WETENSCHAPPELIJK INSTITUUT
VOLKSGEZONDHEID
INSTITUT SCIENTIFIQUE
DE SANTÉ PUBLIQUE

Biosécurité et Biotechnologie

Advice of the Biosafety and Biotechnology Unit (SBB) concerning cases of mutagenesis in plants and animals using transient presence of the CRISPR/Cas9 system delivered as purified ribonucleoprotein with or without a homologous repair DNA template

Summary

- The SBB is of the opinion that the genetic modifications induced in plants and animals by the transient presence of the CRISPR/Cas9 system delivered as purified ribonucleoprotein with or without a homologous repair DNA template, as described in the present request, are similar in type and extent to those that can be obtained via natural or induced (using chemical or physical agents) mutagenesis and can thus be considered as a form of mutagenesis.
- The SBB is of the opinion that mutagenesis using the described applications of the CRISPR/Cas9 system in plants and animals provides for increased specificity and leads to fewer unintended effects compared to conventional mutagenesis techniques and does not raise additional safety concerns in the context of the intended uses.
- The SBB considers that mutagenesis using the described applications of the CRISPR/Cas9 system is a technique of genetic modification that does not involve the use of recombinant nucleic acid molecules or genetically modified organisms (GMOs) in the meaning of the chapeau of Bijlage 15 B of the Decree of the Flemish Government of 6 February 2004 (Annex II Part A of Directive 2009/41/EC).
- **The SBB therefore concludes that the intended uses under containment of animals and plants genetically modified as described in the present request should be considered for exclusion from the scope of the Decree of the Flemish Government of 6 February 2004, according to Annex 15 B of this Decree (Annex II Part A of Directive 2009/41/EC).**
- Generally, with regards to organisms genetically modified using the transient presence of the CRISPR/Cas9 system delivered as purified ribonucleoprotein according to the so-called SDN-1 or SDN-2 approaches, the SBB considers that the exclusion from the scope of the GMO legislation should apply to:
  - all uses (with or without specific containment measures) involving such genetically modified plants;
  - all cases where such genetically modified animals are used as models for research & development under containment.

  In all other cases where such genetically modified animals are used (e.g. in commercial breeding programmes), the SBB considers that the exclusion from the scope of the GMO legislation should only be granted on a case-by-case basis following a preliminary risk assessment, given the lack of history of use of mutagenesis techniques in these cases.
- The rationale presented above needs to be reassessed in case the CRISPR/Cas9 system would be used according to the SDN-1 or SDN-2 approaches to create mutations that go beyond small nucleotide deletions and/or insertions as described in the present request.
- This advice is delivered without prejudice to any further legal interpretation of the terms and provisions of the EU GMO Directives (2009/41/EC and 2001/18/EC) adopted at Belgian or EU level.

Background

On 6 July 2016 the Biosafety and Biotechnology Unit (SBB) received a request to advise the Flemish Competent Authority ("Vlaamse overheid, Departement Leefmilieu, Natuur & Energie, Afdeling Milieuvergunningen") about the regulatory status within the meaning of the GMO legislation of certain plants and animals genetically modified using the transient presence of the CRISPR/Cas9 system delivered as purified ribonucleoprotein with or without a homologous repair DNA template. This request was made following a question from a Belgian research institute whether or not the Decree of the Flemish Government of 6 February 2004 (VL, 2004) transposing Directive 2009/41/EC (EC, 2009) should apply to activities under containment involving these CRISPR/Cas9-modified organisms.
To substantiate its request the Belgian research institute provided a short description of the experimental approaches used to generate the modified organisms to be handled under containment.

To prepare its advice the SBB has taken into consideration the information provided by the Belgian research institute, as well as the scientific literature and other relevant information dealing with genome-editing techniques in plants and animals (summarized in the introduction below).

Introduction

Conventional mutagenesis

Mutations are the basis of genetic variation and mutant populations are indispensable genetic resources in all organisms. This variation can be either naturally occurring or, in plants, animals and lower organisms, induced by chemical or physical treatments.

Conventional mutagenesis techniques are based on the fact that chemicals or irradiation induce damage to DNA, including double-strand breaks (DSBs), that is not always faithfully repaired. Two mechanisms of DSB repair can be used by the cell, the non-homologous end joining (NHEJ) pathway or homologous recombination (HR)-based repair (Wyman and Kanaar, 2006). The NHEJ repair simply re-joins the broken DNA ends without the use of a homologous template. This can result in unfaithful repair, creating nucleotide insertions and/or deletions (indels) at the site of the break. HR is a more precise mechanism in which an undamaged homologous DNA sequence serves as an information source for the repair. HR plays a major role during meiosis. In eukaryotic somatic cells, including plant cells, the NHEJ pathway is generally preferred (EFSA, 2012).

In plants, conventional mutagenesis techniques (using chemicals such as ethyl methane sulphonate – EMS, or irradiation via UV, X-rays or gamma rays) have been widely applied since decades in research & development but are also playing a significant role in plant breeding. The widespread use of mutation techniques in plant breeding programmes throughout the world has generated more than 3200 officially released mutant varieties from 214 different plant species (FAO/IAEA Mutant Variety Database, https://mvd.iaea.org/).
There are two serious limitations to the use of induced physical and chemical mutagenesis in plant breeding (EFSA, 2012): (i) the deleterious effects associated with most newly introduced mutations and (ii) the untargeted and unspecific character of the processes. From a large mutagenized population, extensive selection and backcrossing is subsequently required to identify desirable phenotypes and eliminate the undesirable ones.

In animals, physical and chemical means have been used to induce deletion mutations in animal models such as mouse (Silver, 1995). EMS is used in many invertebrate species for mutagenesis, whereas the chemical mutagen N-ethyl-N-nitrosourea (ENU), which induces point mutations, is used routinely since the 80's in forward (phenotype-driven) genetic screens on animal models for biomedical research (Stottmann and Beier, 2014; Wansleeben *et al.*, 2011).
Contrary to plants the application of physical and chemical mutagenesis in animal breeding is very rare (see e.g. Kuroyanagi *et al.*, 2013).

Genome-editing techniques

During the past decade, genome-editing (or gene-editing) techniques have been developed that allow the direct modification of the genome at specific locations. They generally use nucleases that cleave DNA at specific sites and trigger the cell's own repair mechanisms. These so-called "site-directed nuclease" (SDN) techniques are evolving continuously and rapidly, both in terms of their applications and the types of nucleases used (Hilton and Gersbach, 2015; Podevin *et al.*, 2013). The later include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases (MN) and the clustered regularly interspaced short palindromic repeat (CRISPR/Cas9) system, but new gene-editing systems are under development (Ledford, 2016).

Applications of the SDN techniques are generally grouped in three categories (figure 1):
- In SDN-1 applications, only the SDNs are introduced (stably or transiently), generating site-specific point mutations, short insertions/deletions (indels) or excision by NHEJ.
- In SDN-2 applications, a homologous repair DNA template (donor DNA) is introduced together with the SDN complex to induce specific nucleotide sequence changes by HR. This approach can result in minor or more substantial changes to the nucleotide sequences of the target gene.
- In SDN-3 applications, a large stretch of exogenous donor DNA (up to several kilobases) is introduced together with the SDN complex to target DNA insertion into a predefined genomic locus. The insertion can take place either by HR or by NHEJ.

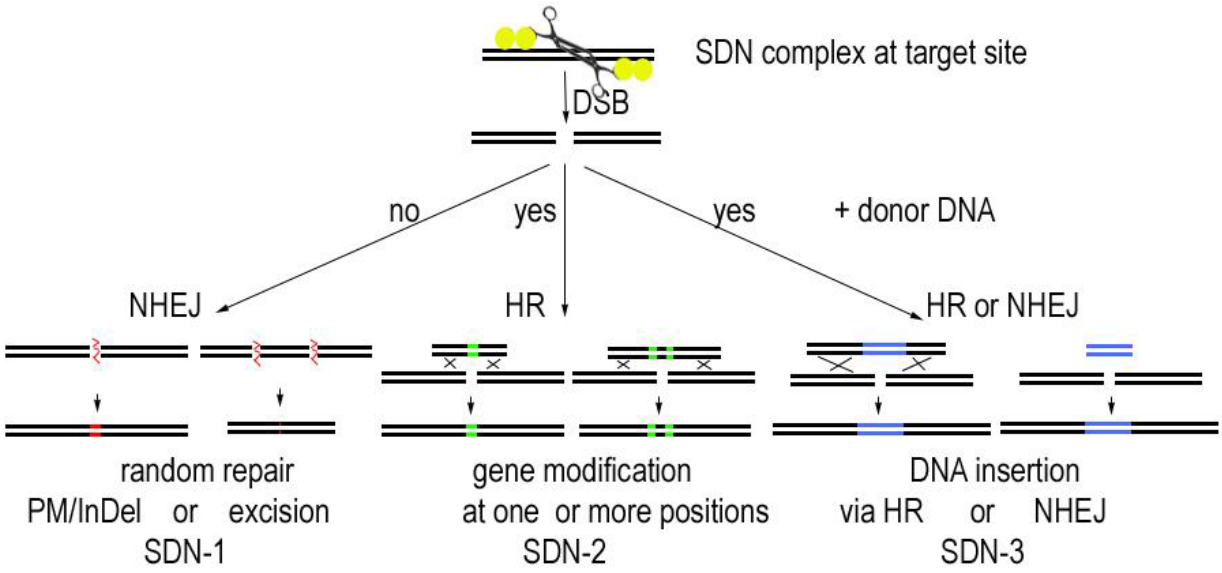

***Figure 1**: Applications of site-directed nuclease (SDN) techniques (Source: EFSA, 2012)*

The components of the SDN system (nuclease and possibly guide RNA) can be delivered into the cells in different ways.

In plants, a commonly used approach involves the stable integration and constitutive expression of the SDN-encoding gene(s) into the host genome. Once the SDN-mediated targeted mutation of the plant genome has been achieved, the introduced SDN gene(s) can be removed by segregation. To circumvent the integration of SDN encoding sequences as foreign DNA, transient delivery can be achieved using for example non-integrative DNA-based expression plasmids, some viral vectors or nuclease encoding mRNAs. In some cases, the components of the SDN system themselves can be delivered directly (see e.g. Woo *et al.*, 2015 and Subburaj *et al.,* 2016 for CRISPR/Cas9).

In animals, delivery can be achieved using viral vectors that do not integrate into the host genome (integrase-deficient lentiviral vectors, adenoviral vectors or adeno-associated virus vectors), or by introducing via lipofection or electroporation nuclease-encoding mRNAs or plasmids that encode the

SDN components (Pauwels *et al.*, 2014). More recently direct injection or electroporation in animal and human cells of the purified ribonucleoprotein CRISPR/Cas9 complex has been successfully applied (Kim *et al.*, 2014; Kouranova *et al.*, 2016).

The CRISPR/Cas9 system

CRISPR is an acronym for "clustered regularly interspaced short palindromic repeats" and Cas9 is a nuclease associated with CRISPR. The CRISPR/Cas9 system is derived from the adaptive immune system of some bacteria and represents the most recent generation of genome editing techniques. Site-specific modification is achieved by a single guide RNA (sgRNA, usually about 20 nucleotides) that is complementary to a target gene or locus and is anchored by a protospacer adjacent motif (PAM). Cas9 nuclease then cleaves the targeted DNA to generate double-strand breaks, eliciting a response from the cells' DNA repair machinery.

In plants, the use of the CRISPR/Cas9 system is recent but the number of applications of this system as a genome engineering tool is increasing rapidly (see e.g. reviews by Bortesi and Fischer, 2015; Luo *et al.*, 2016; Song *et al.*, 2016).
In animals, CRISPR/Cas9 is used more and more extensively in disease modeling, developmental studies and therapeutic applications including gene therapy (see e.g. Riordan et *al.*, 2015; Sander and Joung, 2014; Singh *et al.*, 2015; Yang *et al.*, 2014). Direct microinjection of the editing reagents into the cytoplasm of zygotes has also paved the way for generating founder animals of genetic lines with enhanced productivity or disease resistance traits (Tan *et al*., 2016).

Genome editing and off-target mutations

Specificity is an important endeavor for all genome editing technologies, including CRISPR/Cas9 (Marx, 2014). Sequence-specificity of SDN is not absolute and cleavage can occur at sites similar to but different from the target site. Since this could possibly result in unintended mutations or translocations, efforts have been done to predict and reduce such off-target activity (COGEM, 2014; Pauwels *et al.*, 2014).
In relation to the CRISPR/Cas9 system such efforts focus on the sgRNA and the PAM motif that were shown to predominantly confer target specificity of the system. Off-target effects associated with the CRISPR/Cas9 system can also be minimized by selecting target sequences that have reduced numbers of off-target homologues in the genome. Different approaches (including *in silico* methods) exist to predict genome wide off target cleavage (Kim *et al.*, 2016a; Tsai *et al.*, 2015; Wang *et al.*, 2015a). Novel types of endonucleases are also being discovered that could contribute to reduce 'off-site' targeting (Belhaj *et al.*, 2013; Kim *et al.*, 2016b; Song *et al.*, 2016).

In plants, the few studies published so far reported low to negligible off-target activity (Belhaj *et al.*, 2015, Weeks *et al.*, 2016). In any case, the frequency of off-target mutations caused by the SDN-1 and SDN-2 approaches is considered to be well below the frequency of unwanted mutations resulting from chemical or physical mutagenesis agents; for example, one mutation per 150 kbp can be effected by EMS treatment in *Arabidospsis* (EFSA, 2012; Greene et al., 2003; Podevin *et al.*, 2012). Furthermore, as in conventional breeding, unintended mutations can be segregated away during the selection and breeding process.

In animals, various strategies have been reported to reduce off-target effects, such as the optimization of the concentration and structure of various components of the CRISPR/Cas9 system (Cho *et al.*, 2014; Kleinstiver *et al.*, 2016; Zhang *et al.*, 2015) or shorter duration of Cas9 expression (Hsu *et al.*, 2013). It has also been shown that direct delivery of the purified ribonucleoprotein CRISPR/Cas9 complex contributes to reduce off-target activity without sacrificing on-target cleavage efficiency (Kim *et al.*, 2014; Wang *et al.*, 2015b). Contrary to plants, off-target activity is a major concern in animals, especially for therapeutic, clinical or breeding applications. Selection steps that could eliminate unintended mutations are much more laborious and expensive than in plants. Moreover, for some animals, considerations related to animal health and welfare should be taken into account when dealing with risk assessment of animals for breeding purposes. Therefore approaches that minimize selection steps and undesirable consequences and improve the precision with which gene editing outcomes can be predicted are of even greater importance in animals.

Regulatory considerations

Under the GMO legislation, a genetically modified (micro-)organism is "a (micro-)organism […] in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination" (AR-KB, 2005; EC, 2001; EC, 2009; VL, 2004). This definition is intrinsically linked to:
- A non-exhaustive list of techniques of genetic modification leading to a GMO (Annex I A, Part 1 of AR-KB, 2005; Bijlage 15 A, Deel 1 of VL, 2004). It includes recombinant nucleic acid techniques, techniques in which genetic material prepared outside the organism is introduced directly into the organism (for example by microinjection), and cell fusion or hybridization techniques where live cells with new combinations of heritable genetic material are formed;
- A list of techniques/methods of genetic modification yielding organisms to be excluded from the Directive (Annex I B of AR-KB, 2005; Bijlage 15 B of VL, 2004), which includes mutagenesis. The exclusion of these techniques/methods is possible only on the condition that they do not involve the use of recombinant nucleic acid molecules or GMOs.

(Micro-)organisms genetically modified by conventional mutagenesis techniques are therefore exempted from the EU GMO legislation. An argument underlying this exemption is that these techniques have conventionally been used in a number of applications and have a long safety record (recital 17 of Directive 2001/18/CE).

The regulatory status of genome-edited organisms is a matter of intense discussion by regulators and the scientific community (see e.g. Podevin *et al.*, 2012; Wolt *et al.*, 2016). In relation to the SDN-1 and SDN-2 approaches the question is whether applications of these systems fall under the definition of genetic modification (and therefore under the GMO legislation) or whether the resulting (micro-)organisms could be exempted from the GMO legislation by analogy to those obtained by conventional mutagenesis.

From a science-based perspective, there are arguments supporting the view that the SDN-1 and SDN-2 approaches can be considered a form of mutagenesis (ACRE, 2013; EFSA, 2015; Lusser and Davies, 2013; NTWG, 2012) and that the resulting organisms are not likely to differ from products obtained by conventional breeding or conventional mutagenesis in terms of risks posed to human health or environment, especially if the transgenes for the machinery used for genome editing are absent from the final product (Podevin *et al.*, 2013).
It was also pointed out that in many cases such organisms cannot be distinguished from non-modified organisms, raising questions about the enforceability of the GMO regulations (COGEM, 2014; Lusser *et al.*, 2012; Lusser and Davies, 2013).
In the case of SDN-2 further arguments have been put forward to conclude that the DNA sequence co-delivered with the SDN complex to act as the repair template should not be considered a recombinant nucleic acid molecule (ACRE, 2013; BVL, 2015; EFSA, 2015; Podevin *et al.*, 2012). A same conclusion was reached by the Belgian Biosafety Advisory Council in relation to oligonucleotide-directed mutagenesis (BAC, 2007; Breyer *et al.*, 2009).

These arguments, in addition to others linked to the interpretation of the GMO legislation, led several EU member states to conclude that organisms obtained by the SDN-1 (ACRE, 2013; BVL, 2015; HCB, 2016; Schaart and Visser, 2009; SWE, 2015) and SDN-2 (ACRE, 2013; BVL, 2015; HCB, 2016; Schaart and Visser, 2009) approaches should be considered for exclusion from the GMO legislation. For others a precautionary approach should be adopted, taking into account the uncertainties and limited knowledge on the mode of action of certain types of modifications (Eckerstorfer *et al.*, 2014).

Scientific assessment

Description of the experimental approaches used by the Belgian research institute

The following types of genetic modification are subject to the present request:

1. Purified CRISPR/Cas9 components (protein and sgRNA) are directly injected into rodent zygotes or transferred into plant protoplasts. As a result of the cell's natural DNA-repair process at the double strand breaks induced by the CRISPR/Cas9 system (using non-homologous end joining) point mutations or short deletions (variable length) are created at target loci in the cell genome. The resultant young rodents or plant protoplasts with the most suitable gene modifications are subsequently selected (based on genotyping) and used for further work.

2. Same approach as described in point (1) above with the exception that the purified CRISPR/Cas9 components are injected into rodent zygotes or transferred into plant protoplasts together with a donor DNA (single-stranded or double-stranded) homologous to the target site with the exception of the few base pairs corresponding to the intended gene modification. When this DNA is used as a template during the cell's natural DNA-repair process at the double strand breaks induced by the CRISPR/Cas9 system (using homologous recombination), the intended gene modification is precisely incorporated at the target site.

Concerning the approach as described in point (1)

The SBB is of the opinion that these applications of the CRISPR/Cas9 system fall within the **SDN-1 approach**, i.e. an approach used to generate site-specific mutations (small nucleotide deletions and/or insertions - Indels - at one target site, or deletions, duplications or inversions of DNA sequences between two target sites) via the transient presence of the CRISPR/Cas9 components without the use of an exogenous DNA template.

The alterations of the genetic material (mutations) in the animals and plants occur via non-homologous end joining (NHEJ), which is a cell's own error-prone process that frequently results in small sequence insertions or deletions (indels).

The type of genetic modifications obtained is not similar to the type of genetic modifications that are usually obtained using recombinant nucleic acid techniques, direct introduction of heritable material prepared outside the organism, or cell fusion or hybridization techniques (i.e. techniques listed in Bijlage 15 A of VL, 2004).

The type and extent of genetic modifications obtained is similar to what can be obtained by chemical mutagenesis, by irradiation or by spontaneous natural mutations, and are not distinguishable from them. The genetic modifications are a result of the cellular DNA repair mechanisms of the host and can occur naturally.

Off-target changes that could be induced by these applications of the CRISPR/Cas9 system are of the same type as those changes produced by conventional mutagenesis techniques, therefore not raising additional safety concerns.

These applications of the CRISPR/Cas9 system can be considered a refinement of the conventional mutagenesis (using chemicals or ionizing radiation) with an increased specificity and fewer unintended effects.

Concerning the approach as described in point (2)

The SBB is of the opinion that these applications of the CRISPR/Cas9 system fall within the **SDN-2 approach**, i.e. an approach used to generate site-specific mutations (small nucleotide deletions and/or insertions - Indels - at one target site) via the transient presence of the CRISPR/Cas9 components and of an exogenous DNA serving as template for the DNA repair.

The alterations of the genetic material (mutations) in the animals and plants occur via homologous recombination (HR), which is a cell's own error-free repair of a double strand break in DNA, in which the broken DNA molecule is repaired using a homologous sequence.

The type of genetic modifications obtained is not similar to the type of genetic modifications that are usually obtained using recombinant nucleic acid techniques, direct introduction of heritable material prepared outside the organism, or cell fusion or hybridization techniques (i.e. techniques listed in Bijlage 15 A of VL, 2004).

The exogenous DNA used as template for the DNA repair should not be considered as being a recombinant nucleic acid molecule in the meaning of Bijlage 15 A of VL, 2004. Indeed a recombinant nucleic acid molecule can be defined as a molecule that is generated by joining two or more nucleic acid molecules (EFSA, 2015).

The type and extent of genetic modifications obtained is similar to what can be obtained by chemical mutagenesis, by irradiation or by spontaneous natural mutations, and are not distinguishable from them. The genetic modifications are a result of the cellular DNA repair mechanisms of the host and can occur naturally.

Off-target changes that could be induced by these applications of the CRISPR/Cas9 system are of the same type as those changes produced by conventional mutagenesis techniques, therefore not raising additional safety concerns.

These applications of the CRISPR/Cas9 system can be considered a refinement of the conventional mutagenesis (using chemicals or ionizing radiation) with an increased specificity and fewer unintended effects.

Conclusions

In the light of the above-mentioned considerations the SBB is of the opinion that:

- From a science-based perspective, applications involving the transient presence of the CRISPR/Cas9 system delivered as purified ribonucleoprotein with or without a homologous repair DNA template (corresponding to SDN-1 and SDN-2 approaches) are a form of mutagenesis and do not raise additional safety concerns as compared to conventional mutagenesis techniques.
- The mutagenesis induced by these SDN-1 and SDN-2 approaches is a technique of genetic modification that does not involve the use of recombinant nucleic acid molecules or genetically modified organisms in the meaning of the chapeau of Bijlage 15 B of the Decree of the Flemish Government of 6 February 2004 (Annex II Part A of Directive 2009/41/EC).

The SBB therefore concludes that the intended uses under containment of animals and plants genetically modified as described in the present request should be considered for exclusion from the scope of the Decree of the Flemish Government of 6 February 2004, according to Annex 15 B of this Decree (Annex II Part A of Directive 2009/41/EC).

Generally, the SBB considers that in plants, there is a widespread and long history of use of mutagenesis techniques, both for research & development and in commercial breeding programmes. In animals, a same history of use of mutagenesis techniques exists in the context of the development of animal models in the lab (mainly for biomedical research), but not in breeding programmes.
Therefore, with regards to organisms genetically modified using the transient presence of the CRISPR/Cas9 system delivered as purified ribonucleoprotein according to the SDN-1 or SDN-2 approaches, the SBB considers that exclusion from the scope of the GMO legislation should apply to:

- all uses (with or without specific containment measures) involving such genetically modified plants;
- all cases where such genetically modified animals are used as models for research & development under containment.

In all other cases where such genetically modified animals are used (e.g. in breeding programmes), the SBB considers that the exclusion from the scope of the GMO legislation should only be granted on a case-by-case basis following a preliminary risk assessment.

The rationale presented above needs to be reassessed in case the CRISPR/Cas9 system would be used according to the SDN-1 or SDN-2 approaches to create mutations that go beyond small nucleotide deletions and/or insertions as described in the present request.

This opinion is delivered without prejudice to any further legal interpretation of the terms and provisions of the EU GMO Directives (2001/18/EC and 2009/41/EC) adopted at Belgian or EU level.

References

- ACRE (2013). ACRE advice: New techniques used in plant breeding. Advisory Committee on Releases to the Environment. Available at https://www.gov.uk/government/publications/genetically-modified-organisms-new-plant-growing-methods
- AR-KB (2005). *Arrêté royal du 21 février 2005 réglementant la dissémination volontaire dans l'environnement ainsi que la mise sur le marché d'organismes génétiquement modifiés ou de produits en contenant* (Moniteur belge - 24.02.2005 - Page 7129-7165) / *Koninklijk besluit van 21 februari 2005 tot reglementering van de doelbewuste introductie in het leefmilieu evenals van het in de handel brengen van genetisch gemodificeerde organismen of van producten die er bevatten* (Belgisch Staatsblad - 24.02.2005 - Bz 7129-7165).
- BAC (2007). *Advice of the Belgian Biosafety Advisory Council on the use of "Targeted Gene Repair" as a strategy to develop novel organisms.* 13/06/2007. Ref. WIV-ISP/BAC/2007_SC_529.
- Belhaj et al. (2013). *Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system.* Plant Methods 9, 39.
- Belhaj et al. (2015). *Editing plant genomes with CRISPR/Cas9.* Curr. Opin. Biotechnol., 32:76–78.
- Bortesi and Fischer (2015). *The CRISPR/Cas9 system for plant genome editing and beyond.* Biotechnol Adv. 33(1):41-52.
- Breyer et al. (2009). *Genetic modification through oligonucleotide-mediated mutagenesis. A GMO regulatory challenge?* Environ. Biosafety Res. 8, 57-64.
- BVL (2015). *BVL Opinion on the legal classification of New Plant Breeding Techniques, in particular ODM and CRISPR-Cas9.* December 2015. Available at www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/Opinion_on_the_legal_classification_of_New_Plant_Breeding_Techniques.pdf?__blob=publicationFile&v=2
- Cho et al. (2014). *Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases.* Genome Res. 24(1):132-41.
- COGEM (2014). *CRISPR-Cas - Revolution from the lab.* COGEM Report and advice CGM/141030-01.
- EC (2001). *Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC.* Off. J. Eur. Union L 106, 1-38.
- EC (2009). *Directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified micro-organisms.* Off. J. Eur. Union L 125, 75-97.
- Eckerstorfer et al. (2014). *New Plant Breeding Techniques and Risks Associated with their Application.* Wien, 2014. Reports, Band 0477. ISBN: 978-3-99004-282-3. 94 p. Available at http://www.umweltbundesamt.at/aktuell/publikationen/publikationssuche/publikationsdetail/?pub_id=2054
- EFSA (2012). *EFSA Panel on Genetically modified organisms (GMO); Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function.* EFSA Journal 2012;10(10):2943. [31 pp.] doi:10.2903/j.efsa.2012.2943. Available online: www.efsa.europa.eu/efsajournal
- EFSA (2015). *Answer of the European Food Safety Authority to a request from the European Commission (Mandate M-2015-0183) to provide technical assistance on issues related to the legal analysis of new plant breeding techniques* [Ref. EW/AGe/AGo/MR/lg(2015)].
- Greene et al. (2003). *Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in Arabidopsis.* Genetics, 164, 731-740.
- HCB (2016). Haut Conseil des Biotechnologies. Nouvelles techniques – New plant breeding techniques – Première étape de la réflexion du HCB – Comité scientifique.
- Hilton and Gersbach (2015). *Enabling functional genomics with genome engineering.* Genome Res. 25, 1442–1455.

- Hsu et al. (2014). *Development and applications of CRISPR-Cas9 for genome engineering.* Cell 157: 1262-1278.
- Kim et al. (2014). *Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins.* Genome Res. 24(6): 1012–1019.
- Kim et al. (2016a). *Genome-wide target specificities of CRISPR-Cas9 nucleases revealed by multiplex Digenome-seq.* Genome Res. 26: 406–415.
- Kim et al. (2016b). *Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells.* Nature Biotechnology 34: 863–868.
- Kleinstiver et al. (2016). *High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects.* Nature 529: 490–495.
- Kouranova et al. (2016). *CRISPRs for Optimal Targeting: Delivery of CRISPR Components as DNA, RNA, and Protein into Cultured Cells and Single-Cell Embryos.* Human Gene Therapy 27(6): 464-475.
- Kuroyanagi et al. (2013). *New approach for fish breeding by chemical mutagenesis: establishment of TILLING method in fugu (Takifugu rubripes) with ENU mutagenesis.* BMC Genomics 14:786.
- Ledford (2016). *Beyond CRISPR: A guide to the many other ways to edit a genome.* Nature 536: 136–137.
- Luo et al. (2016). *Applications of CRISPR/Cas9 technology for targeted mutagenesis, gene replacement and stacking of genes in higher plants.* Plant Cell Rep. 35(7):1439-50.
- Lusser et al. (2012). *Deployment of new biotechnologies in plant breeding.* Nature Biotech. 30(3):231.
- Lusser and Davies (2013). Comparative regulatory approaches for groups of new plant breeding techniques. New Biotechnology, Volume 30, Number 5, June 2013, 437.
- Marx (2014). *Gene editing: how to stay on-target with CRISPR.* Nature Methodology 11:1021-1025.
- NTWG (2012). New Techniques Working Group. Final Report.
- Pauwels et al. (2014) *Engineering nucleases for gene targeting: safety and regulatory considerations.* New Biotechnology 31(1):18-27.
- Podevin et al. (2012). *Transgenic or not? No simple answer!* EMBO Rep. 13(12):1057–1061.
- Podevin et al. (2013). *Site-directed nucleases: A paradigm shift in predictable, knowledge-based plant breeding.* Trends in Biotechnology 31, 375-383.
- Riordan et al. (2015). *Application of CRISPR/Cas9 for biomedical discoveries.* Cell Biosci. 2015; 5: 33.
- Sander and Joung (2014). *CRISPR–Cas systems for editing, regulating and targeting genomes.* Nature Biotechnol. 32: 347–355.
- Schaart and Visser (2009). *Novel Plant Breeding Techniques – Consequences of new genetic modification-based plant breeding techniques in comparison to conventional plant breeding.* COGEM Research Report number 2009-02. The Netherlands Commission on Genetic Modification; 2009.
- Silver (1995). *Mouse Genetics. Concepts and Applications.* Oxford University Press. Available online: http://www.informatics.jax.org/silver/index.shtml
- Singh et al. (2015). *A mouse geneticist's practical guide to CRISPR applications.* Genetics 199(1):1-15
- Song et al. (2016). *CRISPR/Cas9: A powerful tool for crop genome editing.* The Crop Journal 4(2)2: 75–82.
- Stottmann and Beier (2014). *ENU Mutagenesis in the Mouse.* Curr Protoc Hum Genet. 82: 15.4.1–15.4.10.
- Subburaj et al. (2016) *Site-directed mutagenesis in Petunia x hybrid protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins.* Plant Cell Rep 35(7):1535-44.
- SWE (2015). Swedish Board of Agriculture, *CRISPR/Cas9 mutated Arabidopsis, 2015*. Available at: www.upsc.se/documents/Information_on_interpretation_on_CRISPR_Cas9_mutated_plants_Final.pdf
- Tan et al. (2016). Gene targeting, genome editing: from Dolly to editors. Transgenic Res. 25(3):273-87.
- Tsai et al. (2015). *GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases.* Nature Biotechnol. 33: 187–197.
- VL (2004). *Besluit van de Vlaamse regering van 6 februari 2004 tot wijziging van het besluit van de Vlaamse regering van 6 februari 1991 houdende vaststelling van het Vlaams reglement betreffende*

de milieuvergunning, en van het besluit van de Vlaamse regering van 1 juni 1995 houdende algemene en sectorale bepalingen inzake milieuhygiëne. Belgisch Staatsbled, 01.04.2004, p. 18281.

- Wang et al. (2015a). *Unbiased detection of off-target cleavage by CRISPR-Cas9 and TALENs using integrase-defective lentiviral vectors.* Nature Biotechnol. 33: 175–178.
- Wang et al. (2015b). *Large genomic fragment deletion and functional gene cassette knock-in via Cas9 protein mediated genome editing in one-cell rodent embryos.* Scientific reports 5:17517.
- Wansleeben et al. (2011) *An ENU-Mutagenesis Screen in the Mouse: Identification of Novel Developmental Gene Functions.* PLoS ONE 6(4): e19357.
- Weeks et al. (2016), *Use of designer nucleases for targeted gene and genome editing in plants.* Plant Biotechnol J, 14: 483–495.
- Wolt et al. (2016). *The Regulatory Status of Genome-edited Crops.* Plant Biotechnol J, 14: 510–518.
- Woo et al. (2015) *DNA-free genome editing in plants with preassembled CRISPR–Cas9 ribonucleoproteins.* Nat Biotechnol 33:1162–1164.
- Wyman and Kanaar (2006). *DNA double-strand break repair: all's well that ends well.* Annu. Rev. Genet. 40: 363-383.
- Yang et al. (2014). *Generating genetically modified mice using CRISPR/Cas-mediated genome engineering.* Nat Protoc. 9(8):1956-68.
- Zhang et al. (2015). *Off-target Effects in CRISPR/Cas9-mediated Genome Engineering.* Molecular Therapy Nucleic Acids 4, e264.