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Advice of the Biosafety and Biotechnology Unit (SBB) concerning genome editing in plants using the CRISPR/Cas9 system

Summary

- The SBB is of the opinion that the genetic modifications achieved in the plants to be released in the field 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. Genome editing in plants using the CRISPR/Cas9 system as described in the present request can thus be considered as a form of mutagenesis.
- The SBB is of the opinion that genome editing in plants using the described CRISPR/Cas9 system does not raise additional safety concerns as compared to the conventional mutagenesis techniques.
- The SBB is of the opinion that the intermediate plants containing an exogenous T-DNA cassette encoding the components of the CRISPR/Cas9 system have been developed using a recombinant nucleic acid technique. They should therefore be considered as GMOs according to the Belgian Royal Decree on GMOs of 21 February 2005.
- When the T-DNA cassette encoding the components of the CRISPR/Cas9 system has been effectively segregated away, the SBB is of the opinion that the resulting plants should not be considered as GMOs in the meaning of the GMO regulatory framework.
- The SBB considers that the mutagenesis induced by the transient presence of the CRISPR/Cas9 system as described in the present request 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 Annex I B of the Belgian Royal Decree on GMOs of 21 February 2005 (Annex I B of Directive 2001/18/EC).
- **Provided that the exogenous DNA used in the intermediate step (T-DNA cassette encoding the components of the CRISPR/Cas9 system) has been effectively removed by segregation, the SBB concludes that the genetically modified plants to be released in the field as described in the present request should be considered for exclusion from the scope of the Belgian Royal Decree on GMOs of 21 February 2005, according to Annex I B of this Decree (Annex I B of Directive 2001/18/EC).**
- This conclusion applies to the field trial under consideration and to all cases where the CRISPR/Cas9 system is used according to the so-called SDN-1 approach, i.e. 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) without the simultaneous delivery of exogenous DNA (DNA template or full gene).
- This conclusion applies only to the above-mentioned applications of the CRISPR/Cas9 technology in plants.
- This advice 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.

Background

On 16 June 2016 the Biosafety and Biotechnology Unit (SBB) received a request to advise the Federal Competent Authority (Federal Public Service (FPS) Health, Food Chain Safety and Environment) about the GMO status within the meaning of the Belgian Royal Decree of 21 February 2005 (AR-KB, 2005) of certain plants genetically modified using the CRISPR/Cas9 technique.
This request was made following a question from a Belgian research institute whether or not the GMO regulatory framework should be applied for field trials with certain CRISPR/Cas9-modified plants.

To substantiate the request a short description of the plants to be tested in the field was provided by the Belgian research institute. The plants described were transformed using disarmed *Agrobacterium tumefaciens* with CRISPR/Cas9 containing T-DNA constructs. As a result of the cell's natural DNA-repair process at the double strand breaks induced by the CRISPR/Cas9 system (and without the use of exogenous homologous DNA template) point mutations or short deletions were created at target loci in the plant genome. Once the targeted genomic changes have been achieved, the introduced T-DNA constructs containing the components of the CRISPR/Cas9 system were removed by segregation to generate plants (those to be tested in the field) that do not contain exogenous DNA anymore.

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 (summarized in the introduction below).

Introduction

Plant breeding: From natural mutations to conventional mutagenesis

Genetic variation is common in plant species as a result of several factors including retrotransposons ("jumping genes"), single base pair polymorphisms (SNPs), horizontal gene transfer and mutations. This extensive genetic variation served as a basis to select new plant varieties.

Mutations occur naturally and sometimes result in the development of new beneficial traits. However, the frequency of such mutations (e.g. not more than 10^{-5} to 10^{-8} per gene or locus in one plant generation) is too low to rely on for accelerated plant breeding (EFSA, 2012).
Since World War II, the application of mutation techniques (mutagenesis) has generated a vast amount of genetic variability and is playing a significant role in plant breeding. Mutation breeding is particularly valuable in those crops with restricted genetic variability or where introgression of genes from wild relatives is difficult and time-consuming. 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/).

Conventional mutagenesis techniques are based on the fact that chemicals (such as ethyl methane sulphonate – EMS) or irradiation (non-ionising - e.g. UV - or ionising radiation - e.g. X-rays, gamma rays) 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. In eukaryotic somatic cells, including plant cells, the NHEJ pathway is generally preferred (EFSA, 2012). 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). HR plays a major role during meiosis. During HR, nucleotide sequences are exchanged between two very similar or identical DNA molecules.

Although conventional mutagenesis has been widely applied, there are two serious limitations to the use of induced physical and chemical mutagenesis (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.

Genome-editing techniques

During the past decade, genome-editing (or gene-editing) techniques have been developed that allow the direct modification of the plant genome at specific locations. They generally use nucleases that cleave DNA at specific sites and trigger the plant'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. 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.

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.

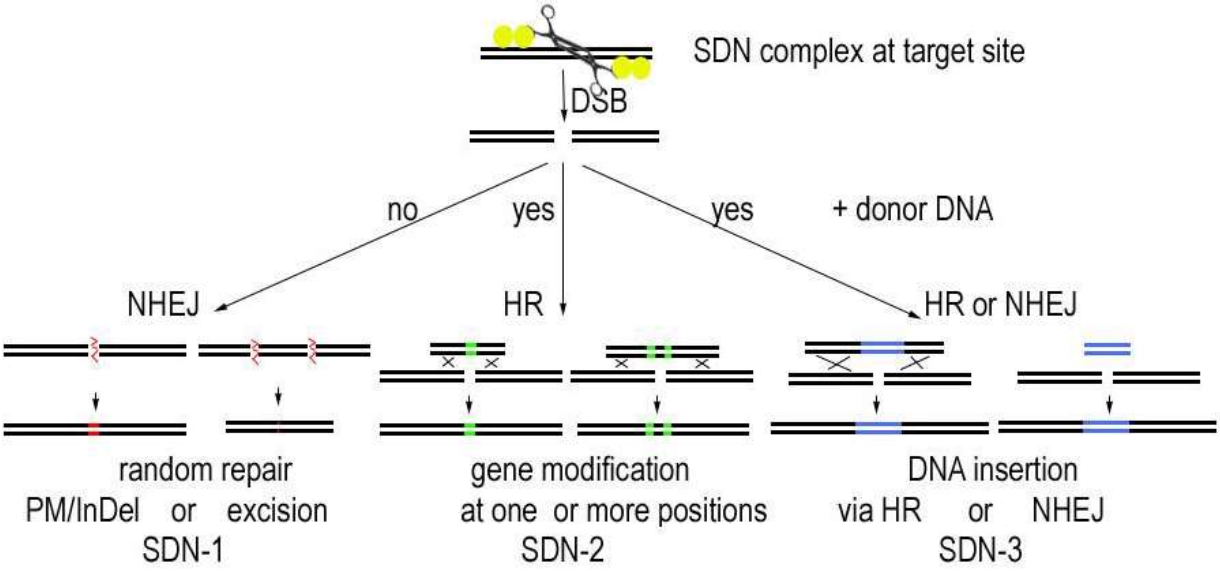

***Figure 1**: Applications of site-directed nuclease (SDN) techniques (Source: EFSA, 2012)*

The components of the SDN system (nuclease and possibly guide RNA) can be delivered into the plant cells in different ways. 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 (see e.g. Woo *et al.*, 2015 and Subburaj *et al.,* 2016 for CRISPR/Cas9).

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).

Plant genome editing and off-target mutations

Specificity is an important endeavour 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 minimised by selecting target sequences that have reduced numbers of off-target homologues in the genome (bioinformatics tools can be used for that purpose). Novel types of Cas9 proteins have also been discovered that could contribute to reduce 'off-site' targeting (Belhaj *et al.*, 2013; Song *et al.*, 2016).

In plants, the few studies published so far reported low to negligible off-target activity compared to animal systems (Belhaj *et al.*, 2015, Weeks *et al.*, 2016). In any case, the frequency of off-target mutations caused by the SDN-1 approach 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; Green et al., 2003; Podevin *et al.*, 2012). Furthermore, as in conventional breeding, unintended mutations can be segregated away during the selection and breeding process.

Regulatory considerations

Under the GMO legislation, a genetically modified organism is "an 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; EU, 2001). 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). 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 hybridisation 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), 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.
Plants genetically modified by conventional mutagenesis techniques are therefore exempted from the EU GMO legislation. The main 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 approach the question is whether applications of the system fall under the definition of genetic modification (and therefore under the GMO legislation) or whether they could be exempted from the GMO legislation by analogy to conventional mutagenesis.

From a science-based perspective, there are arguments supporting the view that the SDN-1 approach 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). 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 approach should be considered for exclusion from the GMO legislation (ACRE, 2013; BVL, 2015; HCB, 2016; Schaart and Visser, 2009; SWE, 2015). 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

Concerning the genetically modified plants to be released in the field

The SBB is of the opinion that the applications of the CRISPR/Cas9 system to develop the plants to be released in the field as described in the present request 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.

The alterations of the genetic material (mutations) in the plants to be released in the field do not make use of an exogenous DNA template and 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 hybridisation techniques.

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 induced by applications of the CRISPR/Cas9 system as described in the present request are of the same type as those changes produced by conventional breeding techniques, therefore not raising additional safety concerns. Moreover, unintended mutations can be segregated away during the selection and breeding process.

Applications of the CRISPR/Cas9 system as described in the present request can be considered a refinement of the conventional mutagenesis (using chemicals or ionizing radiation) with an increased specificity and fewer unintended effects. According to the GMO legislation conventional mutagenesis encompasses techniques of genetic modification that have conventionally been used in a number of applications and have a long safety record.

Concerning the intermediate genetically modified plants containing the T-DNA

At an early and intermediate step in the development of the plants to be released in the field a T-DNA cassette encoding the components of the CRISPR/Cas9 system is stably introduced into the plant genome. The SBB is of the opinion that the corresponding intermediate plants have been developed using a recombinant nucleic acid technique. They should therefore be considered as GMOs and their potential risks for human health and the environment assessed accordingly.

When the T-DNA cassette encoding the components of the CRISPR/Cas9 system has been effectively segregated away, the SBB is of the opinion that the resulting plants should not be considered as GMOs in the meaning of the GMO regulatory framework.
It is the responsibility of the user to ensure that these resulting plants are effectively free of exogenous DNA.

The SBB considers that the development of the GM plants containing the T-DNA cassette on the one hand, and the mutagenesis induced by the transient presence of the CRISPR/Cas9 system on the other hand, are two distinct technical processes. The mutagenesis does not involve *per se* the use of recombinant nucleic acid molecules or genetically modified organisms (the components of the CRISPR/Cas9 system can be delivered by other means into the plant cells).

Conclusions

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

- From a science-based perspective, the applications of the CRISPR/Cas9 system using the SDN-1 approach as described in the present request are a form of mutagenesis and do not raise additional safety concerns as compared to conventional mutagenesis techniques.
- The mutagenesis induced by the transient presence of the CRISPR/Cas9 system as described in the present request 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 Annex I B of the Belgian Royal Decree on GMOs of 21 February 2005 (Annex I B of Directive 2001/18/EC).

The SBB therefore concludes that provided that the exogenous DNA (T-DNA cassette encoding the components of the CRISPR/Cas9 system) used in the intermediate step has been effectively removed by segregation, the genetically modified plants to be released in the field as described in the present request should be considered for exclusion from the scope of the Belgian Royal Decree on GMOs of 21 February 2005, according to Annex I B of this Decree (Annex I B of Directive 2001/18/EC).

This conclusion applies also to all cases where the CRISPR/Cas9 system is used in plants according to the SDN-1 approach.

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).
- 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.
- 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
- 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.
- 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.
- 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.
- Song et al. (2016). *CRISPR/Cas9: A powerful tool for crop genome editing.* The Crop Journal 4(2)2: 75–82.
- 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
- 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.