Public consultation - 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

GenØk Centre for Biosafety Submission
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Item	Opinion
Abstract	The abstract should reflect the suggestions made throughout the document.
1.1 Background as provided by the European Commission	**This text contains mainly EFSA opinion** Three out of the four paragraphs included in this section contains EFSA opinions about the topic. Therefore, is it suggested that this text is moved to the following section "Background as provided by EFSA" and new text on the EC work should be provided. For example, the part concerning risk assessment issues on the ECJ ruling should be described here as the basis for the EC request. "23 According to the referring court, the conventional *in vivo* mutagenesis methods were used for several decades without creating identified risks for the environment or health. By contrast, since the adoption of Directive 2001/18, new varieties, in particular those resistant to herbicides, have been obtained through random mutagenesis techniques applied *in vitro* to plant cells and through directed mutagenesis techniques/methods applying new genetic engineering techniques, such as oligonucleotide-directed mutagenesis or directed nuclease mutagenesis. It is, in the view of the referring court, impossible to determine with certainty the existence and extent of the risks presented by those new herbicide-resistant varieties for the environment and human and animal health, the only risk assessments thus far being carried out in the context of the marketing authorisation procedure for the plant protection products to which those varieties have been made resistant. 24 The referring court considers that those risks are in part similar to those that might result from seeds produced by transgenesis. As regards, in particular, the mutations obtained by the new directed mutagenesis techniques, the direct modification of the genome that they involve would result in the same effects as the introduction of a foreign gene, specific to transgenesis. In addition, since the development of the new techniques of mutagenesis allows the production of modifications of the genetic heritage to increase at a rate out of all proportion to the modifications likely to occur naturally or randomly, the possibility of harm occurring as a result of unintentional modifications of the genome or of the properties of the plant thus obtained would be increased."[1] These issues raised by ECJ are directly linked to EFSA mandate and ToR but were not included neither discussed within the document. **Conventional mutagenesis is not conventional breeding** It is also relevant to clarify in this section that random or chemical mutagenesis are genetic engineering techniques and not conventional breeding techniques. The reason why these techniques are not regulated by the GMO Directive is because of their history of safe use which exempts them from being regulated. There is a clear distinction between what the Court considers conventional breeding techniques and genetic engineering techniques exempt from regulation.

	EFSA provides a correct definition of conventional breeding in footnote #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". However, it is incorrect when it includes conventional mutagenesis techniques as conventional breeding techniques. Mutagenesis techniques are covered by the Directive. EFSA interpretation of mutagenesis techniques is flawed and misleading as it is not clear when it refers to risks related to conventional breeding (crossing and selection of genotypes) and when it refers to risks of chemical mutagenesis. Such flawed statements should be corrected throughout the document: Page 1 - Lines 17 and 24 Page 4 - Lines 84, 85 and 92 Page 5 – Line 135 Page 6 - Lines 144, 154, 167 and 175 Page 10 – Line 279 Page 11 - Lines 341, 347 and 353 Page 12 – Lines 374 and 387 Page 13 – Lines 417 and 418
1.3 **Terms of reference**	**EC asks about the safety of certain nucleases not their final product** There is a fundamental difference in analyzing the safety of a technique and the safety of a product. In this ToR, EC clearly requests advice on the nucleases and not about the outcome. In this regard, EFSA should provide information on how these nucleases work, their activities and functionalities, the techniques that apply such nucleases, etc. On the contrary, EFSA has only focused on a few intended outcomes of such nucleases. I will provide evidence of such narrow approach in the following sections.
2.1.1 **Background information**	**Comparison of techniques not adequate** EFSA has focused its assessment on the comparison of plants developed using SDN1 and SDN2 to mutagenesis approaches. It is unclear why EFSA has focused on mutagenesis approaches and not addressed the safety of the new nucleases as it stands. If the comparison was made with the intention to have a standard and known technique, EFSA should have focused on techniques that are not genetic engineering techniques as per EFSA Guidance on selection of comparators for the risk assessment of genetically modified plants and derived food and feed[2]. In addition, mutagenesis approaches can be many techniques with different applications and outcomes. It is not clear, at any part of this document what techniques have been considered, its characteristics and its safety. On top, EFSA defines mutagenesis techniques as conventional breeding techniques: "[…] plants obtained by conventional breeding techniques focusing mainly on mutagenesis approaches." As discussed in the previous section, not only this is a wrong concept and definition but it is vague and does not help with the assessment of a new technique. It does not provide a basis or standard for comparison. It only misleads the assessment as

	it is not possible to understand at any point of the document to what technique SDN1 and SDN2 are being compared to.
3.1.1 Definition of gene editing: SDN-1, SDN-2 and ODM compared to SDN-3	**Definition of gene editing is out of the scope of this mandate** EFSA should avoid using the term "definition" as it relates to "legal definition" especially since there are current discussions on the topic. In addition, the issue is not in the mandate of EFSA. In fact, according to the latest ECJ ruling on mutagenesis, the definition of gene editing is the same as the GMO definition as per GMO Directive 2001/18/EC. We suggest merging this topic with the following topic and change the title to "Techniques used in SDN-1, SDN-2 and ODM applications".
3.1.2 Technology used in SDN-1, SDN-2 and ODM applications	**EFSA fails in performing a uncertainty analysis for the limited scientific information available** EFSA recognizes the limited amount of scientific evidence towards certain types of new mutagenesis techniques. However, instead of addressing such lack of knowledge by performing an uncertainty analysis, EFSA is satisfied with current scientific information and further provides its opinion on their hazard identification. According to EFSA's own guidance on uncertainty analysis, EFSA should "identify limitations in scientific knowledge and evaluate their implications for scientific conclusions"[3]. Although the current document is not a scientific assessment in itself, its conclusion should provide reliable information for EC decision-making, and therefore, any lack of knowledge should be addressed as uncertainty. References are also made to other reviews of the technologies, such as CRISPR which were not included in EFSA's previous opinion. However, as a technical opinion document, EFSA should summarize the relevant information from the scientific literature to inform EC and provide robust evidence for its conclusions within this document. For instance, it is suggested that EFSA provides a box or a table containing the types of CRISPR systems and available Cas nucleases, their functions and expected outcomes following the below criteria: 1) How are these nucleases or oligonucleotide produced? Do they apply recombinant nucleic acids? 2) What are the delivery systems of these mutagenic agents (link to the next section)? 3) What are the biochemical pathways triggered after the incorporation of these mutagenic agents inside the host cell? 4) Is it capable of continued propagation? Is it a heritable material? **ODM do not only create SDN-2 type modification** Oligonucleotides techniques are characterized by the sequence-specific interaction of nucleic acids, also called hybridization, in vivo. Therefore, parameters, such as the number of nucleotides and range of mutations, are useful guidelines but not exhaustive. There is also the possibility to introduce insertions and deletions.[4] In summary, ODM techniques can create all SDN-1, SDN-3 and SDN-3 outcomes but at the same time it is a technique complete different from those as it does not

	imply the use of any nuclease. EFSA should describe in detail the differences between these techniques. **How to differentiate SDN-2 and SDN-3?** Whereas the main difference for SDN-1 techniques relies on the lack of foreign donor DNA template, it is not clear what are the boundaries for the categorization of SDN-2 and SDN-3.
3.1.3 Methods for delivering or expressing SDN in plants	**Table should focus on the characteristics of the techniques not the final product** EFSA provides a table summarizing the differences in delivery methods for the different techniques of gene editing. Instead, EFSA should present information related to the following criteria which could be included as columns after the column 'delivery methods': 1) What biological material will be delivered inside the cell? Nucleic acids (DNA or RNA or both), proteins or both? 2) What supporting techniques could be applied for efficient delivery (electroporation, lipofectamine, other mutagenic reagent)? Are they mutagenic reagents or techniques? Do they apply genetic engineering techniques? 3) Link the information to the categories SDN-1, SDN-2 and SDN-3. It is also suggested that the two last columns are deleted as they refer to the final product, which should be tested during RA (column 3) or limited to DNA presence and does not include all nucleic acids (column 4). In Annex I, there is a suggestion for a new Table containing the above-mentioned criteria. **Supporting techniques for gene editing** EFSA spends 10 lines out of the 20 lines in this section to discuss DNA-free delivery methods. As suggested in our Annex I, DNA-free delivery methods are only one type of delivery and it can only achieve SDN-1. In addition, EFSA does not explore the supporting techniques used in conjunction to these gene editing technologies. Most of these technologies rely on the use of supporting techniques for their success. And depending on techniques used, the organism might be risk assessed differently. Therefore, it is suggested that EFSA describes all delivery methods available this far, which are the supporting techniques used in these, to which degree they have been applied in plants and which ones are more likely to reach market in the near future. A comprehensive list of the possibilities of delivery methods for each SDN type and the supporting techniques is provided in Table 1 in Annex I of this document.
3.2.1 Introduction	**EFSA focuses on the final product not the techniques used** Differently to what has been requested by the EC in its ToRs, EFSA focuses its assessment on two scenarios described by its final products. In other words, it is not possible to determine which techniques and supporting techniques are being applied in both scenarios.

	EFSA does not fulfills its mandate as it does not provide an assessment of the techniques but rather on fictitious products that could be obtained by many and different techniques of genetic engineering. For example, in lines 281 and 282, EFSA describes scenario #1 as "the full SDN module, part of it, or any exogenous DNA sequence deployed during the genome editing process is present in the plant genome." These products could be obtained by techniques of transgenesis, SDN-2, SDN-3 or ODM. How can EFSA assess the risks of such organism if the techniques applied are not described? How can EFSA assess the risks if it is not described whether a nuclease, a plasmid or an oligonucleotide molecule has been inserted in the cell? The evidence for such flawed assessment is provide in the following sections.
3.2.2.1 Assessment of section 4.1: Source of genes and safety of gene products	**The presence of foreign DNA should not be the only criteria for analysis** EFSA states that "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." Whereas EFSA might be right that a transgene insertion is not expected in SDN-1, SDN-2 and ODM approaches, the integration of exogenous foreign DNA used either as a template or as part of a delivery method (viral vectors, etc) should be verified during RA when these approaches are used. In other words, it cannot be assumed that when using SDN-1, SDN-2 and ODM foreign DNA introgression is not present in the genome. This kind of assumption has lead to the discovery of plasmid sequences in the genome of gene-edited hornless cattle by the Food and Drug Administration Department in the U.S[5]. Neither the developer of the Brazilian Biosafety Authority (CTNBio), which granted non-GMO status to this organism, has detected foreign DNA sequences in the genome of the cattle. Both the company and CTNBio assumed that the non-integrative plasmid containing TALEN and DNA template plasmid sequences were not able to be inserted in the host genome and did not verified that.
3.2.2.2.1 Alteration at the insertion site [section 4.2.1]	**Again: The presence of foreign DNA should not be the only criteria for analysis** In lines 315-317, EFSA states "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." It further confirms that for these reasons, the investigation of several aspects of the insertion site are not relevant for plants developed thought these techniques. EFSA again limited its analysis to the presence or absence or exogenous DNA, omitting the need to verify integration events as well as other aspects of genetic modification. For example, CRISPR is widely used to disrupt gene function by inducing small insertions and deletions like those present in SDN-1 and SDN-2 approaches. There has been evidence that some single-guide RNAs (sgRNAs) can induce small insertions or deletions that partially alter splicing or unexpected larger deletions that remove exons[6]. Exon skipping adds to the unexpected outcomes that must be accounted for in RA.

3.2.2.2.2 Alteration elsewhere in the genome [section 4.2.2]

Off-target activity cannot be reliably predictable

EFSA states that the off-target activity of SDN-1 and SDN-2 are predictable (lines 333 and 334). This statement is wrong for two main reasons:

1) SDN-1 and SDN-2 approaches can use a variety of techniques, as mentioned before, and it is not clear to what techniques is EFSA referring in this statement.
2) It is not correct that in silico analysis can reliably predict off-target activities of gene editing techniques. For example, many of the CRISPR/Cas9 design tools include information about potential off-target sites in the genome of interest, but not every algorithm searches for every kind of off-target effect (e.g., DNA or RNA bulges). It has also been observed that analyses from *in silico* predictions are not always correct and their results don't always align because the CRISPR/Cas9 system is not completely understood[7].

EFSA statement even contradicts its own analysis of such softwares in lines 354-358, where it says prediction softwares are not reliable.

The number of off-target mutations is not relevant for risk assessment

Whereas it is logical to think that the more off-target mutations in the host genome the more probability of risk, it is not correct to think that fewer off-target changes are equivalent to a safe profile. In this regard, we suggest that EFSA deletes lines 340-342 as it does only mislead the risk assessment aspect of off-target activity of site-directed nucleases.

Back-crossing does not remove all off-target changes

EFSA states that "[…] backcrossing following the transformation process, will remove these potential off-targets from the final product […]" (lines 343 and 344).

It is known to any plant breeder that the main challenge in breeding is breaking linkage groups. It is not possible to remove off-target changes performed by nucleases, which overcomes linkage drag effects in plants, by simple cross[8]. For example, genomic analyses in tomato plants have indicated that the linkage drag associated with genome segmentation covers nearly 25.6% of the assembled genome.

Therefore, this statement should be deleted and the need to verify off-target changes should be discussed even when organisms have been back-crossed with untransformed lines.

Conventional breeding does not produce unintended genomic alterations

The following statement from EFSA: "SDN-1 and SDN-2 approaches since they produce only a fraction, if any, of all the unintended genomic alterations introduced by conventional breeding" is false, misleading and purposeful:

1) What SDN-1 and SDN-2 techniques is EFSA referring to? Is it CRISPR? TALEN? What delivery method? DNA-free? Transgenesis?
2) Conventional breeding is not chemical or radiation mutagenesis. Therefore, it does not produce unintended genomic alterations. Conventional breeding as per GMO Directive is simple the natural cross of individuals.
3) How can a quantitative measure, such as a "fraction", informs anyone about the potential risks of a technique? Please see my comments above on the number of off-target mutations.

	The lack of information on off-target activity of ODM should be reported not extrapolated EFSA states "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" (lines 350-352). Again, EFSA limits its analysis of ODM off-target effects on the aspect of sequence similarity whereas the other techniques are completely different and use nucleases that can cause double-stranded breaks, not the case for ODMs. **How can off-target activity of gene editing not be of value to risk assessment?** EFSA states that the off-target effects of SDN-1 and SDN-2 and ODM are negligible and of limited value for the risk analysis. It is unclear why EFSA does not recognizes all the scientific literature on off-target activity of such techniques which has led to a global moratorium on all clinical uses of human germline editing[9]. This entire section is flawed and of no use for the assessment of ToR1. For a recent review on alterations elsewhere in the genome of gene-edited organisms, please read Agapito-Tenfen (2018)[10].
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**	**Conclusions do not reflect the current scientific knowledge on the safety of such techniques** Throughout this document we have shown how the analysis by EFSA was narrow and limited to the assessment of the presence of transgenes at the intended site of modification. With the aim of not being repetitive, we believe that these conclusions and the conclusions under section #4 are not legitimate to the current scientific knowledge presented in this review and we urge that the Panel revises its conclusions according to the review and comments made in this document.

Annex I

Suggested Table 1.

SDN type	Technologies available	Delivery methods	Biological material used	Supporting techniques
SDN-1	CRISPR, TALEN, ZFN and Meganucleases	DNA-free delivery	Ribonucleoprotein complex (enzyme and synthetic guide RNA molecule) or enzymes only	Chemical-based transfection, non-chemical/physical transfection and particle-based transfection
		Transient expression	Recombinant nucleic acids (vector/plasmids) No foreign DNA template for the intended site	Chemical-based transfection, non-chemical/physical transfection Viral/vector-based transfection and agrobacterium-mediated transfection (non-integrative plasmid and vectors)
		Transgene integration	Recombinant nucleic acids (vector/plasmids) No foreign DNA template for the intended site	Chemical-based transfection, non-chemical/physical transfection and particle-based transfection Viral/vector-based transfection and agrobacterium-mediated transfection (integrative plasmid and vectors) Transgenesis
SDN-2	CRISPR, TALEN, ZFN and Meganucleases	Transient expression	Recombinant nucleic acids (vector/plasmids) Foreign DNA template for the intended site	Chemical-based transfection, non-chemical/physical transfection Viral/vector-based transfection and agrobacterium-mediated transfection (non-integrative plasmid and vectors)
		Transgene integration	Recombinant nucleic acids (vector/plasmids) Foreign DNA template for the intended site	Chemical-based transfection, non-chemical/physical transfection and particle-based transfection Viral/vector-based transfection and agrobacterium-mediated transfection (integrative plasmid and vectors) Transgenesis
SDN-3	CRISPR, TALEN, ZFN and Meganucleases	Transient expression	Recombinant nucleic acids (vector/plasmids) Foreign DNA template for the intended site	Chemical-based transfection, non-chemical/physical transfection Viral/vector-based transfection and agrobacterium-mediated transfection (non-integrative plasmid and vectors)
		Transgene integration	Recombinant nucleic acids (vector/plasmids) Foreign DNA template for the intended site	Chemical-based transfection, non-chemical/physical transfection and particle-based transfection Viral/vector-

				based transfection and agrobacterium-mediated transfection (integrative plasmid and vectors) Transgenesis
None	ODM	Oligonucleotide delivery	Foreign DNA, RNA or DNA-RNA hybrid template	Chemical-based transfection, non-chemical/physical transfection and particle-based transfection Viral/vector-based transfection and agrobacterium-mediated transfection (integrative plasmid and vectors) Transgenesis

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