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 Panel on Genetically Modified Organisms (GMO)

Abstract

The European Food Safety Authority (EFSA) published a 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, collectively defined as SDN-3 (EFSA GMO Panel, 2012). The European Commission (EC) requested the EFSA Panel on Genetically Modified Organisms (GMO Panel) to assess whether the section 4 (hazard identification) and the conclusions of the opinion on SDN-3 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. The GMO Panel concluded that, unlike for SDN-3 methods, the application of SDN-1, SDN-2, and ODM approaches results in the modification of plant endogenous genomic sequences without the insertion of exogenous DNA. Consequently, those considerations which are specifically related to the presence of a transgene included in section 4 and conclusions of the opinion on SDN-3 are not relevant to plants obtained via SDN-1, SDN-2, and ODM approaches in case foreign DNA is not present in the final product. Overall, the GMO Panel did not identify new hazards specifically linked to the genomic modification produced via SDN-1, SDN-2 and ODM as compared to 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 (EFSA GMO Panel, 2011) and the Guidance on the environmental risk assessment of genetically modified plants (EFSA GMO Panel, 2010) are sufficient but can be only partially applied to plants generated via SDN-1, SDN-2 and ODM. Indeed, those guidances' requirements which are linked to the presence of foreign DNA are not relevant for the risk assessment of plants developed via SDN-1, SDN-2, and ODM approaches in case the genome of the final product does not contain exogenous DNA.

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35 Keywords

36 Site-directed nuclease, SDN-1, SDN-2, SDN-3, oligonucleotide-directed mutagenesis, transgenesis,
37 off-target, genetically modified plants, risk assessment, EFSA guidance

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1 Introduction

1.1 Background as provided by the European Commission

The judgement of the Court of Justice of the European Union (CJEU) in Case C-528/16 on mutagenesis has clarified that Directive 2001/18/EC 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[1] and in 2011 the Guidance on the risk assessment of food and feed from GM plants[2]. Following a request of the European Commission, EFSA published in 2012 a scientific opinion[3] addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function (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 the 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 of the European Commission (Ref. Ares(2019)2488590-09/04/2019), in April 2019 EFSA assigned the mandate to the molecular characterization working group of the GMO Panel (Ref. BU/GdS/KL/FA/cz_OC-2019-21268932). To allow public consultation, EFSA requested the European Commission to change the scientific opinion deadline (Ref. BU/GdS/EW (2019) OC-2019-22763474) which was then extended from 30th April 2020 to 30th October 2020 (Ref. Ares(2020)250930-15/01/2020).

[1]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, 111 pp. doi:10.2903/j.efsa.2010.1879.

[2]EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2011. Scientific Opinion on Guidance for risk assessment of food and feed from genetically modified plants. EFSA Journal 2011; 9(5): 2150, 37 pp. doi:10.2903/j.efsa.2011.2150.

[3]EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2012. 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.

1.3 Terms of reference

Against this background, the European Commission, in accordance with Article 29 of Regulation (EC) No 178/2002, asked EFSA to address the following two terms of reference (ToR):

1. To advise whether the assessment methodology described in section four 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.

In case 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

EFSA assigned the development of the scientific opinion addressing the safety assessment of plants developed using Site-Directed Nucleases type 1 and 2 (SDN-1 and SDN-2) and Oligonucleotide-Directed Mutagenesis (ODM) to the molecular characterisation (MC) working group (WG) of the GMO Panel. To address the two ToRs, the MC WG took into consideration both the section 4 and the overall conclusions 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 (EFSA GMO Panel, 2012a) (hereafter, "EFSA opinion on SDN-3") and the relevant information reported in the scientific literature. Some background information, the content of the 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 (NPBTs), the first on cisgenesis and intragenesis (EFSA GMO Panel, 2012b) and the second on SDN-3 (EFSA GMO Panel, 2012a). To develop the scientific opinion on SDN-3, the GMO Panel was requested i) to determine the risks in terms of 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 if there was a need for new guidance to risk assess plant 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 the Implementing Regulation (EU) No 503/2013[4] which integrated the guidance's requirements in a legal frame.

[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

To address the requests of the mandate, in the EFSA opinion on SDN-3 the GMO Panel compared plants developed using SDN-3 method with plants obtained by conventional breeding[5] techniques focusing mainly on mutagenesis approaches.

2.1.2 Section 4 of the EFSA opinion on SDN-3

The section 4 of the EFSA opinion on SDN-3 focuses on the hazards associated to plants that are produced using SDN-3 methods which are used to target the insertion of exogenous DNA 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 (Andersson et al., 2012).

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. 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).

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.

[5] Conventional plant breeding is defined as methods used by plant breeders for the improvement of commercial varieties and where the resulting plants/varieties are not covered by the legal definitions of genetic modification (Directive 2001/18/EC) ARPAIA, S., BIRCH, A. N. E., CHESSON, A., DU JARDIN, P., GATHMANN, A., GROPP, J., HERMAN, L., HOEN-SORTEBERG, H. G., JONES, H., KISS, J., KLETER, G., LAGIOU, P., LOVIK, M., MESSEAN, A., NAEGELI, H., NIELSEN, K. M., OVESNA, J., PERRY, J., ROSTOKS, N., TEBBE, C. & MODIFIED, E. P. G. 2012. Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function. *EFSA Journal,* 10.

The EFSA GMO Panel considers that the Guidance for risk assessment of food and feed from genetically modified plants (EFSA, 2011) and the Guidance on the environmental risk assessment of genetically modified plants (EFSA, 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)

3 Assessment

3.1 Introduction.

3.1.1 Definition of gene editing: SDN-1, SDN-2, and ODM compared to SDN-3

The Explanatory Note on New Techniques in Agricultural Biotechnology from the European Commissioner for Health and Food Safety defines site-directed nuclease (SDN) as "*an enzyme (endonuclease) that creates site-specific double-strand breaks (DSBs) at defined sequences. SDN typically recognizes 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 a template DNA to introduce 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 since 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 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).

In general, the application of SDN-1, SDN-2, and ODM methods result either in a random (SDN-1) or in an intended (SDN-2 and ODM) modification of a targeted genomic locus without the insertion of foreign DNA. On the contrary, the aim of the SDN-3 approach is to modify the targeted locus by inserting an exogenous DNA template of various lengths (e.g. a transgene).

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 addition, a literature review on zinc finger nucleases (ZFNs), transcription activator-like effectors (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 requested to deliver an extensive literature review on the technologies deployed in these three approaches. However, considering the advances in genome editing that unfolded in recent years, the GMO Panel deemed appropriate to include some information which is discussed below.

Since 2012, a new RNA-directed SDN-type technology known as 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 of plant genes and these technologies have been used to obtain products that are already on the market in the USA (e.g. Calyno™ High Oleic Soybean Oil and Meal derived from genome edited soybean[6]), 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 (Jiang et al., 2013, Li et al., 2013, Nekrasov et al., 2013) but also to enhance important agronomic traits in crops like maize, sorghum, barley, potato, rice, and wheat (Upadhyay et al., 2013, Liang et al., 2014). So far, the ODM technology has been only used to generate GM plants with relatively simple and easily selectable traits, for example herbicide resistance (Sauer et al., 2016), and the amount of information available in the literature in terms of molecular mechanism, technological aspects, applications, and intrinsic limitations of the system (i.e. efficiency and specificity in different plant species) is considered limited compared to SDN-based technologies such as the CRISPR-Cas system.

It should be noted that while ZFNs, TALENs, meganucleases, and CRISPR-Cas system can all be used to achieve random (SDN-1) and intended (SDN-2) targeted mutations and precise insertion of a transgene (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 introduce 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 fall under the SDN-2 definition (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 (SAM) (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).

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, SDN module). In case of stable integration and for sexually propagated crops, the SDN module can be removed by segregation leaving only the intended sequence mutation in the genome of the final product. This step is not possible in case of non-sexually propagated crops (for example, for vegetatively propagated crops). In this case when the presence of the SDN gene cassette in the final product is not desirable, transient expression is 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 CRISPR-Cas system) are directly delivered into the plant cell without the use of any intermediate sequence of DNA (Metje-Sprink et al., 2019). The possibility to deliver purified sequence-specific nucleases to 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). In case of ODM, the chemically

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

synthetized oligonucleotide is directly delivered to the plant cell without the need of 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 delivering systems have been tested for different plant tissues, including PEG-fusion, electroporation, and biolistics (Metje-Sprink et al., 2019). Table 1 summarizes the different approaches deployed to express or to deliver the module needed to achieve site specific modifications in plants.

Technology used	Delivery methods	Presence of the SDN module in the final product	Exogenous DNA* deployed at any stage during the process
CRISPR-Cas system TALEN ZFN Meganuclease	Stable integration	No if crossed out (only applicable for sexually reproducing plants)	Yes
	Transient expression	No	Yes
	DNA-free delivery	No	No (if synthetized RNA is used)
ODM	Oligonucleotide delivery	No	Yes

Table 1: summary of the delivery methods for the SDN and ODM available in plants. *= DNA originating outside the plant which can be introduced naturally or by technological intervention (European Commission, 2017).

3.2 ToR1 of the mandate: Applicability of the 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 the ToR1 of the mandate, the GMO Panel assessed the section 4 of the EFSA opinion on SDN-3 which compares the hazards associated with plants developed using SDN-3 approaches to those derived from transgenic and conventionally bred plants, 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, or any exogenous DNA sequence deployed during the genome editing process is present in the plant genome (see section 3.1.3 and Table 1). In this case, the product would be risk assessed as a transgenic plant with regards to the exogenous DNA integrated in 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 other exogenous DNA sequence deployed during the genome editing process is not present in the plant genome (see section 3.1.3 and Table 1). In this case, the plant will only be assessed with regards to the modification introduced at the target sequence(s). The assessment of section 4 of 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 result in the insertion of any transgene but rather in the modification of 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 and consumption. For example, 2 extreme scenarios could be envisaged. On one extreme, the new allele obtained through genome editing and the associated trait characterizing the final product are already present in a consumed variety of the same species. In this case, the risk assessment may focus on the knowledge of the consumed variety (history of safe use) and specific data on the edited gene may not be needed. On the other extreme, the modified allele and associated trait present in the final product have never been described before. In this case, specific data on the new allele and the expressed trait would be needed to perform the risk assessment. The GMO Panel considers that a substantial number of different scenarios are possible between these two extremes and 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 the section 4.1 of the EFSA opinion on SDN3 (“Source of genes and safety of gene products”) is applicable only in part to plants developed by SDN1, SDN2, and ODM approaches.

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 the SDN-3. On the contrary, 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) do not induce DSB in the plant genome at any stage during the process. Irrespective of the approach used, the successful application of SDN-1, SDN-2, and ODM results in a sequence modification which is targeted to a specific predetermined genomic locus and no exogenous DNA is inserted. For these reasons, several considerations described in section 4.2.1

of the EFSA opinion on SDN-3 which refers to i) the targeted integration of the transgene 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 not relevant for plants obtained using SDN-1, SDN-2, and ODM approaches.

Because of all the above considerations, the GMO panel concludes that the 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, and ODM approaches.

3.2.2.2.2 Alteration elsewhere in the genome [Section 4.2.2]

In general, the application of SDN-1, SDN-2, and ODM approaches result in the precise and intended modification of predetermined plant genomic sequence(s). However, they can also introduce changes elsewhere in the genome because of the off-target activity associated with these applications (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 homology with the original target locus. In addition, some Base Editing systems have been shown to present a 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 be either predictable (for SDN-1 and SDN-2) or not (for Base Editing). 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)). For example, designing shorter gRNA, lowering intracellular concentration of the Cas-gRNA complex (Pattanayak et al., 2013), and expressing specific anti-CRISPR proteins (Hoffmann et al., 2019) seem to generally reduce off-target effects.

In the EFSA opinion on SDN3, the GMO Panel concluded that the off-target changes induced by the application of SDN-3 approaches are fewer than those occurring with conventional mutagenesis techniques that have been used previously and have a long history of safe use. In addition, backcrossing following the transformation process will remove these potential off-targets from the final product, except for those that are genetically linked to the intentionally modified locus (Hahn and Nekrasov, 2019). The GMO Panel considers that the same conclusions remain valid also for plants generated by the application of both SDN-1 and SDN-2 approaches since they produce only a fraction, if any, of all the unintended genomic alterations introduced by conventional breeding. While an increasing number of publications have investigated off-target effects for SDN-based technologies, information on the off-target mechanism and frequency for ODM is quite limited (Modrzejewski et al., 2019). Despite the lack of information on possible off-target effects, it is reasonable to assume that the same conclusions apply to ODM since this technology is also based on sequence-specific site recognition. Therefore, because off-target effects in SDN- and ODM-based approaches is negligible compared to conventional plant breeding, the GMO Panel considers that the analysis of potential off-targets would be of very limited value for the risk analysis. In addition, although some biochemical and bioinformatic tools are available for off-target prediction (Bae et al., 2014, Tsai et al., 2015, Cameron et al., 2017, Peng et al., 2018), the limited availability and/or completeness of plant genomic sequences and their intra-species and intra-varietal variability would not always allow for a reliable prediction of potential off-target mutations.

When plant transformation is used to introduce the SDN module, the unintended insertion of plasmid DNA or other foreign DNA at off-target positions can happen. Also the application of some methods to achieve SDN-1 and SDN-2 modifications (i.e. DNA free methods) can result in the unintended on-target or off-target integration of exogenous DNA whose sequence is 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), and Solomon (2020)). If the final product is not intended to retain any exogenous DNA, the applicant should demonstrate that the genome of the end product is free from any DNA sequence potentially derived from the methods used to generate the SDN-type of modification (e.g. plasmids or vectors, section 3.1.3).

Because of all the above considerations, the GMO Panel concludes that the section 4.2.2 of the EFSA opinion on SDN3 ("Alteration elsewhere in the genome") is applicable to plants developed by SDN-1, SDN-2, and 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 and ODM approaches

In EFSA GMO Panel (2012a), the EFSA GMO Panel compared plants obtained by the application of 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 and ODM approaches with those associated with plants obtained via SDN-3 approach. In addressing the 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 optimization of the genomic context of the transgene insertion in SDN-3 plants is not applicable to plants obtained via SDN-1, SDN-2, and ODM approaches, since these methods aim at modifying an endogenous DNA sequence without the insertion of any transgene.
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 case of ODM, although very limited amount of 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 since this technology is based on sequence-specific site recognition as for SDN-based methods.
3. The conclusion addressing the risk assessment of the introduced transgene 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.
4. In the EFSA opinion on SDN-3, the GMO Panel concluded that the guidance for 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) were applicable for the risk assessment of plants obtained using SDN-3 method. The GMO Panel considers that the two EFSA guidances are sufficient but can be only partially applied for the risk assessment of plants generated by the application of SDN-1, SDN-2, and ODM methods. Indeed, those requirements related to the presence of transgenes 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 applies also to plant generated via SDN-1, SDN-2, and ODM approaches. Indeed, in the absence of any transgene, the amount of experimental data needed for the risk assessment will mainly depend on the

409 modified trait introduced and even less amount of experimental data would be needed for
410 plants produced via SDN-1, SDN-2,and ODM compared to plants generated via SDN-3.

411 # 4 Conclusions

412 In relation to ToR1, the GMO Panel concludes that the assessment methodology presented in
413 section 4 of the EFSA opinion on SDN-3 is partially applicable to SDN-1, SDN-2, and ODM. Since these
414 approaches aim at modifying an endogenous DNA sequence, in case the final product does not contain
415 any exogenous DNA, these plants will not present any of the potential hazards related to the insertion
416 of a transgene. Moreover, the GMO Panel did not identify any additional hazard associated to the use
417 of the SDN-1, SDN-2 and ODM approaches as compared to both SDN-3 and conventional breeding
418 techniques, including conventional mutagenesis.

419 In relation to ToR2, the GMO Panel concludes that the existing Guidances for food and feed
420 (EFSA GMO Panel, 2011) and environmental risk assessment (EFSA GMO Panel, 2010) are sufficient
421 but can be only partially applied for the risk assessment of plants generated via SDN-1, SDN-2, and
422 ODM approaches. Indeed, as SDN-1, SDN-2 and ODM aim at modifying endogenous DNA sequence(s),
423 a number of requirements of the existing guidances that are linked to the presence of a transgene are
424 not relevant for the assessment of SDN-1, SDN-2 and ODM plants in case the final product does not
425 contain any exogenous DNA.

426

5 Glossary

Backcrossing: cross (a hybrid) with one of its parents or an organism with the same genetic characteristics as one of the parents.

CRISPR: clusters of regularly interspaced short palindromic repeats, a component of a bacterial immunity used to recognize and protect against viruses. It is commonly used as a shorthand for CRISPR/Cas9 system.

Double-strand break: the mechanical, chemical, or enzymatical cleavage of both strands of the DNA.

Exogenous DNA: a fragment of DNA originating outside the organism of interest.

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 organisms which contains all the genetic information necessary for its maintenance.

Homology-directed repair: abbreviated as HDR, a molecular mechanism which allows the repair of DNA double strand breaks using an homologous sequence of DNA as template.

Genomic mutation: permanent alteration of the nucleotide sequence in the genome of a given organism.

Non-homologous end joining: abbreviated as NHEJ, a molecular mechanism which allows the repair of DNA double strand breaks when an homologous sequence of DNA is not available. 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 genome editing techniques.

Oligonucleotide: a stretch of DNA consisting of a relatively low number of nucleotides.

Ribonucleoprotein: a macromolecule complex composed by protein and RNA polymers.

Sequence: usually refers to the linear order of nucleotides in DNA and RNA or amino acids in proteins.

Site-directed Nuclease: abbreviated as SDN, an enzyme which recognizes a specific sequence and cleaves the DNA usually creating a double strand break.

Transformation: in this opinion, the process by which a prokaryotic or eukaryotic cell uptakes exogenous DNA.

Transgene: an exogenous gene used in the transformation process.

Transgenesis: the process of introducing exogenous gene(s) into the genome of a given cell and the propagation of such gene(s) thereafter.

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