices or Brassica or Broccoli or "Brussel Sprout" or Kale or Cauliflower or "Collard Green" or "Collard Greens" or Cabbage or Cabbages or Triticum or Wheat or Wheats or "graminis leaf" or Hordeum or Barley or Avena or Avenas or Oat or Oats or Secale or rye or ryes or Triticale or	306564

	Triticosecale or "Triticum x Secale" or "Triticum aestivum x" or Fragaria or Strawberry or Strawberries or ananassa or Poaceae or Grasses or Graminaceae or Gramineae or Graminae or Grass or Arundo or Imperata or Alopecurus or Camelina or "false flax" or "gold of pleasure" or goldofpleasure).tw,kf.	
4	2 or 3	328574
5	1 and 4	1090
6	2019*.ed,ep,yr,dp,dc.	1074504
7	(201806* or 201807* or 201808* or 201809* or 201810* or 201811* or 201812*).ep,ed,dc.	1041452
8	6 or 7	1881424
9	5 and 8	477

Data base: Embase 1974 to 2018 June 29th

Search date: July 2nd 2018

Articles found: 238

1	(Argonaut* or "Cas 9" or Cas9 or Cpf1 or CRISPR* or dCAS* or ((Gene or genetic or genome) adj (drive* or edit*)) or Meganuclease* or talen or TALENs or "Zinc Finger Nuclease*").tw,kw.	23403

2	tomato/ or apple/ or potato/ or rapeseed/ or soybean/ or maize/ or rice/ or cabbage/ or wheat/ or barley/ or oat/ or rye/ or triticale/ or strawberry/ or Poaceae/ or camelina sativa/	161289
3	("Lycopersicon esculentum" or "Lycopersicum esculentum" or "Solanum lycopersicum" or "Solanum esculentum" or Tomatoes or Tomato or Apple or Apples or Malus or "pome fruit" or "Solanum tuberosum" or Potatoes or Potato or "Brassica rapas" or "Brassica napus" or Rapeseed or Rapeseeds or canola or colza or "oilseed rape" or Soybean or Soybeans or "Soy Beans" or "Soy Bean" or soya or "Glycine max" or Zea or Corn or Maize or Teosinte or Oryza or Rice or Rices or Brassica or Broccoli or "Brussel Sprout" or Kale or Cauliflower or "Collard Green" or "Collard Greens" or Cabbage or Cabbages or Triticum or Wheat or Wheats or "graminis leaf" or Hordeum or Barley or Avena or Avenas or Oat or Oats or Secale or rye or ryes or Triticale or Triticosecale or "Triticum x Secale" or "Triticum aestivum x" or Fragaria or Strawberry or Strawberries or ananassa or Poaceae or Grasses or Graminaceae or Gramineae or Graminae or Grass or Arundo or Imperata or Alopecurus or Camelina or "false flax" or "gold of pleasure" or goldofpleasure).tw,kw.	297172
4	1 and (2 or 3)	621
5	limit 4 to yr="2013 -Current"	444
6	limit 5 to (conference abstracts or embase)	238

Data base: Embase 1974 to 2019 June 14th

Search date: June 17th 2019

Articles found: 165

1	(Argonaut* or "Cas 9" or Cas9 or Cpf1 or CRISPR* or dCAS* or ((Gene or genetic or genome) adj (drive* or edit*)) or Meganuclease* or talen or TALENs or "Zinc Finger Nuclease*").tw,kw.	31659
2	tomato/ or apple/ or potato/ or rapeseed/ or soybean/ or maize/ or rice/ or cabbage/ or wheat/ or barley/ or oat/ or rye/ or triticale/ or strawberry/ or Poaceae/ or camelina sativa/	167365
3	("Lycopersicon esculentum" or "Lycopersicum esculentum" or "Solanum lycopersicum" or "Solanum esculentum" or Tomatoes or Tomato or Apple or Apples or Malus or "pome fruit" or "Solanum tuberosum" or Potatoes or Potato or "Brassica rapas" or "Brassica napus" or Rapeseed or Rapeseeds or canola or colza or "oilseed rape" or Soybean or Soybeans or "Soy Beans" or "Soy Bean" or soya or "Glycine max" or Zea or Corn or Maize or Teosinte or Oryza or Rice or Rices or Brassica or Broccoli or "Brussel Sprout" or Kale or Cauliflower or "Collard Green" or "Collard Greens" or Cabbage or Cabbages or Triticum or Wheat or Wheats or "graminis leaf" or Hordeum or Barley or Avena or Avenas or Oat or Oats or Secale or rye or ryes or Triticale or Triticosecale or "Triticum x Secale" or "Triticum aestivum x" or Fragaria or Strawberry or Strawberries or ananassa or Poaceae or Grasses or Graminaceae or Gramineae or Graminae or Grass or Arundo or Imperata or Alopecurus or Camelina or "false flax" or "gold of pleasure" or goldofpleasure).tw,kw.	313236
4	2 or 3	334356
5	1 and 4	976

6	2019* yr,em,dd,dp.	1588517
7	(201806* or 201807* or 201808* or 201809* or 201810* or 201811* or 201812*).dd.	429365
8	("201826" or "201827" or "201828" or "201829" or "201830" or "201831" or "201832" or "201833" or "201834" or "201835" or "201836" or "201837" or "201838" or "201839" or "201840" or "201841" or "201842" or "201843" or "201844" or "201845" or "201846" or "201847" or "201848" or "201849" or "201850" or "201851" or "201852").em.	898288
9	6 or 7 or 8	2507728
10	5 and 9	395
11	limit 10 to (conference abstracts or embase)	165

Data base: Web of Science

Search date: July 2nd 2018

Articles found: 855

4	2 AND 1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=2013-2018*	855
3	2 AND 1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	1205

2	**TOPIC:** (("Lycopersicon esculentum" or "Lycopersicum esculentum" or "Solanum lycopersicum" or "Solanum esculentum" or "Tomatoes" or "Tomato" or "Apple" or "Apples" or "Malus" or "pome fruit" or "Solanum tuberosum" or "Potatoes" or "Potato" or "Brassica rapas" or "Brassica napus" or "Rapeseed" or "Rapeseeds" or "canola" or "colza" or "oilseed rape" or "Soybean" or "Soybeans" or "Soy Beans" or "Soy Bean" or "soya" or "Glycine max" or "Zea" or "Corn" or "Maize" or "Teosinte" or "Oryza" or "Rice" or "Rices" or "Brassica" or "Broccoli" or "Brussel Sprout" or "Kale" or "Cauliflower" or "Collard Green" or "Collard Greens" or "Cabbage" or "Cabbages" or "Triticum" or "Wheat" or "Wheats" or "graminis leaf" or "Hordeum" or "Barley" or "Avena" or "Avenas" or "Oat" or "Oats" or "Secale" or "rye" or "ryes" or "Triticale" or "Triticosecale" or "Triticum x Secale" or "Triticum aestivum x" or "Fragaria" or "Strawberry" or "Strawberries" or "ananassa" or "Poaceae" or "Grasses" or "Graminaceae" or "Gramineae" or "Graminae" or "Grass" or "Arundo" or "Imperata" or "Alopecurus" or "Camelina" or "false flax" or "gold of pleasure" or "goldofpleasure")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	765421
1	TOPIC: (("Argonaut*" or "Cas 9" or "Cas9" or "Cpf1" or "CRISPR*" or "dCAS*" or (("Gene" or "genetic" or "genome") NEAR/0 ("drive*" or "edit*")) or "Meganuclease*" or "talen" or "TALENs" or "Zinc Finger Nuclease*")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	21343

Data base: Web of Science

Search date: June 17th 2019

Articles found: 551

3	551 #2 AND #1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=2018-2019*	551
2	**TOPIC:** (("Lycopersicon esculentum" or "Lycopersicum esculentum" or "Solanum lycopersicum" or "Solanum esculentum" or "Tomatoes" or "Tomato" or "Apple" or "Apples" or "Malus" or "pome fruit" or "Solanum tuberosum" or "Potatoes" or "Potato" or "Brassica rapas" or "Brassica napus" or "Rapeseed" or "Rapeseeds" or "canola" or "colza" or "oilseed rape" or "Soybean" or "Soybeans" or "Soy Beans" or "Soy Bean" or "soya" or "Glycine max" or "Zea" or "Corn" or "Maize" or "Teosinte" or "Oryza" or "Rice" or "Rices" or "Brassica" or "Broccoli" or "Brussel Sprout" or "Kale" or "Cauliflower" or "Collard Green" or "Collard Greens" or "Cabbage" or "Cabbages" or "Triticum" or "Wheat" or "Wheats" or "graminis leaf" or "Hordeum" or "Barley" or "Avena" or "Avenas" or "Oat" or "Oats" or "Secale" or "rye" or "ryes" or "Triticale" or "Triticosecale" or "Triticum x Secale" or "Triticum aestivum x" or "Fragaria" or "Strawberry" or "Strawberries" or "ananassa" or "Poaceae" or "Grasses" or "Graminaceae" or "Gramineae" or "Graminae" or "Grass" or "Arundo" or "Imperata" or "Alopecurus" or "Camelina" or "false flax" or "gold of pleasure" or "goldofpleasure")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	815693
1	TOPIC: (("Argonaut*" or "Cas 9" or "Cas9" or "Cpf1" or "CRISPR*" or "dCAS*" or (("Gene" or "genetic" or "genome") NEAR/0 ("drive*" or "edit*")) or "Meganuclease*" or "talen" or "TALENs" or "Zinc Finger Nuclease*")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	27680

Data base: Scopus

Search date: July 2nd 2018

Articles found: 823

8	Restricted to 2013-2018	823
7	#1 and (#2 OR #3 OR #4 OR #5 OR #6)	1196
6	TITLE-ABS-KEY (grass OR arundo OR imperata OR alopecurus OR camelina OR "false flax" OR "gold of pleasure" OR goldofpleasure)	117322
5	TITLE-ABS-KEY ("Triticum x Secale" OR "Triticum aestivum x" OR fragaria OR strawberry OR strawberries OR ananassa OR poaceae OR grasses OR graminaceae OR gramineae OR graminae)	149010
4	TITLE-ABS-KEY (cauliflower OR "Collard Green" OR "Collard Greens" OR cabbage OR cabbages OR triticum OR wheat OR wheats OR "graminis leaf" OR hordeum OR barley OR avena OR avenas OR oat OR oats OR secale OR rye OR ryes OR triticale OR triticosecale)	252524
3	TITLE-ABS-KEY (rapeseed OR rapeseeds OR canola OR colza OR "oilseed rape" OR soybean OR soybeans OR "Soy Beans" OR "Soy Bean" OR soya OR "Glycine max" OR zea OR corn OR maize OR teosinte OR oryza OR rice OR rices OR brassica OR broccoli OR "Brussel Sprout" OR kale)	465263
2	TITLE-ABS-KEY ("Lycopersicon esculentum" OR "Lycopersicum esculentum" OR "Solanum lycopersicum" OR "Solanum esculentum" OR tomatoes OR tomato OR apple OR apples OR malus OR "pome fruit" OR "Solanum tuberosum" OR potatoes OR potato OR "Brassica rapas" OR "Brassica napus")	198211
1	TITLE-ABS-KEY ((argonaut* OR "Cas 9" OR cas9 OR cpf1 OR crispr* OR dcas* OR ((gene OR genetic OR genome) PRE/0 (drive* OR edit*)) OR meganuclease* OR talen OR talens OR "Zinc Finger Nuclease*"))	25331

Data base: Scopus

Search date: June 17th 2019

Articles found: 823

8	Restricted to 2018-2019	592
7	#1 and (#2 OR #3 OR #4 OR #5 OR #6)	1629
6	TITLE-ABS-KEY (grass OR arundo OR imperata OR alopecurus OR camelina OR "false flax" OR "gold of pleasure" OR goldofpleasure)	124510
5	TITLE-ABS-KEY ("Triticum x Secale" OR "Triticum aestivum x" OR fragaria OR strawberry OR strawberries OR ananassa OR poaceae OR grasses OR graminaceae OR gramineae OR graminae)	157731
4	TITLE-ABS-KEY (cauliflower OR "Collard Green" OR "Collard Greens" OR cabbage OR cabbages OR triticum OR wheat OR wheats OR "graminis leaf" OR hordeum OR barley OR avena OR avenas OR oat OR oats OR secale OR rye OR ryes OR triticale OR triticosecale)	265797
3	TITLE-ABS-KEY (rapeseed OR rapeseeds OR canola OR colza OR "oilseed rape" OR soybean OR soybeans OR "Soy Beans" OR "Soy Bean" OR soya OR "Glycine max" OR zea OR corn OR maize OR teosinte OR oryza OR rice OR rices OR brassica OR broccoli OR "Brussel Sprout" OR kale)	495078
2	TITLE-ABS-KEY ("Lycopersicon esculentum" OR "Lycopersicum esculentum" OR "Solanum lycopersicum" OR "Solanum esculentum" OR tomatoes OR tomato OR apple OR apples OR malus OR "pome fruit" OR "Solanum tuberosum" OR potatoes OR potato OR "Brassica rapas" OR "Brassica napus")	213575

1	TITLE-ABS-KEY ((argonaut* OR "Cas 9" OR cas9 OR cpf1 OR crispr* OR dcas* OR ((gene OR genetic OR genome) PRE/0 (drive* OR edit*)) OR meganuclease* OR talen OR talens OR "Zinc Finger Nuclease*"))	31875

Contact: Martin Malmstrøm
Librarian: Marita Heinz and Nataliya Byelyey
Comments: Searches restricted to 2013-2018. An updated search with identical criteria was performed for 2018-2019. The first search resulted in a total of 2369 records in Endnote, which was then corrected to 1019 following automated duplicate removal. The second updated search resulted in 1237 records, which was corrected to 858 after automated duplicate removal.

Data base: Ovid MEDLINE(R) Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily, Ovid MEDLINE and Versions(R) 1946 to June 27th, 2018.
Search date: July 3rd2018
Articles found: 526

1	(Argonaut* or "Cas 9" or Cas9 or Cpf1 or CRISPR* or dCAS* or ((Gene or genetic or genome) adj (drive* or edit*)) or Meganuclease* or talen or TALENs or "Zinc Finger Nuclease*").tw,kf.	17582
2	exp Sheep/ or Cattle/ or exp Swine/ or Chickens/ or Turkeys/ or exp Goats/ or Salmo salar/ or Oncorhynchus mykiss/ or Gadus morhua/	748170
3	(Sheep or Ovis or ovine or ovines or Mouflon or Mouflons or ewe or ewes or hogget or hoggets or lamb or lambs or ram or rams or tup or Churra or Corriedale or Dorper or Merino or "Rasa Aragonesa" or Romney or "Santa Ines" or Suffolk or Texel or Cattle or "Bos indicus" or Zebu or Zebus or "Bos taurus" or Cow or Cows or "Bos grunniens" or Yak or Yaks or bovine or bovines or Bovinae or aurochs or aurochsen or "Bos primigenius" or banteng or "Bos javanicus" or "Bos sondaicus" or gaur or gaurs or "Bos gaurus" or "Bos guarus" or "Indian bison" or mithun or mithuns or "Bos frontalis" or gayal or gayals or mithan or "Bos bovis" or "taurine ox" or "taurine oxen" or "Bos mutus" or "Poephagus grunniens" or swine or swines or Suidae or pig or Pigs or Warthog or Warthogs or "Wart Hogs" or "Wart Hog" or "feral hog" or "wild hog" or Phacochoerus or "Sus scrofa" or "Sus domestica" or Boar or Boars or Minipig or Minipigs or suid or suids or porcine or gitt or gitts or piglet or piglets or farrow or farrows or shoat or shoats or sow or sows or Chicken or Chickens or "Gallus gallus" or "Gallus domesticus" or broiler or broilers or capon or capons or cockerel or cockerels or hens or pullet or pullets or rooster or roosters or Turkey or Turkeys or Meleagris or Meleagridinae or Meleagrididae or gobbler or gobblers or "Agriocharis ocellata" or Goat or Goats or capra or capras or caprine or caprines or Bovidae or Rupicapra or Rupicapras or Chamois or ibex or ibexes or aoudad	832355

	or aoudads or Ammotragus or minigoat or minigoats or "Atlantic salmon" or "Atlantic salmons" or "Salmo salar" or "Oncorhynchus mykiss" or "Salmo gairdneri" or "Salmo gairdnerii" or "Rainbow Trout" or Steelhead or Steelheads or "Redband Trout" or "Salmo mykiss" or "Salmo irideus" or Lumpfish or "Cyclopterus lumpus" or Lumpsucker or Lumpsuckers or "Ballan wrasse" or "Labrus berggylta" or "Labrus bergylta" or "Gadus morhua" or "Atlantic cod" or "Atlantic halibut" or "Hippoglossus hippoglossus" or Honeybee or Honeybees or "Honey bee" or "Honey bees" or Apis or Apinae).tw,kf.	
4	1 and (2 or 3)	678
5	limit 4 to yr="2013 -Current"	526

Data base: Ovid MEDLINE(R) Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily, Ovid MEDLINE and Versions(R) 1946 to June 12th, 2019
Search date: June 14th 2019
Articles found: 290

1	(Argonaut* or "Cas 9" or Cas9 or Cpf1 or CRISPR* or dCAS* or ((Gene or genetic or genome) adj (drive* or edit*)) or Meganuclease* or talen or TALENs or "Zinc Finger Nuclease*").tw,kf.	22972
2	exp Sheep/ or Cattle/ or exp Swine/ or Chickens/ or Turkeys/ or exp Goats/ or Salmo salar/ or Oncorhynchus mykiss/ or Gadus morhua/	769977
3	(Sheep or Ovis or ovine or ovines or Mouflon or Mouflons or ewe or ewes or hogget or hoggets or lamb or lambs or ram or rams or tup or Churra or Corriedale or Dorper or Merino or "Rasa Aragonesa" or Romney or "Santa Ines" or Suffolk or Texel or Cattle or "Bos indicus" or Zebu or Zebus or "Bos taurus" or Cow or Cows or "Bos grunniens" or Yak or Yaks or bovine or bovines or Bovinae or aurochs or aurochsen or "Bos primigenius" or banteng or "Bos javanicus" or "Bos sondaicus" or gaur or gaurs or "Bos gaurus" or "Bos guarus" or "Indian bison" or mithun or mithuns or "Bos frontalis" or gayal or gayals or mithan or "Bos bovis" or "taurine ox" or "taurine oxen" or "Bos mutus" or "Poephagus grunniens" or swine or swines or Suidae or pig or Pigs or Warthog or Warthogs or "Wart Hogs" or "Wart Hog" or "feral hog" or "wild hog" or Phacochoerus or "Sus scrofa" or "Sus domestica" or Boar or Boars or Minipig or Minipigs or suid or suids or porcine or gitt or gitts or piglet or piglets or farrow or farrows or shoat or	859483

	shoats or sow or sows or Chicken or Chickens or "Gallus gallus" or "Gallus domesticus" or broiler or broilers or capon or capons or cockerel or cockerels or hens or pullet or pullets or rooster or roosters or Turkey or Turkeys or Meleagris or Meleagridinae or Meleagrididae or gobbler or gobblers or "Agriocharis ocellata" or Goat or Goats or capra or capras or caprine or caprines or Bovidae or Rupicapra or Rupicapras or Chamois or ibex or ibexes or aoudad or aoudads or Ammotragus or minigoat or minigoats or "Atlantic salmon" or "Atlantic salmons" or "Salmo salar" or "Oncorhynchus mykiss" or "Salmo gairdneri" or "Salmo gairdnerii" or "Rainbow Trout" or Steelhead or Steelheads or "Redband Trout" or "Salmo mykiss" or "Salmo irideus" or Lumpfish or "Cyclopterus lumpus" or Lumpsucker or Lumpsuckers or "Ballan wrasse" or "Labrus berggylta" or "Labrus bergylta" or "Gadus morhua" or "Atlantic cod" or "Atlantic halibut" or "Hippoglossus hippoglossus" or Honeybee or Honeybees or "Honey bee" or "Honey bees" or Apis or Apinae).tw,kf.	
4	2 or 3	1093196
5	1 and 4	856
6	2019*.ed,ep,yr,dp,dc.	1074504
7	(201806* or 201807* or 201808* or 201809* or 201810* or 201811* or 201812*).ep,ed,dc.	1041452
8	6 or 7	1881424
9	5 and 8	290

Data base: Embase 1974 to 2018 June 29th
Search date: July 2nd 2018
Articles found: 505

1	(Argonaut* or "Cas 9" or Cas9 or Cpf1 or CRISPR* or dCAS* or ((Gene or genetic or genome) adj (drive* or edit*)) or Meganuclease* or talen or TALENs or "Zinc Finger Nuclease*").tw,kw.	23403
2	exp sheep/ or exp bovine/ or exp suid/ or exp Chicken/ or exp "turkey (bird)"/ or caprinae/ or aoudad/ or exp goat/ or rupicapra/ or Salmo salar/ or exp Oncorhynchus mykiss/ or Atlantic cod/ or Atlantic halibut/ or exp honeybee/	362943

3	(Sheep or Ovis or ovine or ovines or Mouflon or Mouflons or ewe or ewes or hogget or hoggets or lamb or lambs or ram or rams or tup or Churra or Corriedale or Dorper or Merino or "Rasa Aragonesa" or Romney or "Santa Ines" or Suffolk or Texel or Cattle or "Bos indicus" or Zebu or Zebus or "Bos taurus" or Cow or Cows or "Bos grunniens" or Yak or Yaks or bovine or bovines or Bovinae or aurochs or aurochsen or "Bos primigenius" or banteng or "Bos javanicus" or "Bos sondaicus" or gaur or gaurs or "Bos gaurus" or "Bos guarus" or "Indian bison" or mithun or mithuns or "Bos frontalis" or gayal or gayals or mithan or "Bos bovis" or "taurine ox" or "taurine oxen" or "Bos mutus" or "Poephagus grunniens" or swine or swines or Suidae or pig or Pigs or Warthog or Warthogs or "Wart Hogs" or "Wart Hog" or "feral hog" or "wild hog" or Phacochoerus or "Sus scrofa" or "Sus domestica" or Boar or Boars or Minipig or Minipigs or suid or suids or porcine or gitt or gitts or piglet or piglets or farrow or farrows or shoat or shoats or sow or sows or Chicken or Chickens or "Gallus gallus" or "Gallus domesticus" or broiler or broilers or capon or capons or cockerel or cockerels or hens or pullet or pullets or rooster or roosters or Turkey or Turkeys or Meleagris or Meleagridinae or Meleagrididae or gobbler or gobblers or "Agriocharis ocellata" or Goat or Goats or capra or capras or caprine or caprines or Bovidae or Rupicapra or Rupicapras or Chamois or ibex or ibexes or aoudad or aoudads or Ammotragus or minigoat or minigoats or "Atlantic salmon" or "Atlantic salmons" or "Salmo salar" or "Oncorhynchus mykiss" or "Salmo gairdneri" or "Salmo gairdnerii" or "Rainbow Trout" or Steelhead or Steelheads or "Redband Trout" or "Salmo mykiss" or "Salmo irideus" or Lumpfish or "Cyclopterus lumpus" or Lumpsucker or Lumpsuckers or "Ballan wrasse" or "Labrus berggylta" or "Labrus bergylta" or "Gadus morhua" or "Atlantic cod" or "Atlantic halibut" or "Hippoglossus hippoglossus" or Honeybee or Honeybees or "Honey bee" or "Honey bees" or Apis or Apinae).tw,kw.	915153
4	1 and (2 or 3)	800
5	limit 4 to yr="2013 -Current"	624
6	limit 5 to (conference abstracts or embase)	505

Data base: Embase 1974 to 2019 June 13
Search date: June 14th 2019
Articles found: 272

1	(Argonaut* or "Cas 9" or Cas9 or Cpf1 or CRISPR* or dCAS* or ((Gene or genetic or genome) adj (drive* or edit*)) or Meganuclease* or talen or TALENs or "Zinc Finger Nuclease*").tw,kw.	31632
2	exp sheep/ or exp bovine/ or exp suid/ or exp Chicken/ or exp "turkey (bird)"/ or caprinae/ or aoudad/ or exp goat/ or rupicapra/ or Salmo salar/ or exp Oncorhynchus mykiss/ or Atlantic cod/ or Atlantic halibut/ or exp honeybee/	354934
3	(Sheep or Ovis or ovine or ovines or Mouflon or Mouflons or ewe or ewes or hogget or hoggets or lamb or lambs or ram or rams or tup or Churra or Corriedale or Dorper or Merino or "Rasa Aragonesa" or Romney or "Santa Ines" or Suffolk or Texel or Cattle or "Bos indicus" or Zebu or Zebus or "Bos taurus" or Cow or Cows or "Bos grunniens" or Yak or Yaks or bovine or bovines or Bovinae or aurochs or aurochsen or "Bos primigenius" or banteng or "Bos javanicus" or "Bos sondaicus" or gaur or gaurs or "Bos gaurus" or "Bos guarus" or "Indian bison" or mithun or mithuns or "Bos frontalis" or gayal or gayals or mithan or "Bos bovis" or "taurine ox" or "taurine oxen" or "Bos mutus" or "Poephagus grunniens" or swine or swines or Suidae or pig or Pigs or Warthog or Warthogs or "Wart Hogs" or "Wart Hog" or "feral hog" or "wild hog" or Phacochoerus or "Sus scrofa" or "Sus domestica" or Boar or Boars or Minipig or Minipigs or suid or suids or porcine or gitt or gitts or piglet or piglets or farrow or farrows or shoat or shoats or sow or sows or Chicken or Chickens or "Gallus gallus" or "Gallus domesticus" or broiler or broilers or capon or capons or cockerel or cockerels or hens or pullet or pullets or rooster or roosters or Turkey or Turkeys or Meleagris or Meleagridinae or Meleagrididae or gobbler or gobblers or "Agriocharis ocellata" or Goat or Goats or capra or capras or caprine or caprines or Bovidae or Rupicapra or Rupicapras or Chamois or ibex or ibexes or aoudad or aoudads or Ammotragus or minigoat or minigoats or "Atlantic salmon" or "Atlantic salmons" or "Salmo salar" or "Oncorhynchus mykiss" or "Salmo gairdneri" or "Salmo gairdnerii" or "Rainbow Trout" or Steelhead or Steelheads or "Redband Trout" or "Salmo mykiss" or "Salmo irideus" or Lumpfish or "Cyclopterus lumpus" or Lumpsucker or Lumpsuckers or "Ballan wrasse" or "Labrus berggylta" or "Labrus bergylta" or "Gadus morhua" or	897985

	"Atlantic cod" or "Atlantic halibut" or "Hippoglossus hippoglossus" or Honeybee or Honeybees or "Honey bee" or "Honey bees" or Apis or Apinae).tw,kw.	
4	2 or 3	971568
5	1 and 4	1056
6	2019*.yr,em,dd,dp.	1596225
7	(201806* or 201807* or 201808* or 201809* or 201810* or 201811* or 201812*).dd.	429365
8	("201826" or "201827" or "201828" or "201829" or "201830" or "201831" or "201832" or "201833" or "201834" or "201835" or "201836" or "201837" or "201838" or "201839" or "201840" or "201841" or "201842" or "201843" or "201844" or "201845" or "201846" or "201847" or "201848" or "201849" or "201850" or "201851" or "201852").em.	898288
9	6 or 7 or 8	2515436
10	5 and 9	358
11	limit 10 to (conference abstracts or embase)	272

Data base: Web of Science
Search date: July 3rd. 2018
Articles found: 663

4	2 AND 1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=2013-2018*	663
3	2 AND 1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	863
2	**TOPIC:** (("Sheep" or "Ovis" or "ovine" or "ovines" or "Mouflon" or "Mouflons" or "ewe" or "ewes" or "hogget" or "hoggets" or "lamb" or "lambs" or "ram" or "rams" or "tup" or "Churra" or "Corriedale" or "Dorper" or "Merino" or "Rasa Aragonesa" or "Romney" or "Santa Ines" or "Suffolk" or "Texel" or "Cattle" or "Bos indicus" or "Zebu" or "Zebus" or "Bos taurus" or "Cow" or "Cows" or "Bos grunniens" or "Yak" or "Yaks" or "bovine" or "bovines" or "Bovinae" or "aurochs" or "aurochsen" or "Bos primigenius" or "banteng" or "Bos javanicus" or "Bos sondaicus" or "gaur" or "gaurs" or "Bos gaurus" or "Bos guarus" or "Indian bison" or "mithun" or "mithuns" or "Bos frontalis" or "gayal" or "gayals" or "mithan" or "Bos bovis" or "taurine ox" or "taurine oxen" or "Bos mutus" or "Poephagus grunniens" or "swine" or "swines" or "Suidae" or "pig" or "Pigs" or "Warthog" or "Warthogs" or "Wart Hogs" or "Wart Hog" or "feral hog" or "wild hog" or "Phacochoerus" or "Sus scrofa" or "Sus domestica" or "Boar" or "Boars" or "Minipig" or "Minipigs" or "suid" or "suids" or "porcine" or "gitt" or "gitts" or	1113052

	"piglet" or "piglets" or "farrow" or "farrows" or "shoat" or "shoats" or "sow" or "sows" or "Chicken" or "Chickens" or "Gallus gallus" or "Gallus domesticus" or "broiler" or "broilers" or "capon" or "capons" or "cockerel" or "cockerels" or "hens" or "pullet" or "pullets" or "rooster" or "roosters" or "Turkey" or "Turkeys" or "Meleagris" or "Meleagridinae" or "Meleagrididae" or "gobbler" or "gobblers" or "Agriocharis ocellata" or "Goat" or "Goats" or "capra" or "capras" or "caprine" or "caprines" or "Bovidae" or "Rupicapra" or "Rupicapras" or "Chamois" or "ibex" or "ibexes" or "aoudad" or "aoudads" or "Ammotragus" or "minigoat" or "minigoats" or "Atlantic salmon" or "Atlantic salmons" or "Salmo salar" or "Oncorhynchus mykiss" or "Salmo gairdneri" or "Salmo gairdnerii" or "Rainbow Trout" or "Steelhead" or "Steelheads" or "Redband Trout" or "Salmo mykiss" or "Salmo irideus" or "Lumpfish" or "Cyclopterus lumpus" or "Lumpsucker" or "Lumpsuckers" or "Ballan wrasse" or "Labrus berggylta" or "Labrus bergylta" or "Gadus morhua" or "Atlantic cod" or "Atlantic halibut" or "Hippoglossus hippoglossus" or "Honeybee" or "Honeybees" or "Honey bee" or "Honey bees" or "Apis" or "Apinae")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	
1	**TOPIC:** (("Argonaut*" or "Cas 9" or "Cas9" or "Cpf1" or "CRISPR*" or "dCAS*" or (("Gene" or "genetic" or "genome") NEAR/0 ("drive*" or "edit*")) or "Meganuclease*" or "talen" or "TALENs" or "Zinc Finger Nuclease*")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	21343

Data base: Web of Science
Search date: June 14th 2019
Articles found: 189

3	2 AND 1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=2018-2019*	189
2	TI= (("Sheep" or "Ovis" or "ovine" or "ovines" or "Mouflon" or "Mouflons" or "ewe" or "ewes" or "hogget" or "hoggets" or "lamb" or "lambs" or "ram" or "rams" or "tup" or "Churra" or "Corriedale" or "Dorper" or "Merino" or "Rasa Aragonesa" or "Romney" or "Santa Ines" or "Suffolk" or "Texel" or "Cattle" or "Bos indicus" or "Zebu" or "Zebus" or "Bos taurus" or "Cow" or "Cows" or "Bos grunniens" or "Yak" or "Yaks" or "bovine" or "bovines" or "Bovinae" or "aurochs" or "aurochsen" or "Bos primigenius" or "banteng" or "Bos javanicus" or "Bos sondaicus" or "gaur" or "gaurs" or "Bos gaurus" or "Bos guarus" or "Indian bison" or "mithun" or "mithuns" or "Bos frontalis" or "gayal" or "gayals" or "mithan" or "Bos bovis" or "taurine ox" or "taurine oxen" or "Bos mutus" or "Poephagus grunniens" or "swine" or "swines" or "Suidae" or "pig" or "Pigs" or "Warthog" or "Warthogs" or "Wart Hogs" or "Wart Hog" or "feral hog" or "wild hog" or "Phacochoerus" or "Sus scrofa" or "Sus domestica" or "Boar" or "Boars" or "Minipig" or "Minipigs" or "suid" or "suids" or "porcine" or "gitt" or "gitts" or "piglet" or "piglets" or "farrow" or "farrows" or "shoat" or "shoats" or "sow" or "sows" or "Chicken" or "Chickens" or "Gallus gallus" or "Gallus domesticus" or "broiler" or "broilers" or "capon" or "capons" or "cockerel" or "cockerels" or "hens" or "pullet" or	577169

	"pullets" or "rooster" or "roosters" or "Turkey" or "Turkeys" or "Meleagris" or "Meleagridinae" or "Meleagrididae" or "gobbler" or "gobblers" or "Agriocharis ocellata" or "Goat" or "Goats" or "capra" or "capras" or "caprine" or "caprines" or "Bovidae" or "Rupicapra" or "Rupicapras" or "Chamois" or "ibex" or "ibexes" or "aoudad" or "aoudads" or "Ammotragus" or "minigoat" or "minigoats" or "Atlantic salmon" or "Atlantic salmons" or "Salmo salar" or "Oncorhynchus mykiss" or "Salmo gairdneri" or "Salmo gairdnerii" or "Rainbow Trout" or "Steelhead" or "Steelheads" or "Redband Trout" or "Salmo mykiss" or "Salmo irideus" or "Lumpfish" or "Cyclopterus lumpus" or "Lumpsucker" or "Lumpsuckers" or "Ballan wrasse" or "Labrus berggylta" or "Labrus bergylta" or "Gadus morhua" or "Atlantic cod" or "Atlantic halibut" or "Hippoglossus hippoglossus" or "Honeybee" or "Honeybees" or "Honey bee" or "Honey bees" or "Apis" or "Apinae")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	
1	TS =(("Argonaut*" or "Cas 9" or "Cas9" or "Cpf1" or "CRISPR*" or "dCAS*" or (("Gene" or "genetic" or "genome") NEAR/0 ("drive*" or "edit*")) or "Meganuclease*" or "talen" or "TALENs" or "Zinc Finger Nuclease*"))	27656

Data base: Scopus
Search date: July 3rd 2018
Articles found: 675

14	(LIMIT-TO (PUBYEAR , 2018) OR LIMIT-TO (PUBYEAR , 2017) OR LIMIT-TO (PUBYEAR , 2016) OR LIMIT-TO (PUBYEAR , 2015) OR LIMIT-TO (PUBYEAR , 2014) OR LIMIT-TO (PUBYEAR , 2013))	675
13	#1 AND (#2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12)	901
12	TITLE-ABS-KEY ("Atlantic cod" OR "Atlantic halibut" OR "Hippoglossus hippoglossus" OR honeybee OR honeybees OR "Honey bee" OR "Honey bees" OR apis OR apinae)	36422
11	TITLE-ABS-KEY ("Rainbow Trout" OR steelhead OR steelheads OR "Redband Trout" OR "Salmo mykiss" OR "Salmo irideus" OR lumpfish OR "Cyclopterus lumpus" OR lumpsucker OR lumpsuckers OR "Ballan wrasse" OR "Labrus berggylta" OR "Labrus bergylta" OR "Gadus morhua")	31671
10	TITLE-ABS-KEY ("Atlantic salmon" OR "Atlantic salmons" OR "Salmo salar" OR "Oncorhynchus mykiss" OR "Salmo gairdneri" OR "Salmo gairdnerii")	30836
9	TITLE-ABS-KEY (goat OR goats OR capra OR capras OR caprine OR caprines OR bovidae OR rupicapra OR rupicapras OR chamois OR ibex OR ibexes OR aoudad OR aoudads OR ammotragus OR minigoat OR minigoats)	74057

8	TITLE-ABS-KEY (turkey OR turkeys OR meleagris OR meleagridinae OR meleagridae OR gobbler OR gobblers OR "Agriocharis ocellata")	140759
7	TITLE-ABS-KEY (chicken OR chickens OR "Gallus gallus" OR "Gallus domesticus" OR broiler OR broilers OR capon OR capons OR cockerel OR cockerels OR hens OR pullet OR pullets OR rooster OR roosters)	232453
6	TITLE-ABS-KEY (suid OR suids OR porcine OR gitt OR gitts OR piglet OR piglets OR farrow OR farrows OR shoat OR shoats OR sow OR sows)	125780
5	TITLE-ABS-KEY (swine OR swines OR suidae OR pig OR pigs OR warthog OR warthogs OR "Wart Hogs" OR "Wart Hog" OR "feral hog" OR "wild hog" OR phacochoerus OR "Sus scrofa" OR "Sus domestica" OR boar OR boars OR minipig OR minipigs)	471988
4	TITLE-ABS-KEY (gaur OR gaurs OR "Bos gaurus" OR "Bos guarus" OR "Indian bison" OR mithun OR mithuns OR "Bos frontalis" OR gayal OR gayals OR mithan OR "Bos bovis" OR "taurine ox" OR "taurine oxen" OR "Bos mutus" OR "Poephagus grunniens")	932
3	TITLE-ABS-KEY (cattle OR "Bos indicus" OR zebu OR zebus OR "Bos taurus" OR cow OR cows OR "Bos grunniens" OR yak OR yaks OR bovine OR bovines OR bovinae OR aurochs OR aurochsen OR "Bos primigenius" OR banteng OR "Bos javanicus" OR "Bos sondaicus")	574858
2	TITLE-ABS-KEY (sheep OR ovis OR ovine OR ovines OR mouflon OR mouflons OR ewe OR ewes OR hogget OR hoggets OR lamb OR lambs OR ram OR rams OR tup OR churra OR corriedale OR dorper OR merino OR "Rasa Aragonesa" OR romney OR "Santa Ines" OR suffolk OR texel)	257642
1	TITLE-ABS-KEY ((argonaut* OR "Cas 9" OR cas9 OR cpf1 OR crispr* OR dcas* OR ((gene OR genetic OR genome) PRE/0 (drive* OR edit*)) OR meganuclease* OR talen OR talens OR "Zinc Finger Nuclease*"))	25352

Data base: Scopus
Search date: June 14th 2019
Articles found: 486

14	(LIMIT-TO (PUBYEAR , 2019) OR LIMIT-TO (PUBYEAR , 2018))	486
13	#1 AND (#2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12)	1170

12	TITLE-ABS-KEY ("Atlantic cod" OR "Atlantic halibut" OR "Hippoglossus hippoglossus" OR honeybee OR honeybees OR "Honey bee" OR "Honey bees" OR apis OR apinae)	86079
11	TITLE-ABS-KEY ("Rainbow Trout" OR steelhead OR steelheads OR "Redband Trout" OR "Salmo mykiss" OR "Salmo irideus" OR lumpfish OR "Cyclopterus lumpus" OR lumpsucker OR lumpsuckers OR "Ballan wrasse" OR "Labrus berggylta" OR "Labrus bergylta" OR "Gadus morhua")	
10	TITLE-ABS-KEY ("Atlantic salmon" OR "Atlantic salmons" OR "Salmo salar" OR "Oncorhynchus mykiss" OR "Salmo gairdneri" OR "Salmo gairdnerii")	
9	TITLE-ABS-KEY (goat OR goats OR capra OR capras OR caprine OR caprines OR bovidae OR rupicapra OR rupicapras OR chamois OR ibex OR ibexes OR aoudad OR aoudads OR ammotragus OR minigoat OR minigoats)	
8	TITLE-ABS-KEY (turkey OR turkeys OR meleagris OR meleagridinae OR meleagrididae OR gobbler OR gobblers OR "Agriocharis ocellata")	
7	TITLE-ABS-KEY (chicken OR chickens OR "Gallus gallus" OR "Gallus domesticus" OR broiler OR broilers OR capon OR capons OR cockerel OR cockerels OR hens OR pullet OR pullets OR rooster OR roosters)	
6	TITLE-ABS-KEY (suid OR suids OR porcine OR gitt OR gitts OR piglet OR piglets OR farrow OR farrows OR shoat OR shoats OR sow OR sows)	130,982
5	TITLE-ABS-KEY (swine OR swines OR suidae OR pig OR pigs OR warthog OR warthogs OR "Wart Hogs" OR "Wart Hog" OR "feral hog" OR "wild hog" OR phacochoerus OR "Sus scrofa" OR "Sus domestica" OR boar OR boars OR minipig OR minipigs)	
4	TITLE-ABS-KEY (gaur OR gaurs OR "Bos gaurus" OR "Bos guarus" OR "Indian bison" OR mithun OR mithuns OR "Bos frontalis" OR gayal OR gayals OR mithan OR "Bos bovis" OR "taurine ox" OR "taurine oxen" OR "Bos mutus" OR "Poephagus grunniens")	
3	TITLE-ABS-KEY (cattle OR "Bos indicus" OR zebu OR zebus OR "Bos taurus" OR cow OR cows OR "Bos grunniens" OR yak OR yaks OR bovine OR bovines OR bovinae OR aurochs OR aurochsen OR "Bos primigenius" OR banteng OR "Bos javanicus" OR "Bos sondaicus")	
2	TITLE-ABS-KEY (sheep OR ovis OR ovine OR ovines OR mouflon OR mouflons OR ewe OR ewes OR hogget OR hoggets OR lamb OR lambs OR ram OR rams OR tup OR churra OR corriedale OR dorper OR merino OR "Rasa Aragonesa" OR romney OR "Santa Ines" OR suffolk OR texel)	

1	TITLE-ABS-KEY ((argonaut* OR "Cas 9" OR cas9 OR cpf1 OR crispr* OR dcas* OR ((gene OR genetic OR genome) PRE/0 (drive* OR edit*)) OR meganuclease* OR talen OR talens OR "Zinc Finger Nuclease*"))	

Search title: Genome editing in microorganisms

Contact: Siamak Yazdankhah/Martin Malmstrøm
Librarian: Marita Heinz
Comments: Searches restricted to 2013-2018. The search resulted in a total of 2739 records in Endnote, which was then corrected to 1300 following automated duplicate removal.

Data base: Ovid MEDLINE(R) Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily, Ovid MEDLINE and Versions(R) 1946 to June 27th, 2018
Search date: July 3rd 2018
Articles found: 534

1	(Argonaut* or "Cas 9" or Cas9 or Cpf1 or CRISPR* or dCAS* or ((Gene or genetic or genome) adj (drive* or edit*)) or Meganuclease* or talen or TALENs or "Zinc Finger Nuclease*").tw,kf.	17582
2	exp Bacillus/ or exp Bifidobacterium/ or exp Enterococcus/ or exp Lactobacillus/ or exp Lactococcus/ or exp Propionibacterium/ or exp Staphylococcus/ or exp Streptomyces/ or exp Aspergillus/ or exp Candida/ or Fusarium/ or exp Penicillium/ or exp Saccharomyces/	399894
3	(Bacillus or "anthrax bacterium" or "Bacteridium anthracis" or "clostridium licheniforme" or "Denitrobacillus licheniformis" or "b subtilis" or "Bacterium subtilis" or "Vibrio subtilis" or "Natto Bacteria" or Dipel or Thuricide or Bacilan or Bifidobacterium or "Actinobacterium bifidum" or "Actinomyces bifidus" or "Actinomyces parabifidus" or "Bacterium bifidum" or "Bacteroides bifidus" or "Bifidibacterium bifidum" or "Cohnistreptothrix bifidus" or "Nocardia bifida" or "Tissieria bifida" or Enterococcus or "Streptococcus avium" or "Streptococcus casseliflavus" or "Streptococcus durans" or "Micrococcus ovalis" or "Micrococcus zymogenes" or paraghurt or "Streptococcus faecalis" or "streptococcus fecalis" or "Streptococcus glycerinaceus" or "Streptococcus liquefaciens" or "Streptococcus ovalis" or "th 69" or "Streptococcus faecium" or "Streptococcus gallinarum" or "Vancomycin Resistant Enterococci" or "Vancomycinresistant Enterococci" or Lactobacillus or Betabacterium or Lactobacileae or Lactobacilleae or lactobacilli or Lactobacteria or Culturelle or enpac or lacfer or lacteol or lactophil or "Thermobacterium intestinale" or viacil or "Bacterium casei a" or "caseobacterium vulgare" or "Lactobacterium casei" or "streptobacterium casei" or "Bacterium curvatum" or "Lactobacterium fermentum" or "Caseobacterium e" or "Lactobacterium helveticum" or "Plocamobacterium helveticum" or "thermobacterium helveticum" or	439344

	"Lactobacterium plantarum" or "Streptobacterium plantarum" or "Bacterium delbrucki" or "Lactobacterium delbrucki" or "Plocamobacterium delbrucki" or "Thermobacterium cereale" or "Ulvina delbrucki" or Lactococcus or "Streptococcus lactis" or "Bacterium lacti" or "Bacterium lactis" or "Streptococcus garvieae" or Propionibacterium or "bacterium acidi propionici" or "Corynebacterium acnes" or "Corynebacterium parvum" or "Bacillus acnes" or "C parvum" or "Cutibacterium acnes" or "P acnes" or "Propionibacteria acnes" or "Propionicibacterium acnes" or "Propionobacterium acnes" or "Proprionibacterium acnes" or "strain CN 6134" or "Propionicibacterium freudenreichii" or "Bacille granuleux" or "Corynebacterium granulosum" or "Propionicibacterium granulosum" or Staphylococcus or MRSA or MSSA or VRSA or VSSA or MRSE or staphylococci or "Micrococcus hyicus" or "Micrococcus aureus" or "Micrococcus pyogenes" or "Albococcus epidermidis" or "Micrococcus epidermidis" or "S epidermidis" or "Streptococcus epidermidis" or Streptomyces or Chainia or Streptoverticillium or "actinomyces albus" or "streptothrix alba" or "actinomyces antibioticus" or "actinomyces fradii" or "Actinomyces globisporus" or "Actinomyces griseus" or "Actinomyces setonii" or "actinomyces lavendulae" or "Actinomyces viridochromogenes" or Aspergillus or "Eurotium amstelodami" or "Eurotium chevalieri" or "Fennellia flavipes" or "a fumigatus" or "Neosartorya fumigata" or "Emericella nidulans" or "Eurotium repens" or "Eurotium rubrum" or Candida or Candidas or Monilia or Monilias or Torulopsis or "C parapsilosis" or "C orthopsilosis" or "C metapsilosis" or "C albicans" or "Mycotoruloides triadis" or "Oidium albicans" or "Trichosporon oryzae" or "mycotorula dimorpha" or "mycotorula trimorpha" or "oidium tropicale" or Fusarium or Fusariums or Gibberella or "Dichomera saubinetii" or "Microdochium nivale" or "Monographella nivalis" or Penicillium or Penicilliums or Pencillium or Pencilliums or Penicillum or Penicillums or Saccharomyce or Saccharomyces or saccaromyce or saccaromyces or ((baker* or brewer*) adj (yeast or yeasts)) or "S cerevisiae" or "CBS 5926").tw,kf.	
4	1 and (2 or 3)	656
5	limit 4 to yr="2013 -Current"	534

Database: Embase 1974 to 2018 June 29th
Date: July 2nd 2018

Articles found: 540

1	(Argonaut* or "Cas 9" or Cas9 or Cpf1 or CRISPR* or dCAS* or ((Gene or genetic or genome) adj (drive* or edit*)) or Meganuclease* or talen or TALENs or "Zinc Finger Nuclease*").tw,kw.	23403
2	exp Bacillus/ or exp Bifidobacterium/ or exp Enterococcus/ or exp Lactobacillus/ or exp Lactococcus/ or exp Propionibacterium/ or exp Staphylococcus/ or exp Streptomyces/ or exp Aspergillus/ or exp Candida/ or exp Fusarium/ or exp Penicillium/ or exp Saccharomyces/	568143
3	(Bacillus or "anthrax bacterium" or "Bacteridium anthracis" or "clostridium licheniforme" or "Denitrobacillus licheniformis" or "b subtilis" or "Bacterium subtilis" or "Vibrio subtilis" or "Natto Bacteria" or Dipel or Thuricide or Bacilan or Bifidobacterium or "Actinobacterium bifidum" or "Actinomyces bifidus" or "Actinomyces parabifidus" or "Bacterium bifidum" or "Bacteroides bifidus" or "Bifidibacterium bifidum" or "Cohnistreptothrix bifidus" or "Nocardia bifida" or "Tissieria bifida" or Enterococcus or "Streptococcus avium" or "Streptococcus casseliflavus" or "Streptococcus durans" or "Micrococcus ovalis" or "Micrococcus zymogenes" or paraghurt or "Streptococcus faecalis" or "streptococcus fecalis" or "Streptococcus glycerinaceus" or "Streptococcus liquefaciens" or "Streptococcus ovalis" or "th 69" or "Streptococcus faecium" or "Streptococcus gallinarum" or "Vancomycin Resistant Enterococci" or "Vancomycinresistant Enterococci" or Lactobacillus or Betabacterium or Lactobacileae or Lactobacilleae or lactobacilli or Lactobacteria or Culturelle or enpac or lacfer or lacteol or lactophil or "Thermobacterium intestinale" or viacil or "Bacterium casei a" or "caseobacterium vulgare" or "Lactobacterium casei" or "streptobacterium casei" or "Bacterium curvatum" or "Lactobacterium fermentum" or "Caseobacterium e" or "Lactobacterium helveticum" or "Plocamobacterium helveticum" or "thermobacterium helveticum" or "Lactobacterium plantarum" or "Streptobacterium plantarum" or "Bacterium delbrucki" or "Lactobacterium delbrucki" or "Plocamobacterium delbrucki" or "Thermobacterium cereale" or "Ulvina delbrucki" or Lactococcus or "Streptococcus lactis" or "Bacterium lacti" or "Bacterium lactis" or "Streptococcus garvieae" or Propionibacterium or "bacterium acidi propionici" or "Corynebacterium acnes" or "Corynebacterium parvum" or "Bacillus acnes" or "C parvum" or "Cutibacterium acnes" or "P acnes" or "Propionibacteria acnes" or "Propionicibacterium acnes" or "Propionobacterium acnes" or	514085

	"Proprionibacterium acnes" or "strain CN 6134" or "Propionicibacterium freudenreichii" or "Bacille granuleux" or "Corynebacterium granulosum" or "Propionicibacterium granulosum" or Staphylococcus or MRSA or MSSA or VRSA or VSSA or MRSE or staphylococci or "Micrococcus hyicus" or "Micrococcus aureus" or "Micrococcus pyogenes" or "Albococcus epidermidis" or "Micrococcus epidermidis" or "S epidermidis" or "Streptococcus epidermidis" or Streptomyces or Chainia or Streptoverticillium or "actinomyces albus" or "streptothrix alba" or "actinomyces antibioticus" or "actinomyces fradii" or "Actinomyces globisporus" or "Actinomyces griseus" or "Actinomyces setonii" or "actinomyces lavendulae" or "Actinomyces viridochromogenes" or Aspergillus or "Eurotium amstelodami" or "Eurotium chevalieri" or "Fennellia flavipes" or "a fumigatus" or "Neosartorya fumigata" or "Emericella nidulans" or "Eurotium repens" or "Eurotium rubrum" or Candida or Candidas or Monilia or Monilias or Torulopsis or "C parapsilosis" or "C orthopsilosis" or "C metapsilosis" or "C albicans" or "Mycotoruloides triadis" or "Oidium albicans" or "Trichosporon oryzae" or "mycotorula dimorpha" or "mycotorula trimorpha" or "oidium tropicale" or Fusarium or Fusariums or Gibberella or "Dichomera saubinetii" or "Microdochium nivale" or "Monographella nivalis" or Penicillium or Penicilliums or Pencillium or Pencilliums or Penicillum or Penicillums or Saccharomyce or Saccharomyces or saccaromyce or saccaromyces or ((baker* or brewer*) adj (yeast or yeasts)) or "S cerevisiae" or "CBS 5926").tw,kw.	
4	1 and (2 or 3)	747
5	limit 4 to yr="2013 -Current"	614
6	limit 5 to (conference abstracts or embase)	540

Database: Web of Science
Search date: July 3rd. 2018
Articles found: 894

4	2 AND 1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=2013-2018*	894
3	2 AND 1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	1106
2	TS=(("Bacillus" or "anthrax bacterium" or "Bacteridium anthracis" or "clostridium licheniforme" or "Denitrobacillus licheniformis" or "b subtilis" or "Bacterium subtilis" or "Vibrio subtilis" or "Natto Bacteria" or "Dipel" or "Thuricide" or "Bacilan" or "Bifidobacterium" or "Actinobacterium bifidum" or "Actinomyces bifidus" or "Actinomyces parabifidus" or "Bacterium bifidum" or "Bacteroides bifidus" or "Bifidibacterium bifidum" or "Cohnistreptothrix bifidus" or "Nocardia bifida" or "Tissieria bifida" or "Enterococcus" or "Streptococcus avium" or "Streptococcus casseliflavus" or "Streptococcus durans" or "Micrococcus ovalis" or "Micrococcus zymogenes" or "paraghurt" or "Streptococcus faecalis" or "streptococcus fecalis" or "Streptococcus glycerinaceus" or "Streptococcus liquefaciens" or "Streptococcus ovalis" or "th 69" or "Streptococcus faecium" or "Streptococcus gallinarum" or "Vancomycin Resistant Enterococci" or "Vancomycinresistant Enterococci" or "Lactobacillus" or "Betabacterium" or "Lactobacileae" or "Lactobacilleae" or "lactobacilli" or "Lactobacteria" or "Culturelle" or "enpac" or "lacfer" or "lacteol" or "lactophil" or "Thermobacterium intestinale" or "viacil" or "Bacterium casei a" or "caseobacterium vulgare" or "Lactobacterium casei" or "streptobacterium casei" or "Bacterium curvatum" or "Lactobacterium fermentum" or "Caseobacterium e" or "Lactobacterium helveticum" or "Plocamobacterium helveticum" or "thermobacterium helveticum" or "Lactobacterium plantarum" or "Streptobacterium plantarum" or "Bacterium delbrucki" or "Lactobacterium delbrucki" or "Plocamobacterium delbrucki" or "Thermobacterium cereale" or "Ulvina delbrucki" or "Lactococcus" or "Streptococcus lactis" or "Bacterium lacti" or "Bacterium lactis" or "Streptococcus garvieae" or "Propionibacterium" or "bacterium acidi propionici" or "Corynebacterium acnes" or "Corynebacterium parvum" or "Bacillus acnes" or "C parvum" or "Cutibacterium acnes" or "P acnes" or "Propionibacteria acnes" or "Propionicibacterium acnes" or "Propionobacterium acnes" or "Proprionibacterium acnes" or "strain CN 6134" or "Propionicibacterium freudenreichii" or "Bacille granuleux" or "Corynebacterium granulosum" or "Propionicibacterium granulosum" or "Staphylococcus" or "MRSA" or "MSSA" or "VRSA" or "VSSA" or "MRSE" or "staphylococci" or "Micrococcus hyicus" or "Micrococcus aureus" or "Micrococcus pyogenes" or "Albococcus epidermidis" or "Micrococcus epidermidis" or "S epidermidis" or "Streptococcus epidermidis" or "Streptomyces" or "Chainia" or "Streptoverticillium" or "actinomyces albus" or "streptothrix alba" or "actinomyces antibioticus" or "actinomyces fradii" or "Actinomyces globisporus" or "Actinomyces griseus" or "Actinomyces setonii" or "actinomyces lavendulae" or "Actinomyces viridochromogenes" or "Aspergillus" or "Eurotium amstelodami" or "Eurotium chevalieri" or "Fennellia flavipes" or "a fumigatus" or "Neosartorya fumigata" or "Emericella nidulans" or "Eurotium repens" or "Eurotium rubrum" or "Candida" or "Candidas" or "Monilia" or "Monilias" or "Torulopsis" or "C parapsilosis" or "C orthopsilosis" or "C metapsilosis" or "C albicans" or "Mycotoruloides triadis" or "Oidium albicans" or "Trichosporon oryzae" or "mycotorula dimorpha" or "mycotorula trimorpha"	604162

	or "oidium tropicale" or "Fusarium" or "Fusariums" or "Gibberella" or "Dichomera saubinetii" or "Microdochium nivale" or "Monographella nivalis" or "Penicillium" or "Penicilliums" or "Pencillium" or "Pencilliums" or "Penicillum" or "Penicillums" or "Saccharomyce" or "Saccharomyces" or "saccaromyce" or "saccaromyces" or (("baker*" or "brewer*") NEAR/0 ("yeast" or "yeasts")) or "S cerevisiae" or "CBS 5926")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	
1	21,343 **TOPIC:** (("Argonaut*" or "Cas 9" or "Cas9" or "Cpf1" or "CRISPR*" or "dCAS*" or (("Gene" or "genetic" or "genome") NEAR/0 ("drive*" or "edit*")) or "Meganuclease*" or "talen" or "TALENs" or "Zinc Finger Nuclease*")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	21343

Data base: Scopus
Search date: July 3rd.018
Articles found: 768

23	(LIMIT-TO (PUBYEAR , 2018) OR LIMIT-TO (PUBYEAR , 2017) OR LIMIT-TO (PUBYEAR , 2016) OR LIMIT-TO (PUBYEAR , 2015) OR LIMIT-TO (PUBYEAR , 2014) OR LIMIT-TO (PUBYEAR , 2013))	768
22	#1 AND (#2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17 OR #18 OR #19 OR #20 OR #21)	1,012
21	TITLE-ABS-KEY (saccharomyce OR saccharomyces OR saccaromyce OR saccaromyces OR ((baker* OR brewer*) PRE/0 (yeast OR yeasts)) OR "S cerevisiae" OR "CBS 5926")	
20	TITLE-ABS-KEY (penicillium OR penicilliums OR pencillium OR pencilliums OR penicillum OR penicillums)	
19	TITLE-ABS-KEY (fusarium OR fusariums OR gibberella OR "Dichomera saubinetii" OR "Microdochium nivale" OR "Monographella nivalis")	
18	TITLE-ABS-KEY ("mycotorula trimorpha" OR "oidium tropicale")	
17	TITLE-ABS-KEY (candida OR candidas OR monilia OR monilias OR torulopsis OR "C parapsilosis" OR "C orthopsilosis" OR "C metapsilosis" OR "C albicans" OR "Mycotoruloides triadis" OR "Oidium albicans" OR "Trichosporon oryzae" OR "mycotorula dimorpha")	
16	TITLE-ABS-KEY (aspergillus OR "Eurotium amstelodami" OR "Eurotium chevalieri" OR "Fennellia flavipes" OR "a fumigatus" OR "Neosartorya fumigata" OR "Emericella nidulans" OR "Eurotium repens" OR "Eurotium rubrum")	
15	TITLE-ABS-KEY ("Actinomyces viridochromogenes")	
14	TITLE-ABS-KEY (streptomyces OR chainia OR streptoverticillium OR "actinom	

	yces albus" OR "streptothrix alba" OR "actinomyces antibioticus" OR "actinomyces fradii" OR "Actinomyces globisporus" OR "Actinomyces griseus" OR "Actinomyces setonii" OR "actinomyces lavendulae")	
13	TITLE-ABS-KEY (staphylococcus OR mrsa OR mssa OR vrsa OR vssa OR mrse OR staphylococci OR "Micrococcus hyicus" OR "Micrococcus aureus" OR "Micrococcus pyogenes" OR "Albococcus epidermidis" OR "Micrococcus epidermidis" OR "S epidermidis" OR "Streptococcus epidermidis")	
12	TITLE-ABS-KEY ("Propionobacterium acnes" OR "Proprionibacterium acnes" OR "strain CN 6134" OR "Propionicibacterium freudenreichii" OR "Bacille granuleux" OR "Corynebacterium granulosum" OR "Propionicibacterium granulosum")	
11	TITLE-ABS-KEY (propionibacterium OR "bacterium acidi propionici" OR "Corynebacterium acnes" OR "Corynebacterium parvum" OR "Bacillus acnes" OR "C parvum" OR "Cutibacterium acnes" OR "P acnes" OR "Propionibacteria acnes" OR "Propionicibacterium acnes")	
10	TITLE-ABS-KEY (lactococcus OR "Streptococcus lactis" OR "Bacterium lacti" OR "Bacterium lactis" OR "Streptococcus garvieae")	
9	TITLE-ABS-KEY ("Bacterium delbrucki" OR "Lactobacterium delbrucki" OR "Plocamobacterium delbrucki" OR "Thermobacterium cereale" OR "Ulvina delbrucki")	
8	TITLE-ABS-KEY ("Lactobacterium helveticum" OR "Plocamobacterium helveticum" OR "thermobacterium helveticum" OR "Lactobacterium plantarum" OR "Streptobacterium plantarum")	
7	TITLE-ABS-KEY ("caseobacterium vulgare" OR "Lactobacterium casei" OR "streptobacterium casei" OR "Bacterium curvatum" OR "Lactobacterium fermentum" OR "Caseobacterium e")	
6	TITLE-ABS-KEY (lactobacillus OR betabacterium OR lactobacileae OR lactobacilleae OR lactobacilli OR lactobacteria OR culturelle OR enpac OR lacfer OR lacteol OR lactophil OR "Thermobacterium intestinale" OR viacil OR "Bacterium casei a")	
5	TITLE-ABS-KEY ("Streptococcus glycerinaceus" OR "Streptococcus liquefaciens" OR "Streptococcus ovalis" OR "th 69" OR "Streptococcus faecium" OR "Streptococcus gallinarum" OR "Vancomycin Resistant Enterococci" OR "Vancomycinresistant Enterococci")	
4	TITLE-ABS-KEY (enterococcus OR "Streptococcus avium" OR "Streptococcus casseliflavus" OR "Streptococcus durans" OR "Micrococcus ovalis" OR "Micrococcus zymogenes" OR paraghurt OR "Streptococcus faecalis" OR "streptococcus fecalis")	
3	TITLE-ABS-KEY (bifidobacterium OR "Actinobacterium bifidum" OR "Actinomyces bifidus" OR "Actinomyces	

	parabifidus" OR "Bacterium bifidum" OR "Bacteroides bifidus" OR "Bifidibacterium bifidum" OR "Cohnistreptothrix bifidus" OR "Nocardia bifida" OR "Tissieria bifida")	
2	TITLE-ABS-KEY (bacillus OR "anthrax bacterium" OR "Bacteridium anthracis" OR "clostridium licheniforme" OR "Denitrobacillus licheniformis" OR "b subtilis" OR "Bacterium subtilis" OR "Vibrio subtilis" OR "Natto Bacteria" OR dipel OR thuricide OR bacilan)	
1	TITLE-ABS-KEY ((argonaut* OR "Cas 9" OR cas9 OR cpf1 OR crispr* OR dcas* OR ((gene OR genetic OR genome) PRE/0 (drive* OR edit*)) OR meganuclease* OR talen OR talens OR "Zinc Finger Nuclease*"))	

New methods in genome editing - microorganisms

Contact:	**Martin Malmstrøm**
Librarian:	**Nataliya Byelyey**
Comments:	**2018-2019**
Doublets in EndNote:	**Prior to doublet subtraction: 1694**
	After doublet subtraction: 763

Data base: **Ovid MEDLINE(R)** Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily, Ovid MEDLINE and Versions(R) 1946 to June 14th, 2019
Search date: June 17th 2019
Articles found 371

1	(Argonaut* or "Cas 9" or Cas9 or Cpf1 or CRISPR* or dCAS* or ((Gene or genetic or genome) adj (drive* or edit*)) or Meganuclease* or talen or TALENs or "Zinc Finger Nuclease*").tw,kf.	23120
2	exp Bacillus/ or exp Bifidobacterium/ or exp Enterococcus/ or exp Lactobacillus/ or exp Lactococcus/ or exp Propionibacterium/ or exp Staphylococcus/ or exp Streptomyces/ or exp Aspergillus/ or exp Candida/ or Fusarium/ or exp Penicillium/ or exp Saccharomyces/	415618
3	(Bacillus or "anthrax bacterium" or "Bacteridium anthracis" or "clostridium licheniforme" or "Denitrobacillus licheniformis" or "b subtilis" or "Bacterium subtilis" or "Vibrio subtilis" or "Natto Bacteria" or Dipel or Thuricide or Bacilan or Bifidobacterium or "Actinobacterium bifidum" or "Actinomyces bifidus" or "Actinomyces parabifidus" or "Bacterium bifidum" or "Bacteroides bifidus" or "Bifidibacterium bifidum" or "Cohnistreptothrix bifidus" or "Nocardia bifida" or "Tissieria bifida" or Enterococcus or "Streptococcus avium" or "Streptococcus casseliflavus" or "Streptococcus durans" or "Micrococcus ovalis" or "Micrococcus zymogenes" or paraghurt or "Streptococcus faecalis" or "streptococcus fecalis" or "Streptococcus glycerinaceus" or "Streptococcus liquefaciens" or "Streptococcus ovalis" or "th 69" or "Streptococcus faecium" or "Streptococcus gallinarum" or "Vancomycin Resistant Enterococci" or "Vancomycinresistant Enterococci" or Lactobacillus or Betabacterium or Lactobacileae or Lactobacilleae or lactobacilli or Lactobacteria or Culturelle or enpac or lacfer or lacteol or lactophil or "Thermobacterium intestinale" or viacil or "Bacterium casei a" or "caseobacterium vulgare" or "Lactobacterium casei" or "streptobacterium casei" or "Bacterium curvatum" or "Lactobacterium fermentum" or "Caseobacterium e" or "Lactobacterium helveticum" or "Plocamobacterium	463140

	helveticum" or "thermobacterium helveticum" or "Lactobacterium plantarum" or "Streptobacterium plantarum" or "Bacterium delbrucki" or "Lactobacterium delbrucki" or "Plocamobacterium delbrucki" or "Thermobacterium cereale" or "Ulvina delbrucki" or Lactococcus or "Streptococcus lactis" or "Bacterium lacti" or "Bacterium lactis" or "Streptococcus garvieae" or Propionibacterium or "bacterium acidi propionici" or "Corynebacterium acnes" or "Corynebacterium parvum" or "Bacillus acnes" or "C parvum" or "Cutibacterium acnes" or "P acnes" or "Propionibacteria acnes" or "Propionicibacterium acnes" or "Propionobacterium acnes" or "Proprionibacterium acnes" or "strain CN 6134" or "Propionicibacterium freudenreichii" or "Bacille granuleux" or "Corynebacterium granulosum" or "Propionicibacterium granulosum" or Staphylococcus or MRSA or MSSA or VRSA or VSSA or MRSE or staphylococci or "Micrococcus hyicus" or "Micrococcus aureus" or "Micrococcus pyogenes" or "Albococcus epidermidis" or "Micrococcus epidermidis" or "S epidermidis" or "Streptococcus epidermidis" or Streptomyces or Chainia or Streptoverticillium or "actinomyces albus" or "streptothrix alba" or "actinomyces antibioticus" or "actinomyces fradii" or "Actinomyces globisporus" or "Actinomyces griseus" or "Actinomyces setonii" or "actinomyces lavendulae" or "Actinomyces viridochromogenes" or Aspergillus or "Eurotium amstelodami" or "Eurotium chevalieri" or "Fennellia flavipes" or "a fumigatus" or "Neosartorya fumigata" or "Emericella nidulans" or "Eurotium repens" or "Eurotium rubrum" or Candida or Candidas or Monilia or Monilias or Torulopsis or "C parapsilosis" or "C orthopsilosis" or "C metapsilosis" or "C albicans" or "Mycotoruloides triadis" or "Oidium albicans" or "Trichosporon oryzae" or "mycotorula dimorpha" or "mycotorula trimorpha" or "oidium tropicale" or Fusarium or Fusariums or Gibberella or "Dichomera saubinetii" or "Microdochium nivale" or "Monographella nivalis" or Penicillium or Penicilliums or Pencillium or Pencilliums or Penicillum or Penicillums or Saccharomyce or Saccharomyces or saccaromyce or saccaromyces or ((baker* or brewer*) adj (yeast or yeasts)) or "S cerevisiae" or "CBS 5926").tw,kf.	
4	2 or 3	573960
5	1 and 4	908
6	2019*.ed,ep,yr,dp,dc.	1074504

7	(201806* or 201807* or 201808* or 201809* or 201810* or 201811* or 201812*).ep,ed,dc	1041452
8	6 or 7	1881424
9	5 and 8	371

Database: Ovid Embase 1974 to 2019 June 14th
Search date: Juje 17th 2019
Antall treff: 347

1	(Argonaut* or "Cas 9" or Cas9 or Cpf1 or CRISPR* or dCAS* or ((Gene or genetic or genome) adj (drive* or edit*)) or Meganuclease* or talen or TALENs or "Zinc Finger Nuclease*").tw,kw.	31659
2	exp Bacillus/ or exp Bifidobacterium/ or exp Enterococcus/ or exp Lactobacillus/ or exp Lactococcus/ or exp Propionibacterium/ or exp Staphylococcus/ or exp Streptomyces/ or exp Aspergillus/ or exp Candida/ or exp Fusarium/ or exp Penicillium/ or exp Saccharomyces/	562875
3	(Bacillus or "anthrax bacterium" or "Bacteridium anthracis" or "clostridium licheniforme" or "Denitrobacillus licheniformis" or "b subtilis" or "Bacterium subtilis" or "Vibrio subtilis" or "Natto Bacteria" or Dipel or Thuricide or Bacilan or Bifidobacterium or "Actinobacterium bifidum" or "Actinomyces bifidus" or "Actinomyces parabifidus" or "Bacterium bifidum" or "Bacteroides bifidus" or "Bifidibacterium bifidum" or "Cohnistreptothrix bifidus" or "Nocardia bifida" or "Tissieria bifida" or Enterococcus or "Streptococcus avium" or "Streptococcus casseliflavus" or "Streptococcus durans" or "Micrococcus ovalis" or "Micrococcus zymogenes" or paraghurt or "Streptococcus faecalis" or "streptococcus fecalis" or "Streptococcus glycerinaceus" or "Streptococcus liquefaciens" or "Streptococcus ovalis" or "th 69" or "Streptococcus faecium" or "Streptococcus gallinarum" or "Vancomycin Resistant Enterococci" or "Vancomycinresistant Enterococci" or Lactobacillus or Betabacterium or Lactobacileae or Lactobacilleae or lactobacilli or Lactobacteria or Culturelle or enpac or lacfer or lacteol or lactophil or "Thermobacterium intestinale" or viacil or "Bacterium casei a" or "caseobacterium vulgare" or "Lactobacterium casei" or "streptobacterium casei" or "Bacterium curvatum" or "Lactobacterium fermentum" or "Caseobacterium e" or "Lactobacterium helveticum" or "Plocamobacterium helveticum" or "thermobacterium helveticum" or "Lactobacterium plantarum" or "Streptobacterium plantarum"	524633

	or "Bacterium delbrucki" or "Lactobacterium delbrucki" or "Plocamobacterium delbrucki" or "Thermobacterium cereale" or "Ulvina delbrucki" or Lactococcus or "Streptococcus lactis" or "Bacterium lacti" or "Bacterium lactis" or "Streptococcus garvieae" or Propionibacterium or "bacterium acidi propionici" or "Corynebacterium acnes" or "Corynebacterium parvum" or "Bacillus acnes" or "C parvum" or "Cutibacterium acnes" or "P acnes" or "Propionibacteria acnes" or "Propionicibacterium acnes" or "Propionobacterium acnes" or "Proprionibacterium acnes" or "strain CN 6134" or "Propionicibacterium freudenreichii" or "Bacille granuleux" or "Corynebacterium granulosum" or "Propionicibacterium granulosum" or Staphylococcus or MRSA or MSSA or VRSA or VSSA or MRSE or staphylococci or "Micrococcus hyicus" or "Micrococcus aureus" or "Micrococcus pyogenes" or "Albococcus epidermidis" or "Micrococcus epidermidis" or "S epidermidis" or "Streptococcus epidermidis" or Streptomyces or Chainia or Streptoverticillium or "actinomyces albus" or "streptothrix alba" or "actinomyces antibioticus" or "actinomyces fradii" or "Actinomyces globisporus" or "Actinomyces griseus" or "Actinomyces setonii" or "actinomyces lavendulae" or "Actinomyces viridochromogenes" or Aspergillus or "Eurotium amstelodami" or "Eurotium chevalieri" or "Fennellia flavipes" or "a fumigatus" or "Neosartorya fumigata" or "Emericella nidulans" or "Eurotium repens" or "Eurotium rubrum" or Candida or Candidas or Monilia or Monilias or Torulopsis or "C parapsilosis" or "C orthopsilosis" or "C metapsilosis" or "C albicans" or "Mycotoruloides triadis" or "Oidium albicans" or "Trichosporon oryzae" or "mycotorula dimorpha" or "mycotorula trimorpha" or "oidium tropicale" or Fusarium or Fusariums or Gibberella or "Dichomera saubinetii" or "Microdochium nivale" or "Monographella nivalis" or Penicillium or Penicilliums or Pencillium or Pencilliums or Penicillum or Penicillums or Saccharomyce or Saccharomyces or saccaromyce or saccaromyces or ((baker* or brewer*) adj (yeast or yeasts)) or "S cerevisiae" or "CBS 5926").tw,kw.	
4	2 or 3	666383
5	1 and 4	1064
6	2019*.yr,em,dd,dp.	429365
7	(201806* or 201807* or 201808* or 201809* or 201810* or 201811* or 201812*).dd.	429365

8	("201826" or "201827" or "201828" or "201829" or "201830" or "201831" or "201832" or "201833" or "201834" or "201835" or "201836" or "201837" or "201838" or "201839" or "201840" or "201841" or "201842" or "201843" or "201844" or "201845" or "201846" or "201847" or "201848" or "201849" or "201850" or "201851" or "201852").em.	898288
9	6 or 7 or 8	2515436
10	5 and 9	440
11	limit 10 to (conference abstracts or embase)	347

Data base: Web of Science
Search date: June 17th. 2019
Articles found: 457

4	2 AND 1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=2018-2019*	457
3	1,349 2 AND 1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	1349
2	TS=(("Bacillus" or "anthrax bacterium" or "Bacteridium anthracis" or "clostridium licheniforme" or "Denitrobacillus licheniformis" or "b subtilis" or "Bacterium subtilis" or "Vibrio subtilis" or "Natto Bacteria" or "Dipel" or "Thuricide" or "Bacilan" or "Bifidobacterium" or "Actinobacterium bifidum" or "Actinomyces bifidus" or "Actinomyces parabifidus" or "Bacterium bifidum" or "Bacteroides bifidus" or "Bifidibacterium bifidum" or "Cohnistreptothrix bifidus" or "Nocardia bifida" or "Tissieria bifida" or "Enterococcus" or "Streptococcus avium" or "Streptococcus casseliflavus" or "Streptococcus durans" or "Micrococcus ovalis" or "Micrococcus zymogenes" or "paraghurt" or "Streptococcus faecalis" or "streptococcus fecalis" or "Streptococcus glycerinaceus" or "Streptococcus liquefaciens" or "Streptococcus ovalis" or "th 69" or "Streptococcus faecium" or "Streptococcus gallinarum" or "Vancomycin Resistant Enterococci" or "Vancomycinresistant Enterococci" or "Lactobacillus" or "Betabacterium" or "Lactobacileae" or "Lactobacilleae" or "lactobacilli" or "Lactobacteria" or "Culturelle" or "enpac" or "lacfer" or "lacteol" or "lactophil" or "Thermobacterium intestinale" or "viacil" or "Bacterium casei a" or "caseobacterium vulgare" or "Lactobacterium casei" or "streptobacterium casei" or "Bacterium curvatum" or "Lactobacterium fermentum" or "Caseobacterium e" or "Lactobacterium helveticum" or "Plocamobacterium helveticum" or "thermobacterium helveticum" or "Lactobacterium plantarum" or "Streptobacterium plantarum" or "Bacterium delbrucki" or "Lactobacterium delbrucki" or "Plocamobacterium delbrucki" or "Thermobacterium cereale" or "Ulvina delbrucki" or "Lactococcus" or "Streptococcus lactis" or "Bacterium lacti" or "Bacterium lactis" or "Streptococcus garvieae" or "Propionibacterium" or "bacterium acidi propionici" or "Corynebacterium acnes" or "Corynebacterium parvum" or "Bacillus acnes" or "C parvum" or "Cutibacterium acnes" or "P acnes" or "Propionibacteria acnes" or "Propionicibacterium acnes" or "Propionobacterium acnes" or "Proprionibacterium acnes" or "strain CN 6134" or	637322

	"Propionicibacterium freudenreichii" or "Bacille granuleux" or "Corynebacterium granulosum" or "Propionicibacterium granulosum" or "Staphylococcus" or "MRSA" or "MSSA" or "VRSA" or "VSSA" or "MRSE" or "staphylococci" or "Micrococcus hyicus" or "Micrococcus aureus" or "Micrococcus pyogenes" or "Albococcus epidermidis" or "Micrococcus epidermidis" or "S epidermidis" or "Streptococcus epidermidis" or "Streptomyces" or "Chainia" or "Streptoverticillium" or "actinomyces albus" or "streptothrix alba" or "actinomyces antibioticus" or "actinomyces fradii" or "Actinomyces globisporus" or "Actinomyces griseus" or "Actinomyces setonii" or "actinomyces lavendulae" or "Actinomyces viridochromogenes" or "Aspergillus" or "Eurotium amstelodami" or "Eurotium chevalieri" or "Fennellia flavipes" or "a fumigatus" or "Neosartorya fumigata" or "Emericella nidulans" or "Eurotium repens" or "Eurotium rubrum" or "Candida" or "Candidas" or "Monilia" or "Monilias" or "Torulopsis" or "C parapsilosis" or "C orthopsilosis" or "C metapsilosis" or "C albicans" or "Mycotoruloides triadis" or "Oidium albicans" or "Trichosporon oryzae" or "mycotorula dimorpha" or "mycotorula trimorpha" or "oidium tropicale" or "Fusarium" or "Fusariums" or "Gibberella" or "Dichomera saubinetii" or "Microdochium nivale" or "Monographella nivalis" or "Penicillium" or "Penicilliums" or "Pencillium" or "Pencilliums" or "Penicillum" or "Penicillums" or "Saccharomyce" or "Saccharomyces" or "saccaromyce" or "saccaromyces" or (("baker*" or "brewer*") NEAR/0 ("yeast" or "yeasts")) or "S cerevisiae" or "CBS 5926")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	
1	**TOPIC:** (("Argonaut*" or "Cas 9" or "Cas9" or "Cpf1" or "CRISPR*" or "dCAS*" or (("Gene" or "genetic" or "genome") NEAR/0 ("drive*" or "edit*")) or "Meganuclease*" or "talen" or "TALENs" or "Zinc Finger Nuclease*")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	27731

Data base: Scopus
Search date: June 10th. 2019
Articles found: 519

23	(LIMIT-TO (PUBYEAR , 2019) OR LIMIT-TO (PUBYEAR , 2018))	519
22	#1 AND (#2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17 OR #18 OR #19 OR #20 OR #21)	1,387
21	TITLE-ABS-KEY (saccharomyce OR saccharomyces OR saccaromyce OR saccaromyces OR ((baker* OR brewer*) PRE/0 (yeast OR yeasts)) OR "S cerevisiae" OR "CBS 5926")	
20	TITLE-ABS-KEY (penicillium OR penicilliums OR pencillium OR pencilliums OR penicillum OR penicillums)	
19	TITLE-ABS-KEY (fusarium OR fusariums OR gibberella OR "Dichomera	

	saubinetii" OR "Microdochium nivale" OR "Monographella nivalis")	
18	TITLE-ABS-KEY ("mycotorula trimorpha" OR "oidium tropicale")	
17	TITLE-ABS-KEY (candida OR candidas OR monilia OR monilias OR tor ulopsis OR "C parapsilosis" OR "C orthopsilosis" OR "C metapsilosis" OR "C albicans" OR "Mycotoruloides triadis" OR "Oidium albicans" OR "Trichosporon oryzae" OR "mycotorula dimorpha")	
16	TITLE-ABS-KEY (aspergillus OR "Eurotium amstelodami" OR "Eurotium chevalieri" OR "Fennellia flavipes" OR "a fumigatus" OR "Neosartorya fumigata" OR "Emericella nidulans" OR "Eurotium repens" OR "Eurotium rubrum")	
15	TITLE-ABS-KEY ("Actinomyces viridochromogenes")	
14	TITLE-ABS-KEY (streptomyces OR chainia OR streptoverticillium OR "a ctinomyces albus" OR "streptothrix alba" OR "actinomyces antibioticus" OR "actinomyces fradii" OR "Actinomyces globisporus" OR "Actinomyces griseus" OR "Actinomyces setonii" OR "actinomyces lavendulae")	
13	TITLE-ABS-KEY (staphylococcus OR mrsa OR mssa OR vrsa OR vssa OR mrse OR staphylococci OR "Micrococcus hyicus" OR "Micrococcus aureus" OR "Micrococcus pyogenes" OR "Albococcus epidermidis" OR "Micrococcus epidermidis" OR "S epidermidis" OR "Streptococcus epidermidis")	
12	TITLE-ABS-KEY ("Propionobacterium acnes" OR "Proprionibacterium acnes" OR "strain CN 6134" OR "Propionicibacterium freudenreichii" OR "Bacille granuleux" OR "Corynebacterium granulosum" OR "Propionicibacterium granulosum")	
11	TITLE-ABS-KEY (propionibacterium OR "bacterium acidi propionici" OR "Corynebacterium acnes" OR "Corynebacterium parvum" OR "Bacillus acnes" OR "C parvum" OR "Cutibacterium acnes" OR "P acnes" OR "Propionibacteria acnes" OR "Propionicibacterium acnes")	
10	TITLE-ABS-KEY (lactococcus OR "Streptococcus lactis" OR "Bacterium lacti" OR "Bacterium lactis" OR "Streptococcus garvieae")	
9	TITLE-ABS-KEY ("Bacterium delbrucki" OR "Lactobacterium delbrucki" OR "Plocamobacterium	

	delbrucki" OR "Thermobacterium cereale" OR "Ulvina delbrucki")	
8	TITLE-ABS-KEY ("Lactobacterium helveticum" OR "Plocamobacterium helveticum" OR "thermobacterium helveticum" OR "Lactobacterium plantarum" OR "Streptobacterium plantarum")	
7	TITLE-ABS-KEY ("caseobacterium vulgare" OR "Lactobacterium casei" OR "streptobacterium casei" OR "Bacterium curvatum" OR "Lactobacterium fermentum" OR "Caseobacterium e")	
6	TITLE-ABS-KEY (lactobacillus OR betabacterium OR lactobacileae OR lactobacilleae OR lactobacilli OR lactobacteria OR culturelle OR enpac OR lacfer OR lacteol OR lactophil OR "Thermobacterium intestinale" OR viacil OR "Bacterium casei a")	
5	TITLE-ABS-KEY ("Streptococcus glycerinaceus" OR "Streptococcus liquefaciens" OR "Streptococcus ovalis" OR "th 69" OR "Streptococcus faecium" OR "Streptococcus gallinarum" OR "Vancomycin Resistant Enterococci" OR "Vancomycinresistant Enterococci")	
4	TITLE-ABS-KEY enterococcus OR "Streptococcus avium" OR "Streptococcus casseliflavus" OR "Streptococcus durans" OR "Micrococcus ovalis" OR "Micrococcus zymogenes" OR paraghurt OR "Streptococcus faecalis" OR "streptococcus fecalis")	
3	TITLE-ABS-KEY (bifidobacterium OR "Actinobacterium bifidum" OR "Actinomyces bifidus" OR "Actinomyces parabifidus" OR "Bacterium bifidum" OR "Bacteroides bifidus" OR "Bifidibacterium bifidum" OR "Cohnistreptothrix bifidus" OR "Nocardia bifida" OR "Tissieria bifida")	
2	TITLE-ABS-KEY (bacillus OR "anthrax bacterium" OR "Bacteridium anthracis" OR "clostridium licheniforme" OR "Denitrobacillus licheniformis" OR "b subtilis" OR "Bacterium subtilis" OR "Vibrio subtilis" OR "Natto Bacteria" OR dipel OR thuricide OR bacilan)	
1	TITLE-ABS-KEY ((argonaut* OR "Cas 9" OR cas9 OR cpf1 OR crispr* OR dcas* OR ((gene OR genetic OR genome) PRE/0 (drive* OR edit*)) OR meganuclease* OR talen OR talens OR "Zinc Finger Nuclease*"))	