

ADOPTED: 14 October 2020

doi: 10.2903/j.efsa.2020.6299

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

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Abstract

The European Commission requested the EFSA Panel on Genetically Modified Organisms (GMO) to assess whether section 4 (hazard identification) and the conclusions of EFSA's *Scientific opinion on the risk assessment of plants developed using zinc finger nuclease type 3 technique (ZFN-3) and other site-directed nucleases (SDN) with similar function* are valid for plants developed via SDN-1, SDN-2 and oligonucleotide-directed mutagenesis (ODM). In delivering this Opinion, the GMO Panel compared the hazards associated with plants produced via SDN-1, SDN-2 and ODM with those associated with plants obtained via both SDN-3 and conventional breeding. Unlike for SDN-3 methods, the application of SDN-1, SDN-2 and ODM approaches aims to modify genomic sequences in a way which can result in plants not containing any transgene, intragene or cisgene. Consequently, the GMO Panel concludes that those considerations which are specifically related to the presence of a transgene, intragene or cisgene included in section 4 and the conclusions of the Opinion on SDN-3 are not relevant to plants obtained via SDN-1, SDN-2 or ODM as defined in this Opinion. Overall, the GMO Panel did not identify new hazards specifically linked to the genomic modification produced via SDN-1, SDN-2 or ODM as compared with both SDN-3 and conventional breeding. Furthermore, the GMO Panel considers that the existing *Guidance for risk assessment of food and feed from genetically modified plants* and the *Guidance on the environmental risk assessment of genetically modified plants* are sufficient but are only partially applicable to plants generated via SDN-1, SDN-2 or ODM. Indeed, those guidance documents' requirements that are linked to the presence of exogenous DNA are not relevant for the risk assessment of plants developed via SDN-1, SDN-2 or ODM approaches if the genome of the final product does not contain exogenous DNA.

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Keywords: site-directed nuclease, SDN-1, SDN-2, SDN-3, oligonucleotide-directed mutagenesis, transgenesis, off-target, genetically modified plants, risk assessment, EFSA guidance

Requestor: European Commission

Question number: EFSA-Q-2019-00297

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Acknowledgements: The GMO Panel wishes to thank the following for the support provided to this scientific output: the Molecular Characterisation Working Group of the GMO Panel and the EFSA staff Nikoletta Papadopoulou and Elisabeth Waigmann.

Suggested citation: EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Naegeli H, Bresson J-L, Dalmay T, Dewhurst IC, Epstein MM, Firbank LG, Guerche P, Hejatko J, Moreno FJ, Mullins E, Nogué F, Sánchez Serrano JJ, Savoini G, Veromann E, Veronesi F, Casacuberta J, Gennaro A, Paraskevopoulos K, Raffaello T and Rostoks N, 2020. 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 Journal* 2020;18(11):6299, 14 pp. <https://doi.org/10.2903/j.efsa.2020.6299>

ISSN: 1831-4732

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The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.



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1. Introduction

1.1. Background as provided by the European Commission¹

The judgment of the Court of Justice of the European Union in Case C-528/16 on mutagenesis has clarified that Directive 2001/18/EC² is applicable to genetically modified organisms (GMOs) obtained by mutagenesis techniques that have emerged since its adoption ('new mutagenesis techniques').

Directive 2001/18/EC regulates the deliberate release of GMOs into the environment. In 2010, the EFSA Panel on Genetically Modified Organisms issued the *Guidance on the environmental risk assessment of genetically modified (GM) plants* (EFSA GMO Panel 2010) and in 2011 the *Guidance on the risk assessment of food and feed from GM plants* (EFSA GMO Panel 2011). Following a request of the European Commission, in 2012 EFSA published a *Scientific opinion addressing the safety assessment of plants developed using zinc finger nuclease 3 and other site-directed nucleases with similar function* (SDN-3) (EFSA GMO Panel (2012a), hereafter 'EFSA Scientific Opinion on SDN-3'). In this Scientific Opinion, the assessment methodology applied by the EFSA GMO Panel was to compare the hazards associated with plants produced by the SDN-3 technique with those obtained by conventional plant breeding techniques and by currently used transgenesis. Among the conventional plant breeding techniques, the EFSA GMO Panel considered certain mutation breeding techniques that emerged before the adoption of Directive 2001/18/EC and that are used as a tool to create genetic variation.

The Scientific Opinion concluded that 'the SDN-3 technique can minimise hazards associated with the disruption of genes and/or regulatory elements in the recipient genome. Whilst the SDN-3 technique can induce off-target changes in the genome of the recipient plant, these would be fewer than those occurring with most mutagenesis techniques. Furthermore, where such changes occur, they would be of the same types as those produced by conventional breeding techniques.'

The EFSA GMO Panel also concluded that its 2010 and 2011 guidance documents 'are applicable for the evaluation of food and feed products derived from plants developed using the SDN-3 technique and for performing an environmental risk assessment. However, on a case-by-case basis lesser amounts of event-specific data may be needed for the risk assessment of plants developed using the SDN-3 technique.'

1.2. Background as provided by EFSA

Following a request from the European Commission (Ref. Ares(2019)2488590-09/04/2019), in April 2019 EFSA assigned the mandate to the molecular characterisation working group of the GMO Panel (Ref. BU/GdS/KL/FA/cz_OC-2019-21268932). To allow for public consultation, EFSA asked the European Commission to change the Scientific Opinion deadline (Ref. BU/GdS/EW(2019)OC-2019-22763474) which was then extended from 30 April 2020 to 30 October 2020 (Ref. Ares(2020)250930-15/01/2020).

1.3. Terms of reference¹

Against this background, the European Commission, in accordance with Article 29 of Regulation (EC) No 178/2002³, asked EFSA to address the following two terms of reference (ToR):

- 1) To advise whether the assessment methodology described in section 4 of the *EFSA scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function* may be applicable, in whole or in part, to plants developed with type 1 and type 2 site-directed nucleases and with oligonucleotide-directed mutagenesis.

¹ The text of this section was provided by the European Commission as part of the mandate's documentation. The document [Annex to letter (ToR)] can be found in the EFSA register of questions at <http://registerofquestions.efsa.europa.eu/roqFrontend/questionsListLoader?mandate=M-2019-0095>

² 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 - Commission Declaration. OJ L 106, 17.4.2001, p. 1–39.

³ Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

If the advice to ToR1 is affirmative, the Commission would ask EFSA, in accordance with Article 29 of Regulation (EC) No 178/2002:

- 2) To advise whether the conclusions of the EFSA 2012 *Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function* are valid, in whole or in part, to plants developed with type 1 and type 2 site-directed nucleases and with oligonucleotide-directed mutagenesis.

2. Data and methodologies

To address the two terms of reference, the molecular characterisation working group (MC WG) took into consideration both section 4 and the overall conclusions of the EFSA Opinion on SDN-3 and the relevant information reported in the scientific literature. Background information, the content of section 4, and the overall conclusions of the EFSA Opinion on SDN-3 are provided in Section 2.1 below.

2.1. EFSA Opinion on SDN-3

2.1.1. Background information

In 2012, EFSA issued two Opinions on new plant breeding techniques, the first on cisgenesis and intragenesis (EFSA GMO Panel, 2012b) and the second on SDN-3 (EFSA GMO Panel, 2012a). To develop the Opinion on SDN-3, the GMO Panel was asked: i) to determine the risks in terms of the impact on humans, animals and the environment that ZFN-3 could pose, by comparing plants developed using ZFN-3 techniques with plants obtained by conventional plant breeding techniques and with plants obtained with currently used genetic modification techniques; and ii) to determine whether there was a need for new guidance on the risk assessment of plants generated using ZFN-3 techniques or whether the existing guidance on food and feed risk assessment (EFSA GMO Panel, 2011) and on environmental risk assessment of GM plants (EFSA GMO Panel, 2010) should be updated or further elaborated. It should be noted that the guidance on food and feed risk assessment (EFSA GMO Panel, 2011) was superseded by Implementing Regulation (EU) No 503/2013⁴.

To develop the Scientific Opinion on SDN-3, the GMO Panel compared plants developed using SDN-3 methods with plants obtained by conventional breeding, including the use of both spontaneous and induced mutations (EFSA GMO Panel 2012a).

2.1.2. Section 4 of the EFSA Opinion on SDN-3

Section 4 of the EFSA Opinion on SDN-3 focuses on the hazards associated with plants that are produced using SDN-3 methods that are used to target the insertion of transgenes, intragenes or cisgenes to a specific plant genomic locus. The GMO Panel concluded that:

'Hazards that might result from various plant breeding techniques are related to the source of genes used, the genes and traits deployed and changes to the structure, organisation and sequence of the recipient genome. The primary drivers are the genetic alterations that various breeding processes introduce into the plants, as all other changes that take place are direct or indirect consequences of these changes. Hazards regarding these alterations may arise both in conventional breeding and in transgenesis.

'The ZFN-3 technique, and SDN-3 in general, is used for targeted insertion of DNA. With respect to the genes introduced, the SDN-3 technique does not differ from the other genetic modification techniques currently used, and can be used to introduce transgenes, intragenes or cisgenes. The hazards related to the source of genes have been described by EFSA (EFSA GMO Panel 2012b).

'The SDN-3 technique makes use of the same transformation techniques as transgenesis, although both transient and stable expression of the SDN can be used to introduce the site-specific DSB [double-strand break]. In the case of stable integration of the SDN genes, they can subsequently be removed by segregation to obtain plants containing only the integrated gene' (EFSA GMO Panel, 2012a).

⁴ Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. OJ L 157, 8.6.2013, p. 1–48.

2.1.3. Conclusions of the EFSA Opinion on SDN-3

In the overall conclusions of the EFSA Opinion on SDN-3, the GMO Panel stated that:

'The EFSA GMO Panel compared the hazards associated with plants produced by the SDN-3 technique with those associated with plants obtained by conventional plant breeding techniques and by currently used transgenesis.

'The main difference between the SDN-3 technique and transgenesis is that the insertion of DNA is targeted to a predefined region of the genome. Therefore, the SDN-3 technique can optimise the genomic environment for gene expression and minimise hazards associated with the disruption of genes and/or regulatory elements in the recipient genome.

'The SDN-3 technique can induce off-target changes but these would be fewer than those occurring with most mutagenesis techniques. Where they do occur, the changes would be the same types as those produced by conventional breeding techniques.

'With respect to the genes introduced, the SDN-3 technique does not differ from the other genetic modification techniques currently used, and can be used to introduce transgenes, intragenes or cisgenes.

'The EFSA GMO Panel considers that the Guidance for risk assessment of food and feed from genetically modified plants (EFSA GMO Panel, 2011) and the Guidance on the environmental risk assessment of genetically modified plants (EFSA GMO Panel, 2010) are applicable for the evaluation of food and feed products derived from plants developed using the SDN-3 technique and for performing an environmental risk assessment. However, on a case-by-case basis, lesser amounts of event-specific data may be needed for the risk assessment of plants developed using the SDN-3 technique. There is therefore a need for flexibility in the data requirements for risk assessments.' (EFSA GMO Panel, 2012a)

2.2. Consultation

In line with its policy on openness and transparency, EFSA consulted EU Member States and its stakeholders via an online public consultation. Between April and May 2020, interested persons were invited to submit their comments on the draft GMO Panel Scientific Opinion.⁵ Following this consultation process, the document was revised by the GMO Panel and the experts of the MC WG.

The outcome of the online public consultation is reported in an EFSA Technical report that will be published on EFSA's website together with the final Scientific Opinion.

3. Assessment

3.1. Introduction

3.1.1. Comparison of SDN-1, SDN-2 and ODM with SDN-3

An operational definition of site-directed nuclease (SDN) is provided by the Explanatory Note on New Techniques in Agricultural Biotechnology from the Group of Chief Scientific Advisors, Scientific Advice Mechanism (SAM) of the European Commission, as 'an enzyme (endonuclease) that creates site-specific double-strand breaks (DSBs) at defined sequences. SDN typically recognises a specific DNA sequence and 'cleaves' DNA within such a sequence or nearby. The recognition of the DNA target is mediated by the protein molecule itself (in protein-directed SDNs) or by an associated guide RNA molecule (in RNA-directed SDNs).' (European Commission, 2017).

Depending on the approach chosen when using an SDN, different outcomes are possible. In the SDN-1 application, the plant non-homologous end-joining (NHEJ) repair pathway is exploited to introduce random mutations (substitutions, insertions and deletions) at the target DSB site. Conversely, the SDN-2 approach makes use of template DNA to generate a predicted modification (i.e. intended sequence modification) at the target DSB site by exploiting the plant homology-directed repair (HDR) pathway. Finally, the SDN-3 approach can exploit both NHEJ and HDR to insert a large stretch of DNA in a targeted genomic location (EFSA GMO Panel, 2012a, Podevin et al., 2013).

ODM is set apart from SDN-based techniques because it does not rely on exogenous nucleases. The Explanatory Note on New Techniques in Agricultural Biotechnology defines ODM as an approach which is '[...] based on the use of oligonucleotides for the introduction of targeted mutations in the

⁵ Published at <http://www.efsa.europa.eu/en/calls/consultations>

genome, usually of one or a few adjacent nucleotides. The genetic changes that can be obtained using ODM include substitutions, insertions or deletions.’ (European Commission, 2017).

Overall, the application of SDN-1, SDN-2 and ODM methods results either in random (SDN-1) or predicted (SDN-2 and ODM) mutations of a targeted genomic locus without the insertion of exogenous DNA at the targeted locus. The aim of the SDN-3 approach is to modify the targeted locus by inserting an exogenous DNA template of various lengths.

3.1.2. Technology used in SDN-1, SDN-2 and ODM applications

The EFSA Opinion on SDN-3 addressed the development and the application of technologies in the area of plant genome editing up to the year 2012. In this regard, a literature review on zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and meganucleases was included (section 2.1 of EFSA GMO Panel (2012a)). In this Scientific Opinion on SDN-1, SDN-2 and ODM, the GMO Panel was not asked to deliver an extensive literature review on the technologies deployed in these three approaches. However, considering the advances in genome editing that have unfolded in recent years, the GMO Panel deemed it appropriate to include some information on them, which is discussed below.

Since 2012, a new RNA-directed SDN-type technology known as the CRISPR/Cas system (clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease) has emerged (Jinek et al., 2012). Although there are still reports describing the use of ZFNs and TALENs for editing plant genes and these technologies have been used to obtain products that are already on the market in the USA (e.g. Calyso High Oleic Soybean Oil and Meal derived from genome-edited soybean using the TALENs approach⁶), the CRISPR/Cas system has become *de facto* the preferred technology for genome editing (Chen et al., 2019). The CRISPR/Cas system has been applied in genome editing across multiple plant species, including model plants (see, e.g. Jiang et al. (2013), Li et al. (2013), Nekrasov et al. (2013)) and also crops like maize, sorghum, barley, potato, rice and wheat (see, e.g. Upadhyay et al. (2013), Liang et al. (2014), Modrzejewski et al. (2019), Afzal et al. (2020)). So far, the ODM technology has only been used to generate GM plants with relatively simple and easily selectable traits; e.g. herbicide resistance (Sauer et al., 2016), and the amount of information available in the literature in terms of the molecular mechanism, technological aspects, applications and intrinsic limitations of the system (i.e. efficiency and specificity in different plant species) is considered limited compared with SDN-based technologies such as the CRISPR/Cas system (Modrzejewski et al., 2019).

It should be noted that while ZFNs, TALENs, meganucleases and the CRISPR/Cas system can all be used to achieve random (SDN-1) and predicted (SDN-2) mutations of a targeted genomic locus and precise insertion of a DNA sequence (SDN-3), ODM is practically applied only to generate targeted gene modifications which resemble those of the SDN-2 type. Other techniques which have recently emerged such as base editing and prime editing (Komor et al., 2016; Anzalone et al., 2019; Lin et al., 2020) can be used to generate specific nucleotide changes in a targeted sequence without deploying any template DNA and without inducing DSB in the target locus. In this Scientific Opinion, the GMO Panel considers that the genetic modifications obtained using base editing and prime editing are comparable to those created by SDN-2 technology (see Section 3.1.1).

For a more extensive review of the technologies applied to generate genome-edited plants, the GMO Panel refers the reader to the explanatory note of the EU Scientific Advice Mechanism (European Commission, 2017) and to several comprehensive recent reviews (Doudna and Charpentier, 2014; Komor et al., 2017; Chen et al., 2019; Hua et al., 2019; Zhang, 2020).

3.1.3. Methods for delivering or expressing SDN in plants

In plants, site-directed mutagenesis can be achieved by the stable integration, the transient expression, or the ‘DNA-free’ delivery of the molecular components necessary to achieve the genetic mutation (hereafter, the SDN module). In the case of stable integration and for sexually propagated crops, the SDN module can be removed by segregation. This step will probably not be performed for commonly asexually (vegetatively) propagated crops. In general, in all those cases where the presence of the SDN gene cassette in the final product is not desirable, transient expression could be a valid alternative method to express the SDN module (Ma et al., 2017). For ‘DNA-free’ delivery, either the messenger RNA expressing the nuclease, the protein itself (for TALENs, ZFNs and meganucleases) or the ribonucleoprotein complex (for the CRISPR/Cas system) are directly delivered into the plant cell

⁶ <https://calyxt.com/first-commercial-sale-of-calyxt-high-oleic-soybean-oil-on-the-u-s-market/>

without the use of any intermediate sequence of DNA (Metje-Sprink et al., 2019). The possibility to deliver purified sequence-specific nucleases to a plant cell was first described in *Nicotiana tabacum* using meganucleases and TALENs (Luo et al., 2015). Since then, DNA-free delivery methods have been applied to several plant species including important crops like rice (Woo et al., 2015), wheat (Zhang et al., 2016; Bilichak et al., 2019), maize (Svitashev et al., 2016) and soybean (Kim et al., 2017). For ODM, the chemically synthesised oligonucleotide is directly delivered to the plant cell without the need for any stable or transient expression system. ODM has been successfully applied to several crops like maize (Zhu et al., 2000), rice (Okuzaki and Toriyama, 2004) and oilseed rape (Gocal et al., 2015). Multiple delivery systems for SDN components have been tested for different plant tissues, including PEG-fusion, electroporation and biolistics (for a review of CRISPR/Cas9 component delivery methods see (Sandhya et al., 2020)).

3.2. ToR1 of the mandate: applicability of section 4 of the EFSA Opinion on SDN-3 to plants obtained using SDN-1, SDN-2 and ODM approaches

3.2.1. Introduction

In addressing ToR1 of the mandate, the GMO Panel assessed section 4 of the EFSA Opinion on SDN-3, which compares the hazards associated with plants developed using SDN-3 approaches with those associated with transgenic and conventionally bred plants,⁷ and assessed its applicability to plants developed using SDN-1, SDN-2 and ODM applications. The GMO Panel envisages two possible scenarios. In the first one, the full SDN module, part of it and any nucleic acid sequence intentionally deployed during the genome editing process is present in whole or in part in the plant genome (see section 3.1.3). In this case, the product would be risk assessed as a transgenic plant with regard to the exogenous DNA integrated into the genome and as a gene-edited plant in relation to the target sequence(s) which was modified via SDN-1, SDN-2 or ODM approaches. In the second scenario, the SDN module and any nucleic acid sequence intentionally deployed during the genome editing process is not present in the genome of the edited plant (see Section 3.1.3). In this case, the assessment will only focus on the modification(s) resulting from the SDN activity. The assessment of section 4 of the EFSA Opinion on SDN-3 is described in Section 3.2.2 below.

3.2.2. Assessment of section 4 of the EFSA Opinion on SDN-3

3.2.2.1. Assessment of section 4.1: source of genes and safety of gene products

SDN-1, SDN-2 and ODM approaches differ from SDN-3 and transgenesis in that they do not aim to insert any DNA sequence but rather to modify an already existing endogenous sequence (see Section 3.1.1). Depending on the nature of the gene/locus modified and the origin of the allele and trait associated with the final product, the risk assessment process will necessarily take into consideration the history of safe use. For example, two very different scenarios, in terms of knowledge on the newly brought function, could be envisaged. On the one end, the new allele obtained through genome editing and the associated trait characterising the final product are already present in a consumed and/or cultivated variety of the same species. In this case, the risk assessment may focus on the knowledge of that variety (the history of safe use) and specific data on the edited gene and its product may not be needed. On the other end, the modified allele and associated trait present in the final product have never been described before. In this case, data on the new allele and the associated trait would be needed to perform the risk assessment. The GMO Panel considers that a substantial number of different scenarios can be envisaged between these two possibilities, and consequently, a range of data requirements may apply depending on the specific case. Depending on the product under assessment, in some cases only a subset of the data required for SDN-3 would be needed.

Because of all the above considerations, the GMO Panel concludes that section 4.1 of the EFSA Opinion on SDN-3 ('Source of genes and safety of gene products') is applicable only in part to plants developed by SDN-1, SDN-2 or ODM approaches.

⁷ From the EFSA Opinion on SDN-3: conventional breeding methods include a wide range of techniques (van der Wiel, et al. 2010). The EFSA GMO Panel considers the following techniques relevant for a comparison with plants developed by the SDN-3 technique: sexual crosses, bridge crosses, embryo rescue, somatic hybridisation, translocation breeding and mutation breeding.

3.2.2.2. Assessment of section 4.2: alteration to the genome

3.2.2.2.1. Alteration at the insertion site (section 4.2.1)

SDN-1 and SDN-2 approaches use the same molecular mechanisms to induce DSBs as SDN-3. Unlike the SDN-1 and SDN-2 approaches, the ODM approach is not designed to induce DSBs, and in this respect, it is different from SDN-3. Moreover, the application of recent technological developments in the area of genome editing (i.e. prime and base editing, see Section 3.1.2) does not induce DSB in the plant genome at any stage during the process; rather, they induce in general a single strand break at the target site. Irrespective of the approach used, the successful application of SDN-1, SDN-2 or ODM results in a sequence modification which is targeted to a specific predetermined genomic locus where no exogenous DNA is inserted. For these reasons, the considerations described in section 4.2.1 of the EFSA Opinion on SDN-3 which refers to: i) the targeted integration of the transgene, intragene or cisgene mediated by SDN-3; ii) the possibility to add or exchange specific genes at their native loci; and iii) the optimisation of the newly created junctions between the plant DNA and the inserted DNA, are all irrelevant for plants obtained using SDN-1, SDN-2 or ODM approaches.

Because of all the above considerations, the GMO Panel concludes that section 4.2.1 of the EFSA Opinion on SDN-3 ('Alteration at the insertion site') is not applicable to plants developed by SDN-1, SDN-2 or ODM approaches.

3.2.2.2.2. Alteration elsewhere in the genome (section 4.2.2)

The application of SDN-1, SDN-2 or ODM approaches aims to modify a predetermined plant genomic sequence. However, it can also introduce changes elsewhere in the genome because of the off-target activity associated with these applications and their processes (Hahn and Nekrasov, 2019). The off-target activity depends not only on the specificity of the technology used but also on the presence and accessibility of sequences which share a certain level of sequence similarity with the original target locus. In addition, some base editing systems have been shown to present Cas9-independent off-target effects linked to the base editor activity itself (Jin et al., 2019; Zuo et al., 2019). For these reasons, SDN-1, SDN-2 or base editing off-target activity might result in unintended mutations outside the original target sequence that can either be predictable (for SDN-1 and SDN-2) or not (for some base-editing systems) (Jin et al., 2019; Naeem et al., 2020). In recent years, considerable effort has been directed to the improvement of the efficiency and specificity of SDN-based technologies, particularly for the CRISPR/Cas system (including base editing; see Doman et al. (2020), Deng et al. (2020), Anzalone et al. (2020)). For example, designing shorter gRNAs or their improved design (Young et al., 2019), lowering intracellular concentration of the Cas-gRNA complex (Pattanayak et al., 2013), expressing specific anti-CRISPR proteins (Hoffmann et al., 2019), or RNP delivery (Svitashev et al., 2016) seem to generally reduce off-target effects. Moreover, the development (and/or the identification) of other CRISPR-associated nucleases has helped to improve efficiency and specificity and reduce off-target effects (Veillet et al., 2020).

In the EFSA Opinion on SDN-3, the GMO Panel concluded that the off-target mutations induced by the application of SDN-3 approaches are fewer than those occurring when applying conventional mutagenesis techniques that have been used previously and have a long history of safe use. In addition, backcrossing following the transformation process can be used to remove these potential off-target mutations from the final product, except for those that are genetically linked to the intentionally modified locus (EFSA GMO Panel, 2012a). Following the publication of the SDN-3 Opinion, experimental evidence has been published describing the type and number of off-target mutations generated by the application of SDN-based methods (Tang et al., 2018; Lee et al., 2019; Li et al., 2019). These publications confirmed that the off-target mutations potentially induced by SDNs are of the same type as those mutations used in conventional breeding, including spontaneous mutations and those produced by physical and chemical mutagenesis. Moreover, these publications also confirmed that the number of off-target mutations generated by SDN-based methods is lower than the number of mutations observed in conventional breeding due to spontaneous or induced mutations (Tang et al., 2018; Lee et al., 2019; Li et al., 2019). Therefore, the GMO Panel considers that the analysis of potential off-targets would be of very limited value for the risk assessment. In addition, although some biochemical and bioinformatic tools are available to identify potential off-target mutations (Bae et al., 2014; Tsai et al., 2015; Cameron et al., 2017; Akcakaya et al., 2018; Peng et al., 2018; Naeem et al., 2020), the limited availability and/or completeness of plant genomic sequences and their intraspecies and intravarietal variability would not always allow for a reliable prediction of potential off-target mutations (Tang et al., 2018; Lee et al., 2019). While an increasing number of publications have

investigated off-target effects for SDN-based technologies, the GMO Panel noticed that information on the off-target mechanism and frequency for ODM is quite limited (Modrzejewski et al., 2019). Finally, the EFSA Opinion on SDN-3 stated that backcrossing steps which follow the transformation process would likely remove off-target mutations from the genome of the final product (see above). The GMO Panel considers this aspect still applicable to plants generated via SDN-1, SDN-2 and ODM approaches.

When plant transformation is used to introduce the SDN module, the unintended insertion of plasmid DNA or other exogenous DNA into the plant genome can happen. Furthermore, the application of some methods (e.g. transient expression and DNA-free methods) to achieve SDN-1 and SDN-2 modifications can result in the unintended integration of exogenous DNA whose sequence may be known *a priori* (examples of unintended on-target insertion of exogenous DNA can be found in Clasen et al. (2016), Andersson et al. (2018), Norris et al. (2020), Solomon (2020)). If the final product is not intended to retain any exogenous DNA, the applicant should assess the potential presence of a DNA sequence derived from the methods used to generate the SDN modification (e.g. plasmids or vectors, see Section 3.1.3). It should be noted that the assessment of the unintentional integration of exogenous DNA is already part of the molecular characterisation in the risk assessment of GM plants, under EU Regulations. Therefore, this is not to be considered a new requirement for risk-assessing genome-edited plants.

Because of all the above considerations, the GMO Panel concludes that section 4.2.2 of the EFSA Opinion on SDN-3 ('Alteration elsewhere in the genome') is applicable to plants developed by SDN-1, SDN-2 or ODM approaches.

3.3. ToR2 of the mandate: applicability of the conclusions of the EFSA Opinion on SDN-3 to plants obtained using SDN-1, SDN-2 or ODM approaches

The EFSA GMO Panel (2012a) compared plants obtained by the application of the SDN-3 approach with plants produced by conventional breeding techniques and by currently used transgenesis. In the following section, the GMO Panel compared the hazards associated with plants produced via SDN-1, SDN-2 or ODM approaches with those associated with plants obtained via the SDN-3 approach. In addressing ToR2 of the mandate, the GMO Panel also considered its assessment reported in Section 3.2 and evaluated the conclusions of the EFSA Opinion on SDN-3 (EFSA GMO Panel, 2012a). The following considerations are raised:

- 1) The conclusion referring to the optimisation of the genomic context of the transgene, cisgene or intragene insertion in SDN-3 plants is not applicable to plants obtained via SDN-1, SDN-2 or ODM approaches, since these methods aim to modify an endogenous DNA sequence without the insertion of any transgene, intragene or cisgene.
- 2) The EFSA Opinion on SDN-3 concluded that the application of SDN-3 can induce off-target mutations but these would be fewer than those occurring with most mutagenesis techniques. Where they do occur, these changes would be the same types as those derived by conventional breeding techniques (EFSA GMO Panel, 2012a). As SDN-1 and SDN-2 techniques use the same molecular mechanisms to generate DSB as SDN-3, the conclusions for SDN-3 are also applicable to SDN-1 and SDN-2. In the case of ODM, although very limited information on the mechanisms and frequency of off-target effect is available in the literature, it is reasonable to assume that the same conclusions also apply because this technology is based on sequence-specific site recognition, as are SDN-based methods.
- 3) The conclusion addressing the risk assessment of the introduced transgene, intragene or cisgene is not applicable because of the reason outlined in point 1. However, the GMO Panel considers that in some cases, the SDN module could be stably introduced as a transgene in the plant genome. In these cases, the obtained plant should be considered a transgenic plant and the presence of the transgene will be risk-assessed according to all the provisions laid down in the EU regulation of GMOs.
- 4) In the EFSA Opinion on SDN-3, the GMO Panel concluded that the guidance for the risk assessment of food and feed from GM plants (EFSA GMO Panel, 2011) and the guidance on the environmental risk assessment of GM plants (EFSA GMO Panel, 2010) are applicable for the risk assessment of plants obtained using the SDN-3 method. The GMO Panel considers that the two EFSA guidance documents are sufficient but are only partially applicable to the risk assessment of plants generated by the application of SDN-1, SDN-2 or ODM methods.

Indeed, those requirements that relate to the presence of transgenes, intragenes or cisgenes are not relevant because of the reason outlined in point 1. In the EFSA Opinion on SDN-3, the GMO Panel also concluded that 'on a case-by-case basis lesser amounts of event-specific data are needed for the risk assessment' (EFSA GMO Panel, 2012a). The GMO Panel considers that this conclusion also applies to plants generated via SDN-1, SDN-2 or ODM approaches. Indeed, in the absence of any transgene, intragenes or cisgenes, the amount of experimental data needed for the risk assessment will mainly depend on the modified trait introduced and even less experimental data would be needed for plants produced via SDN-1, SDN-2 or ODM compared with plants generated via SDN-3.

4. Conclusions

In relation to ToR1, the GMO Panel concludes that the assessment methodology presented in section 4 of the EFSA Opinion on SDN-3 is partially applicable to SDN-1, SDN-2 and ODM. Since these approaches aim to modify an endogenous DNA sequence in a targeted manner, if the final product does not contain any transgene, intragene or cisgene, these plants will not present any of the hazards potentially associated with the inserted transgene, intragene or cisgene found in plants obtained using the SDN-3 approach. Moreover, the GMO Panel did not identify any additional hazard associated with the use of the SDN-1, SDN-2 or ODM approaches as compared with both SDN-3 and conventional breeding techniques which include conventional mutagenesis. The SDN-1 and SDN-2 approaches can induce off-target changes but, like for SDN3, these would be fewer than those occurring with classical mutagenesis techniques, decreasing the risk of alteration or interruption of genes.

In relation to ToR2, the GMO Panel concludes that the existing guidance for food and feed (EFSA GMO Panel, 2011) and environmental risk assessment (EFSA GMO Panel, 2010) is sufficient but is only partially applicable for the risk assessment of plants generated via SDN-1, SDN-2 or ODM approaches. Indeed, as SDN-1, SDN-2 and ODM aim to modify endogenous DNA sequence(s) without integrating exogenous DNA, a number of requirements in the existing guidance that are linked to the presence of a transgene are not relevant for the assessment of SDN-1, SDN-2 or ODM plants. The amount of experimental data needed for the risk assessment will mainly depend on the modified trait introduced and, therefore, the GMO Panel considers that the principle of a case-by-case approach to a risk assessment is particularly relevant for SDN-1, SDN-2 and ODM plants.

References

- Afzal S, Sirohi P and Singh NK, 2020. A review of CRISPR associated genome engineering: application, advances and future prospects of genome targeting tool for crop improvement. *Biotechnology Letters*, 42, 1611–1632.
- Akçakaya P, Bobbin ML, Guo JA, Malagon-Lopez J, Clement K, Garcia SP, Fellows MD, Porritt MJ, Firth MA, Carreras A, Baccega T, Seeliger F, Bjursell M, Tsai SQ, Nguyen NT, Nitsch R, Mayr LM, Pinello L, Bohlooly YM, Aryee MJ, Maresca M and Joung JK, 2018. *In vivo* CRISPR editing with no detectable genome-wide off-target mutations. *Nature*, 561, 416–419.
- Andersson M, Tureson H, Olsson N, Falt AS, Ohlsson P, Gonzalez MN, Samuelsson M and Hofvander P, 2018. Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. *Physiologia Plantarum*, 164, 378–384.
- Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, Chen PJ, Wilson C, Newby GA, Raguram A and Liu DR, 2019. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*, 576, 149–157.
- Anzalone AV, Koblan LW and Liu DR, 2020. Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. *Nature Biotechnology*, 38, 824–844.
- Bae S, Park J and Kim JS, 2014. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics*, 30, 1473–1475.
- Bilichak A, Sastry-Dent L, Sriram S, Simpson M, Samuel P, Webb S, Jiang FY and Eudes F, 2019. Genome editing in wheat microspores and haploid embryos mediated by delivery of ZFN proteins and cell-penetrating peptide complexes. *Plant Biotechnology Journal*, 18, 1307–1316.
- Cameron P, Fuller CK, Donohoue PD, Jones BN, Thompson MS, Carter MM, Gradia S, Vidal B, Garner E, Slorach EM, Lau E, Banh LM, Lied AM, Edwards LS, Settle AH, Capurso D, Llaca V, Deschamps S, Cigan M, Young JK and May AP, 2017. Mapping the genomic landscape of CRISPR-Cas9 cleavage. *Nature Methods*, 14, 600–606.
- Chen KL, Wang YP, Zhang R, Zhang HW and Gao CX, 2019. CRISPR/Cas genome editing and precision plant breeding in agriculture. *Annual Review of Plant Biology*, 70, 667–697.
- Clasen BM, Stoddard TJ, Luo S, Demorest ZL, Li J, Cedrone F, Tibebu R, Davison S, Ray EE, Daulhac A, Coffman A, Yabandith A, Retterath A, Haun W, Baltes NJ, Mathis L, Voytas DF and Zhang F, 2016. Improving cold storage and processing traits in potato through targeted gene knockout. *Plant Biotechnology Journal*, 14, 169–176.

- Deng C, Lv X, Li J, Liu Y, Du G and Liu L, 2020. Development of a DNA double-strand break-free base editing tool in *Corynebacterium glutamicum* for genome editing and metabolic engineering. *Metab Eng Commun*, 11, e00135.
- Doman JL, Raguram A, Newby GA and Liu DR, 2020. Evaluation and minimization of Cas9-independent off-target DNA editing by cytosine base editors. *Nature Biotechnology*, 38, 620–628.
- Doudna JA and Charpentier E, 2014. The new frontier of genome engineering with CRISPR-Cas9. *Science*, 346, 1077–+.
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2010. Guidance on the environmental risk assessment of genetically modified plants. *EFSA Journal* 2010;8(11):1879. <https://doi.org/10.2903/j.efsa.2010.1879>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2011. Guidance for risk assessment of food and feed from genetically modified plants EFSA Panel on Genetically Modified Organisms (GMO). *EFSA Journal* 2011;9(6):2193. <https://doi.org/10.2903/j.efsa.2011.2193>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2012a. 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. <https://doi.org/10.2903/j.efsa.2012.2943>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2012b. Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. *EFSA Journal* 2012;10(2):2561.
- European Commission, 2017. New techniques in agricultural biotechnology. CEU. SAM_ADV, Directorate-General for Research and Innovation, 28 April 2017.
- Gocal GFW, Schöpke C and Beetham PR, 2015. Oligo-Mediated Targeted Gene Editing. In: Zhang F, Puchta H and Thomson JG, eds. *Advances in New Technology for Targeted Modification of Plant Genomes*. Springer, New York, New York, NY.
- Hahn F and Nekrasov V, 2019. CRISPR/Cas precision: do we need to worry about off-targeting in plants? *Plant Cell Reports*, 38, 437–441.
- Hoffmann MD, Aschenbrenner S, Grosse S, Rapti K, Domenger C, Fakhiri J, Mastel M, Borner K, Eils R, Grimm D and Niopek D, 2019. Cell-specific CRISPR-Cas9 activation by microRNA-dependent expression of anti-CRISPR proteins. *Nucleic Acids Research*, 47.
- Hua K, Zhang JS, Botella JR, Ma CL, Kong FJ, Liu BH and Zhu JK, 2019. Perspectives on the application of genome-editing technologies in crop breeding. *Molecular Plant*, 12, 1047–1059.
- Jiang WZ, Zhou HB, Bi HH, Fromm M, Yang B and Weeks DP, 2013. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice. *Nucleic Acids Research*, 41.
- Jin S, Zong Y, Gao Q, Zhu ZX, Wang YP, Qin P, Liang CZ, Wang DW, Qiu JL, Zhang F and Gao CX, 2019. Cytosine, but not adenine, base editors induce genome-wide off-target mutations in rice. *Science*, 364, 292–+.
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA and Charpentier E, 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, 337, 816–821.
- Kim H, Kim ST, Ryu J, Kang BC, Kim JS and Kim SG, 2017. CRISPR/Cpf1-mediated DNA-free plant genome editing. *Nature. Communications*, 8.
- Komor AC, Kim YB, Packer MS, Zuris JA and Liu DR, 2016. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*, 533, 420–+.
- Komor AC, Badran AH and Liu DR, 2017. CRISPR-based technologies for the manipulation of eukaryotic genomes. *Cell*, 168, 20–36.
- Lee K, Zhang Y, Kleinstiver BP, Guo JA, Aryee MJ, Miller J, Malzahn A, Zarecor S, Lawrence-Dill CJ, Joung JK, Qi Y and Wang K, 2019. Activities and specificities of CRISPR/Cas9 and Cas12a nucleases for targeted mutagenesis in maize. *Plant Biotechnology Journal*, 17, 362–372.
- Li JF, Norville JE, Aach J, McCormack M, Zhang DD, Bush J, Church GM and Sheen J, 2013. Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nature Biotechnology*, 31, 688–691.
- Li J, Manghwar H, Sun L, Wang P, Wang G, Sheng H, Zhang J, Liu H, Qin L, Rui H, Li B, Lindsey K, Daniell H, Jin S and Zhang X, 2019. Whole genome sequencing reveals rare off-target mutations and considerable inherent genetic or/and somaclonal variations in CRISPR/Cas9-edited cotton plants. *Plant Biotechnology Journal*, 17, 858–868.
- Liang Z, Zhang K, Chen KL and Gao CX, 2014. Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. *Journal of Genetics and Genomics*, 41, 63–68.
- Lin Q, Zong Y, Xue C, Wang S, Jin S, Zhu Z, Wang Y, Anzalone AV, Raguram A, Doman JL, Liu DR and Gao C, 2020. Prime genome editing in rice and wheat. *Nature Biotechnology*, 38, 582–585.
- Luo S, Li J, Stoddard TJ, Baltes NJ, Demorest ZL, Clasen BM, Coffman A, Retterath A, Mathis L, Voytas DF and Zhang F, 2015. Non-transgenic plant genome editing using purified sequence-specific nucleases. *Molecular Plant*, 8, 1425–1427.
- Ma J, Xiang H, Donnelly DJ, Meng FR, Xu HM, Durnford D and Li XQ, 2017. Genome editing in potato plants by agrobacterium-mediated transient expression of transcription activator-like effector nucleases. *Plant Biotechnology Reports*, 11, 249–258.

- Metje-Sprink J, Menz J, Modrzejewski D and Sprink T, 2019. DNA-free genome editing: past, present and future. *Frontiers. Plant Science*, 9.
- Modrzejewski D, Hartung F, Sprink T, Krause D, Kohl C and Wilhelm R, 2019. What is the available evidence for the range of applications of genome-editing as a new tool for plant trait modification and the potential occurrence of associated off-target effects: a systematic map. *Environmental Evidence*, 8.
- Naeem M, Majeed S, Hoque MZ and Ahmad I, 2020. Latest developed strategies to minimize the off-target effects in CRISPR-Cas-mediated genome editing. *Cells*, 9.
- Nekrasov V, Staskawicz B, Weigel D, Jones JDG and Kamoun S, 2013. Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nature Biotechnology*, 31, 691–693.
- Norris AL, Lee SS, Greenlees KJ, Tadesse DA, Miller MF and Lombardi HA, 2020. Template plasmid integration in germline genome-edited cattle. *Nature Biotechnology*, 38, 163–164.
- Okuzaki A and Toriyama K, 2004. Chimeric RNA/DNA oligonucleotide-directed gene targeting in rice. *Plant Cell Reports*, 22, 509–512.
- Pattanayak V, Lin S, Guiling JP, Ma EB, Doudna JA and Liu DR, 2013. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature Biotechnology*, 31, 839–+.
- Peng H, Zheng Y, Zhao Z, Liu T and Li J, 2018. Recognition of CRISPR/Cas9 off-target sites through ensemble learning of uneven mismatch distributions. *Bioinformatics*, 34, i757–i765.
- Podevin N, Davies HV, Hartung F, Nogue F and Casacuberta JM, 2013. Site-directed nucleases: a paradigm shift in predictable, knowledge-based plant breeding. *Trends in Biotechnology*, 31, 375–383.
- Sandhya D, Jogam P, Allini VR, Abbagani S and Alok A, 2020. The present and potential future methods for delivering CRISPR/Cas9 components in plants. *J Genet Eng Biotechnol*, 18, 25.
- Sauer NJ, Mozoruk J, Miller RB, Warburg ZJ, Walker KA, Beetham PR, Schopke CR and Gocal GFW, 2016. Oligonucleotide-directed mutagenesis for precision gene editing. *Plant Biotechnology Journal*, 14, 496–502.
- Solomon SM, 2020. Genome editing in animals: why FDA regulation matters. *Nature Biotechnology*, 38, 142–143.
- Svitashev S, Schwartz C, Lenderts B, Young JK and Cigan AM, 2016. Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. *Nature. Communications*, 7.
- Tang X, Liu G, Zhou J, Ren Q, You Q, Tian L, Xin X, Zhong Z, Liu B, Zheng X, Zhang D, Malzahn A, Gong Z, Qi Y, Zhang T and Zhang Y, 2018. A large-scale whole-genome sequencing analysis reveals highly specific genome editing by both Cas9 and Cpf1 (Cas12a) nucleases in rice. *Genome Biology*, 19, 84.
- Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP, Aryee MJ and Joung JK, 2015. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nature Biotechnology*, 33, 187–197.
- Upadhyay SK, Kumar J, Alok A and Tuli R, 2013. RNA-guided genome editing for target gene mutations in wheat. *G3-Genes Genomes. Genetics*, 3, 2233–2238.
- Van der Wiel C, Schaart J, Niks R and Visser R, 2010. Traditional plant breeding methods. Report 338, Available from <http://edepot.wur.nl/141713> (Wageningen UR Plant Breeding).
- Veillet F, Kermarrec MP, Chauvin L, Chauvin JE and Nogue F, 2020. CRISPR-induced indels and base editing using the *Staphylococcus aureus* Cas9 in potato. *PLoS ONE*, 15, e0235942.
- Woo JW, Kim J, Il Kwon S, Corvalan C, Cho SW, Kim H, Kim SG, Kim ST, Choe S and Kim JS, 2015. DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nature Biotechnology*, 33, 1162–U156.
- Young J, Zastrow-Hayes G, Deschamps S, Svitashev S, Zaremba M, Acharya A, Paulraj S, Peterson-Burch B, Schwartz C, Djukanovic V, Lenderts B, Feigenbutz L, Wang L, Alarcon C, Siksnys V, May G, Chilcoat ND and Kumar S, 2019. CRISPR-Cas9 editing in maize: systematic evaluation of off-target activity and its relevance in crop improvement. *Scientific Reports*, 9, 6729.
- Zhang B, 2020. CRISPR/Cas9: a robust genome-editing tool with versatile functions and endless application. *International Journal of Molecular Sciences*, 21.
- Zhang Y, Liang Z, Zong Y, Wang YP, Liu JX, Chen KL, Qiu JL and Gao CX, 2016. Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. *Nature. Communications*, 7.
- Zhu T, Mettenburg K, Peterson DJ, Tagliani L and Baszczynski CL, 2000. Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides. *Nature Biotechnology*, 18, 555–558.
- Zuo EW, Sun YD, Wei W, Yuan TL, Ying WQ, Sun H, Yuan LY, Steinmetz LM, Li YX and Yang H, 2019. Cytosine base editor generates substantial off-target single-nucleotide variants in mouse embryos. *Science*, 364, 289–+.

Abbreviations

DSBs	double-strand breaks
GMO	Genetically Modified Organisms
HDR	homology-directed repair
MC WG	molecular characterisation working group
NHEJ	non-homologous end-joining

ODM	oligonucleotide-directed mutagenesis
SAM	Scientific Advice Mechanism
SDN	site-directed nuclease
TALENs	transcription activator-like effector nucleases
ToR	terms of reference
ZFNs	zinc finger nucleases

Glossary

Backcrossing	Cross (a hybrid) with one of its parents or an organism with the same genetic characteristics as one of the parents
Cisgenesis	The genetic modification of a recipient organism with a gene from a crossable – sexually compatible – organism (same species or closely related species) (EFSA GMO Panel, 2012b)
CRISPR	Clustered regularly interspaced short palindromic repeats, a component of a bacterial immunity used to recognise and protect against viruses. It is commonly used as a shorthand for the CRISPR/Cas9 system
Double-strand break (DSB)	The mechanical, chemical or enzymatical cleavage of both strands of the DNA
Exogenous DNA	DNA originating outside the plant being modified which can be introduced naturally or by technological intervention
Genetically linked	Genomic loci which are located in such close proximity that they are inherited together during the meiosis in sexually propagated organisms
Genome	The haploid set of chromosomes of a given organism which contains all the genetic information necessary for its maintenance
Genomic mutation	Permanent change of the nucleotide sequence in the genome of a given organism
Homology-directed repair	Abbreviated to HDR, a molecular mechanism which allows the repair of DNA double-strand breaks using a homologous sequence of DNA as template
Intragenesis	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 (EFSA GMO Panel, 2012b)
Non-homologous end joining	Abbreviated to NHEJ, a molecular mechanism which allows the repair of DNA double-strand breaks when a homologous sequence of DNA is not available. In some cases, NHEJ results in genomic mutations, usually insertion or deletion of fragments of DNA
Off-target mutation	A genomic mutation which occurs in a genomic locus other than the intended one as a result of the application of genetic engineering techniques
Oligonucleotide Ribonucleoprotein Sequence	A stretch of nucleic acid consisting of a relatively low number of nucleotides A macromolecule complex composed of protein and RNA polymers Usually refers to the linear order of nucleotides in DNA and RNA or amino acids in proteins
Site-directed mutagenesis	In this Opinion, a molecular biology method that is used to make specific and intentional changes (insertions, deletions and substitutions) to a genomic locus
Site-directed nuclease	Abbreviated to SDN, an enzyme which recognises a specific sequence and cleaves the DNA usually creating a double-strand break
SDN module	In this Opinion, molecular components necessary to achieve the genetic mutation
Transformation	In this Opinion, the process by which a prokaryotic or eukaryotic cell takes up exogenous DNA
Transgenesis	The process of introducing gene(s) from a different, sexually incompatible, species into the genome of a given cell and the propagation of such gene (s) thereafter