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Review

Implications of the EFSA Scientific Opinion on Site Directed Nucleases 1 and 2 for Risk Assessment of Genome-Edited Plants in the EU

Nils Rostoks [1,2]

[1] Faculty of Biology, University of Latvia, LV-1004 Riga, Latvia; nils.rostoks@lu.lv or nils.rostoks@biomed.lu.lv
[2] Latvian Biomedical Research and Study Centre, LV-1067 Riga, Latvia

Abstract: Genome editing is a set of techniques for introducing targeted changes in genomes. It may be achieved by enzymes collectively called site-directed nucleases (SDN). Site-specificity of SDNs is provided either by the DNA binding domain of the protein molecule itself or by RNA molecule(s) that direct SDN to a specific site in the genome. In contrast to transgenesis resulting in the insertion of exogenous DNA, genome editing only affects specific endogenous sequences. Therefore, multiple jurisdictions around the world have exempted certain types of genome-edited organisms from national biosafety regulations completely, or on a case-by-case basis. In the EU, however, the ruling of the Court of Justice on the scope of mutagenesis exemption case C-528/16 indicated that the genome-edited organisms are subject to the GMO Directive, but the practical implications for stakeholders wishing to develop and authorize genome-edited products in the EU remain unclear. European Food Safety Authority in response to a request by European Commission has produced a scientific opinion on plants developed by SDN-1, SDN-2, and oligonucleotide-directed mutagenesis (ODM) genome editing techniques. In this review, I will (1) provide a conceptual background on GMO risk assessment in the EU; (2) will introduce the main conclusions of the EFSA opinion, and (3) will outline the potential impact on the risk assessment of genome-edited plants.

Keywords: site-directed nuclease; SDN-1; SDN-2; EFSA opinion; genome-edited organism; genetically modified organism; risk assessment

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1. Introduction

Plant breeding harnesses existing genetic diversity and makes use of different tools to increase it. Conventional plant breeding utilizes existing genetic diversity in breeders' gene pool, but also creates new variants using chemical and radiation mutagenesis or takes advantage of somaclonal variation during plant tissue culture. Genetic engineering (transgenesis) allows the direct creation of novel traits by adding genes from different species, or rapid transfer of traits among varieties of the same species. In the case of cisgenesis, the inserted genes and regulatory sequences are from the same or cross-compatible species and have the same native structure, while in intragenesis the inserted DNA can represent a new combination of genes and regulatory sequences from the same or cross-compatible species [1]. In the EU, these forms of genetic modification all fall under the GMO legislation. Genome editing is a collection of methods for introducing targeted changes in genomes [2,3]. Unlike conventional chemical and radiation mutagenesis, specific regions in genomes are targeted by using enzymes that recognize specific DNA sequences, while trying to limit off-target mutations to a very low level. Editing of a genome can be done using different techniques, but they usually involve enzymes that can introduce double-stranded DNA breaks and are collectively called site-directed nucleases (SDN). The operational definition of SDNs is provided by the High-Level Group of Scientific Advisors to the European Commission: " . . . . 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" [4]. However, modifications of these enzymes exist that do not produce dsDNA breaks but allow for sequence-specific DNA recognition, binding, and target site modification using base or prime editors [5]. The earlier attempts of genome editing were done by zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), or meganuclease enzymes, which were proteins engineered to have both sequence-specific DNA recognition domain and nuclease (often *Fok*I restriction enzyme) domain. However, most of the genome editing is now done by RNA-directed SDNs, of which the Cas9 nuclease representing the type II-C clustered regularly interspaced short palindromic repeat (CRISPR) system from *Streptococcus pyogenes* is the most commonly used. SDNs can be arbitrarily divided into three types depending on the presence of exogenous DNA, the mechanism for the repair of dsDNA breaks, and the resulting modification in the genome [6]. The SDN-3 scenario results in the insertion of an exogenous DNA (transgene, cisgene, or intragene) into the target genome at a predefined locus determined by the specificity of SDN-3, and as such organisms obtained using SDN-3 are considered as genetically modified organisms (GMO). However, the SDN-1 and SDN-2 scenarios result in plants that contain no exogenous DNA at the target locus, and genome modifications, such as nucleotide substitutions and small deletions and insertions are technically indistinguishable from natural genetic variation. Provided that genome-edited plants do not contain DNA constructs for Cas9 nuclease and single-guide RNA (sgRNA) or other genome-editing reagents, certain types of genome-edited plants are considered as non-GMO by some jurisdictions, e.g., USA, Australia, and Japan [7–9]. In the European Union (EU), however, the European Court of Justice ruling in the case C-528/16 affirmed that the "organisms obtained by mutagenesis are GMOs within the meaning of the Directive" [10], which was taken as a suggestion that the EU GMO legislation may apply to genome-edited organisms. While the applicability of ruling to genome-edited organisms has been addressed recently [11], the potential consequences of considering genome-edited plants as GMOs are of tremendous economic and scientific importance, and the full impact of the ruling may not be immediately apparent, but will certainly have a long-term impact on the competitiveness of European plant breeding industry [12]. Per request of the Council of the European Union (Council Decision (EU) 2019/1904), the EC is conducting an ongoing study involving input from the Member States and different stakeholders regarding the status of novel genomic techniques including genome editing. Within this framework, the European Commission (EC) mandated the European Food Safety Authority (EFSA) to issue a scientific opinion on the risk assessment of plants produced by the SDN-1, SDN-2, and oligonucleotide-directed mutagenesis (ODM) techniques. As the ODM technique does not rely on DNA cleavage at the target site as required by SDN definition, it will be mentioned only briefly here. In this review, I will outline the context of the EFSA opinion on SDN-1, SDN-2, and ODM techniques in relation to SDN-3 opinion. Furthermore, I will outline the main conclusions of the EFSA opinion and then discuss the implications for risk assessment of genome-edited plant products in the EU.

2. Context of Genome Editing within the Existing GMO Risk Assessment Framework

A historical review of the regulatory framework of GMOs and new plant breeding techniques (NBTs) can be found in a recent review [11]. Currently, GMO legal framework in the EU is based on several Directives and Regulations, among which the Directive 2001/18 on the deliberate release into the environment, and the Regulation 1829/2003 that outlines the authorization of genetically modified food and feed are the most relevant. Detailed instructions on the application of Regulation 1829/2003 are provided in the Commission Implementing Regulation 503/2013 (IR503) on applications for authorization of genetically modified food and feed. Thus, GMOs are regulated products in the EU, meaning that a specific risk assessment and management framework is provided. Scientific risk assessment of each new GMO product is delegated to EFSA that issues scientific opinions on products submitted by agricultural biotechnology companies for authorization in the EU based on detailed dossiers. A scientific GMO risk assessment by EFSA is based on (1)

molecular characterization; (2) comparative assessment including agronomic, phenotypic, and compositional characterization; (3) food and feed safety assessment; (4) environmental risk assessment. A set of guidance documents facilitating the preparation of application dossiers is provided by the EFSA, e.g., a Guidance document on environmental risk assessment of GMOs [13] and a Guidance document on risk assessment of GM food and feed [14]. In practice, GMO food and feed risk assessment guidance document is superseded by the Commission Implementing Regulation 503/2013, which is rather specific and quite inflexible. Even though a possibility for derogation is foreseen in Article 5 of the Implementing Regulation, in practice, it is not applied, even though scientific arguments may be available indicating no need for specific information. For example, the mandatory requirement for a 90-day feeding study in rodents was found to be excessive and of little use for risk assessment by GRACE and G-TWYST projects [15,16], nevertheless, it is still required by the EC for all applications containing single transformation events and, in practice, also for applications containing stacked events.

However, the existing guidance documents and the IR503 do not directly address the requirements for risk assessment of genome-edited organisms. Since the early 2000s, when the GMO legislation in the EU was updated, a diverse set of new plant breeding methods was identified [17]. Since these required new approaches for risk assessment, the EC mandated EFSA to provide scientific opinions on some of these techniques. In 2012, EFSA issued an opinion on genetically modified plants developed using cisgenesis and intragenesis [17], and SDN-3 [6]. Specifically, the SDN-3 opinion addressed the new hazards associated with the plants created with SDN-3 techniques and compared these with conventional breeding and established methods of genetic modification. The main hazards were linked to the presence of exogenous DNA at the target site and to the potential off-target effects of SDN-3 enzymes [6]. In Section 4 of the SDN-3 opinion, EFSA identified and characterized the hazards linked to the SDN-3 techniques, such as the source of genes, the safety of gene products and traits, intended alterations to the genome, and possible off-target alterations [6]. The main difference of the SDN-3 technique compared to transgenesis was the ability to direct the insertion to a specific region in the genome. This was considered important because it would allow optimizing the genomic environment for gene expression and minimizing potential disruption of endogenous genes and regulatory sequences. The SDN-3 technique was found to introduce fewer off-target changes than traditional and widely used random mutagenesis techniques. Moreover, it was concluded that, when such off-target mutations occurred, they would be the same as those produced by conventional breeding. In addition, EFSA considered that the Guidance for risk assessment of food and feed from GM plants and the Guidance on the environmental risk assessment of GM plants were applicable to SDN-3 plants. As a consequence, EFSA concluded that 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, and reinforced the need for flexibility in the data requirements for risk assessment [6]. Importantly, the EFSA SDN-3 opinion only reviewed Zinc finger nucleases, TALENs, and meganucleases.

SDN-1 and SDN-2 scenarios were outlined in the 2012 EFSA opinion on the SDN-3 technique and were technically feasible also using ZFN, TALEN, and meganuclease protein-directed genome editing systems [6]. SDN-1 and SDN-2 techniques were rapidly advanced with the Nobel prize-winning application of RNA-directed CRISPR/Cas system for genome editing [18,19], which made it accessible to the broader scientific community and soon resulted in the demonstration of genome editing in many model and crop plant species [20–25]. These demonstrations soon turned into targeted applications with the aim of crop improvement [26–28]. As the purpose of this paper is not to review CRISPR/Cas9 techniques *per se*, the readers are directed to recent papers that comprehensively cover the technology [29–31], applications in different crop plant species [32,33], and regulation [34–37].

Considering the rapid development of genome editing techniques based on CRISPR/Cas9, and the growing number of agricultural applications, as well as the European Court of

Justice ruling in case C-528/16, EC requested EFSA to provide a scientific opinion on organisms developed using SDN-1, SDN-2, and ODM techniques. Even though the ODM technique does not rely on protein- or RNA-guided target site recognition, it was included in the mandate because the outcome is very similar to the SDN-2 scenario, and, in fact, the oilseed rape product developed using ODM by the company Cibus precipitated the ECJ case [37]. Since these organisms could now be considered as GMOs in the EU, the EC needed to know if the existing GMO risk assessment guidance documents including the SDN-3 opinion may be used for risk assessment of genome-edited organisms. The EC mandate M-2019-0095 provided to EFSA specifically asked EFSA to address two terms of reference: (1) the applicability of the risk assessment methodology for hazards described in Section 4 of the SDN-3 opinion for plants developed with SDN-1 and SDN-2; and, if the opinion was affirmative, (2) the validity of conclusions addressing the safety assessment of SDN-3 plants in comparison to plants developed with SDN-1 and SDN-2 [38]. Because of the specifics of the mandate, the Molecular Characterization Working Group of the EFSA GMO Panel was tasked with the preparation of the scientific opinion. EFSA MC WG interpreted the terms of reference as a request to compare the hazards of SDN-1 and SDN-2 plants with those of conventional breeding and established techniques of genetic modification. In parallel, EFSA received from the EC mandate M-2018-0205 to develop a scientific opinion on organisms produced by synthetic biology techniques. Considering the extremely broad scope of synthetic biology mandate, it was split into six work packages covering microbial characterization and environmental risk assessment (ERA) of genetically modified microorganisms (GMM) (WP1), molecular characterization (MC), and an ERA of genetically modified plants (GMP) (WP2), food and feed risk assessment of GMMs (WP3), food and feed risk assessment of GMPs (WP4), MC and ERA of genetically modified animals (WP5) and food and feed risk assessment of genetically modified animals (WP6). Recently, EFSA published its opinion on MC and ERA of GMPs, which focused on three case study including a low-gluten wheat example produced by targeted mutations of multiple α-gliadin genes using CRISPR/Cas9 genome editing [39].

3. EFSA Risk Assessment of Plants Obtained with SDN-1, SDN-2, and ODM Techniques

The main conclusions of the EFSA opinion on plants developed with SDN-1 and SDN-2 techniques are aligned with the specific terms of reference [38], but they also take into account the latest knowledge on genome editing techniques, i.e., the absence of exogenous DNA, lower rates of off-target mutations and the similarity of genome editing mutations to all other mutations used in plant breeding, including natural and induced variation, as well as comparison with conventional plant breeding and established techniques of genome modification. Even though formally the terms of reference for SDN-1 and SDN-2 mandate asked for a comparison with SDN-3, it is clear that, by extension, SDN-1 and SDN-2 also need to be compared to conventional breeding including traditional random mutagenesis, and established methods of genetic modification. The main focus of the assessment of plants obtained with SDN-1 and SDN-2 techniques is on changes in molecular characterization of these plants; however, it is also clear that these mutations occurring at the DNA level may have implications for other pillars of GMO risk assessment, i.e., comparative assessment, food and feed safety assessment and environmental risk assessment.

3.1. Targeted Mutations in Genomes

The main difference between the plants developed with SDN-3, on the one hand, and the plants developed with SDN-1 and SDN-2, on the other hand, is that no exogenous DNA is present in the final SDN-1 and SDN-2 genome-edited plant. Thus, genome editing in SDN-1 and SDN-2 plants is purely done on endogenous sequences, and the desired traits are resulting from changes in endogenous sequences. However, to achieve the editing, it may be necessary to introduce a construct expressing SDN enzyme and sgRNA, and perhaps also to introduce a DNA fragment that is used as a template for homology-directed DNA repair (HDR) in SDN-2 scenario.

SDN-1 and SDN-2 techniques can introduce a multitude of changes in target genomes. Typical for SDN-1 are single nucleotide insertions or deletions of a few nucleotides that occur due to the error-prone NHEJ process [40]. Such deletions can result in frame-shifts, which in turn can result in truncated or mistranslated proteins. Other strategies include the use of two sgRNAs targeting adjacent regions in the same gene, resulting in deletion of part of a gene and its complete inactivation [41]. SDN-2 scenario involves the use of an external DNA fragment that is used by the internal HDR system to introduce small, precisely defined changes, such as single nucleotide substitutions in the target sequence which was subjected to a dsDNA break [31]. The external DNA fragment is presumed to be degraded after the editing occurs and/or eliminated through subsequent cell divisions [42,43]. However, certain techniques of genome editing, such as base editing and prime editing relying on ssDNA breaks or no DNA cleavage at all at the target site, do not require an external DNA template and still result in SDN-2 type changes [5]. ODM technique, which is also covered by the EFSA opinion [38], does not rely on DNA cleavage at the target site. Nevertheless, ODM and different variations of the SDN-2 scenario produce similar types of small changes in the target DNA sequence [44,45]. Since the predicted changes at the target site are the same under the SDN-2 scenario, the expected risks for the same types of mutations are considered similar. As observed with natural or induced random mutations, CRISPR/Cas9 mutations can be of any type and may have many different outcomes, e.g., at the protein level they can lead to an altered amino acid sequence of proteins resulting in non-functional proteins or proteins with altered function [46], or they can also result in spatial or temporal changes in gene expression through the editing of gene regulatory sequences [47] and alternative splicing [48]. Other possibilities for regulating gene expressions, such as epigenetic modification system [49], Cas9-based synthetic transcription factors [50], or direct targeting of specific cellular RNAs [51] are not considered here, because they do not fall under SDN-1 or SDN-2 mode of action.

Some of the alleles and traits created by genome editing mimic alleles that are already present in the breeding gene pool of the same species, e.g., mutations in acetohydroxyacid synthase (AHAS, also known as acetolactate synthase (ALS)) gene conferring herbicide tolerance in oilseed rape [44,52]. Similarly, mutations in all six homeoalleles of the *TaMLO* gene providing powdery mildew resistance in wheat [53] mimic mutations in the barley *MLO* gene [54]. However, there are also possibilities to create completely new mutations that do not yet exist in any crop species, e.g., editing of multiple α-gliadin genes in wheat resulting in low gluten content [55] or deletion of C-terminal autoinhibitory domain of two fruit-specific glutamate decarboxylase genes resulting in a high level of γ-aminobutyric acid in tomato [56]. The low-gluten wheat example also illustrates the power to multiplex mutations either by using the same sgRNA on paralogous sequences or by introducing multiple sgRNAs. CRISPR/Cas9 genome editing differs from random chemical and physical mutagenesis techniques in the sense that it is targeting specific genome sequences. Even though there is some evidence that CRISPR/Cas9 DSBs are repaired in a mostly error-prone manner (reviewed in [57], the type of mutations is nevertheless the same as with other types of mutagenesis. In addition to multiplex genome editing, CRISPR/Cas9 technology permits the editing of gene alleles that are otherwise inaccessible to breeding, because they are located in low recombination regions in genomes [57]. Clearly, these few examples show the multitude of options available to plant breeders, as well as the extreme variability of data that EFSA would need to carry out the risk assessment of these different products. Thus, the case-by-case approach is the only viable option for risk assessment of different genome-edited organisms and the resulting trait should be clearly considered for risk assessment. In fact "case-by-case" principle in risk assessment of GMOs has been applied since the very beginning of recombinant DNA technology [58]. However, even considering only novel mutations and different options for multiplexing, which could give rise to organisms with complex editing patterns, it is clear that the nature of each mutation remains essentially the same as in traditional breeding, while combining different

mutations in a genome-edited organism is undoubtedly speedier, but still essentially the same as combining multiple gene alleles by conventional crop breeding.

An additional point which is relevant for risk assessment is that the editing agent which is either protein or a ribonucleoprotein complex, i.e., sgRNA–Cas9 complex, must be present in the cell at some time to carry out the editing. The presence of CRISPR/Cas9 constructs in a plant genome would make it a GMO, thus, subject to GMO regulation. For sexually reproducing crop plants stably transformed with CRISPR/Cas9 constructs using, e.g., *Agrobacterium* transformation, the constructs can be segregated away, while for vegetatively propagated plants this option may not be available. A number of techniques are available that employ either a transient expression of CRISPR/Cas9 constructs or directly introduce the ribonucleoprotein complexes into cells, which are capable of yielding genome-edited organisms without exogenous DNA [59]. In addition, the SDN-2 approach makes use of an external DNA fragment that serves as a template for homology-directed repair. While this approach is not considered to introduce exogenous DNA into the genome, it still requires some additional testing to prove the absence of these DNA fragments due to accidental incorporation in the genome at random locations due to off-target dsDNA breaks and NHEJ (see Section 3.2).

The EFSA opinion does not address the risk assessment of all the possible types of mutations in detail, mostly because these mutations are the same as those occurring naturally. Nevertheless, technological advancements can result in novel capabilities, e.g., base editing can now introduce transversion mutations [60], while prime editing can result in a rewrite of a part of a gene, which will require flexibility in data requirements for risk assessment. Instead, EFSA has provided a framework for risk assessment of genome editing mutations, which concentrates on the origin of mutations (known alleles versus novel alleles) and the traits of phenotypes resulting from these mutations. Clearly, if alleles of genes obtained by classical mutagenesis or resulting from natural genetic variation, which are used in plant breeding, are mimicked by genome editing, the resulting traits will be identical, and usually, a substantial amount of information on the trait will be available already. Therefore, risk assessment of such a product should require a lesser amount of information. As it is impossible to provide a set of risk assessment instructions for each type of mutation, a more general approach has been taken to allow for case-by-case risk assessment and flexibility of data requirements. Implicitly, the risk assessment of edited endogenous genes requires also the assessment of translated proteins resulting from edited genes and possible newly created open reading frames (ORFs) but again depending on the specific mutations. CRISPR/Cas9 gene knock-outs resulting from NHEJ in the SDN-1 scenario can yield aberrant protein products in human cell lines [46], but presumably also in other cells and organisms. This again is not different from conventional plant breeding, which often intentionally uses germplasm with nonsense mutations in specific genes, e.g., deletions and point mutations in barley (*Hordeum vulgare* L.) *MLO* gene conferring resistance to powdery mildew [54] or deletion in *FAE1* gene in rapeseed (*Brassica napus* L.) conferring low erucic acid trait [61]. No specific risk assessment of such varieties is required, and the potentially detrimental mutations on plant fitness are eliminated through the breeding and variety testing process, as could be done also in the case of genome-edited organisms. Therefore, bioinformatic analyses of modified endogenous protein sequences including putative ORFs in genome-edited organisms should be sufficient in most cases, and experimental data may be needed only in individual cases when specific hazards are identified.

3.2. Off-Target Mutations in Genomes

Similar to the SDN-3 technique, the SDN-1 and SDN-2 are capable of off-target DNA cleavage [62]. The factors that affect the occurrence of off-target effects in plants have been recently reviewed, indicating that the most profound effect on off-target frequency is exerted by the number of mismatches and their position/distance with respect to the protospacer adjacent motif (PAM) [63]. Strategies to reduce the number of off-target mu-

tations, thus, depend primarily on the design of specific sgRNAs. In addition, off-target site cleavage can be reduced by selecting more specific natural enzymes, e.g., *Staphylococcus aureus* Cas9 [64] and Cpf1 (Cas12a) [65] or engineering SpCas9 enzymes with higher specificity [66,67], modifying sgRNAs, limiting exposure to Cas9 nuclease through transient expression or RNP delivery and other approaches (reviewed [68]). In terms of risk assessment, potential off-target changes can be predicted using bioinformatics tools, if a suitable reference genome sequence is available. Unfortunately, this is not always the case, since the number of available genome sequences is still relatively limited for crop plants, and there is no guarantee that the genomes of the germplasm used for genome editing will be closely related to the reference genomes. For example, the genomes of four European maize lines showed pronounced differences compared to the US germplasm [69], and eight rapeseed genomes showed substantial differences among themselves and to a reference genome [70]. Thus, bioinformatic analyses for predicting potential off-target editing have limited value. Moreover, some genome editing techniques that use base editors can induce off-target mutations in random locations [71], which cannot be predicted by bioinformatics tools. Thus, instead of mandating a bioinformatics search, the EFSA recognizes the limited use of off-target searches and recommends that these are used only on a case-by-case basis, when a specific risk hypothesis, e.g., editing members of a gene family, can be formulated. This is particularly valid since generally, and in line with what has been found also for the SDN-3 techniques [6], the off-target changes in SDN-1 and SDN-2 occur substantially less frequently than in the case of random mutagenesis techniques, and where they do occur they are of the same type as any other genetic variation. A recent review summarizes evidence that DSBs resulting from CRISPR/Cas9 cleavage may be repaired in the predominantly error-prone way in human cell lines [57]; however, even if such phenomenon is observed also in plants, and even considering the capacity of the CRISPR/Cas9 technique for multiplex genome editing, the resulting number of off-target mutations would be much lower than in random mutagenesis. Therefore, there are no specific risks associated with off-target mutations caused by the SDN-1 and SDN-2 techniques. These mutations can be easily removed through backcrossing, which is a common breeding technique, while the effects of remaining off-target mutations can be assessed through the agronomic, phenotypic, and compositional characterization of genome-edited organisms, or through regular field trials for variety testing which also in the case of conventional non-GM crops serve to eliminate unfavorable genotypes.

In addition to off-target mutations, dsDNA breaks at off-target sites may occasionally incorporate random pieces of DNA, such as fragments of SDN-encoding plasmids. Such insertions in on-target sites have been demonstrated in potatoes for TALEN-induced dsDNA breaks [72] and CRISPR/Cas9 [73], and in cattle edited with TALENs [74]; therefore it is reasonable to assume that they may occur also at off-target sites. Such insertions are also common in established methods of genetic engineering, which often result in the insertion of exogenous fragments in random locations in the genome, both using particle bombardment techniques and *Agrobacterium* transformation [75,76]. In general, however, the possibility of such insertions is reduced in genome-edited organisms, because the number of off-target dsDNA breaks is small. Nevertheless, the presence of such vector sequences should be controlled in the genome-edited organisms using standard techniques such as sequencing, Southern blot analyses, etc. The presence of other possibly inserted DNA fragments, such as chloroplast DNA, would be much harder to detect, but also would cause little concern from the safety point of view. Any adverse effects from such insertions would be identified and, if necessary, eliminated during breeding.

Thus, in general, off-target effects in SDN-1 and SDN-2 plants are expected to occur at low frequency, similarly to SDN-3 plants. There are strategies to minimize off-target occurrence during the genome editing process, as well as measures to reduce them after genome editing through backcrossing. On a case-by-case basis, if a potential hazard can be identified, bioinformatic analyses can be employed to predict potential off-target sites and subsequently to check them experimentally for the presence of off-target mutations. Most

importantly, however, the type of mutations introduced at off-target sites would be the same as produced by any other breeding technique including random mutagenesis. Therefore, a case-by-case approach is recommended for risk assessment of potential off-target mutations depending on specific risk assessment hypotheses.

4. Impact on the Risk Assessment of Genome-Edited Plants in the EU

The European Court of Justice ruling in 2018 established that organisms obtained through new mutagenesis techniques are subject to GMO legislation in the EU. The EFSA Scientific Opinion on plants obtained by SDN-1, SDN-2, and ODM techniques describes hazards and risk assessment approaches applicable to genome-edited plants, although it does not deliver an itemized list of required information for risk assessment [38]. These are provided by the EFSA guidance documents on environmental risk assessment and risk assessment of GM food and feed [13,14], as well as by the IR503. Considering the wide range of applications and the rapid development of genome editing technology, it is likely that the risk assessment will always be on a case-by-case basis and may range from straightforward to very complex. In simple SDN-2 cases with a single nucleotide substitution(s) leading to herbicide-tolerant enzyme variants which mimic those that are present in conventional herbicide-tolerant crops, the risk assessment would be straightforward. However, in cases involving previously uncharacterized mutations or multiplexed mutations, or complex combinations of both, the risk assessment procedure is far from clear. For instance, the characterization of multiple mutations in α-gliadin genes in low-gluten wheat [55] would be complicated even from the molecular characterization point of view, but it would be even more difficult for food and feed risk assessment considering the potential impact on the allergenicity of the product. Recently, this case was considered by the EFSA scientific opinion on MC and ERA of GMPs, which concluded that the requirements of the EU regulatory framework and existing EFSA guidelines are adequate for the risk assessment of SynBio products to be developed in the next 10 years, although specific requirements may not apply to all products [39]. Specifically, the opinion addressed the highly multiplex editing in low-gluten genome-edited wheat which would require detailed molecular characterization of all the α-gliadin and glutenin genes in hexaploidy wheat genome, as well as the expression level of the α-gliadins. A more detailed evaluation of requirements specific for food and feed risk assessment will be developed by EFSA as part of the WP4 on GMOs developed through SynBio and their implications for risk assessment methodologies [39].

The EFSA was not mandated to develop a new guidance document for risk assessment of genome-edited organisms in the EU. Even though specific sections of existing risk assessment guidance documents are either not applicable to genome-edited organisms or only applicable in specific cases, the general risk assessment approach and the guiding principles are still broadly applicable and sufficient. The development of a new risk assessment framework would be desirable, but it is hindered by the general uncertainty in relation to the GMO regulation in the EU. Moreover, considering the rapid advances in genome editing technology, the development of prescriptive risk assessment procedures is best avoided, while a trait-based risk assessment strategy is definitely worth considering.

The scope of this review was limited to risk assessment of genome-edited organisms. Therefore, risk management, such as detection methods for genome-edited crops, was not considered, even though the detection depends on the type of modification achieved by genome editing enzymes. These are in detail considered in the Joint Research Centre publication [77] and elsewhere [78]. Clearly, the issue is not simply the ability to detect a specific change in the nucleic acid sequence, but rather the ability to do so in a complex mixture, such as food or feed, in a quantitative manner, and to distinguish natural genetic variation from deliberate changes in nucleic acid sequences achieved by genome editing.

One might ask what happens next and what would be the consequences of the EFSA Scientific opinion. First, the opinion was limited in scope, and, second, it was limited by the need to operate within the existing legal framework, without the possibility to consider

risk assessment strategies from other jurisdictions. Therefore, the data requirements of the existing guidance documents and IR503 still apply, as long as the required data can be obtained from a specific genome-edited plant. The EFSA is yet to receive an application for risk assessment of genome-edited plants for food and feed in the EU. It is possible that the agricultural biotechnology companies developing genome-edited plants are waiting for the rules to become clear, and it is hoped that the EFSA Scientific opinion [38] and the study requested by the Council of the European Union on novel genomic techniques will eventually clarify the rules. Increasing numbers of stakeholders including farmers, biotech companies, and scientists in the EU are calling for reform of the EU legislation on GMOs [79–81].

5. Conclusions

In summary, the EFSA opinion concluded that the risk assessment methodology and the existing guidelines are sufficient, although only partially applicable for risk assessment of genome-edited organisms. The literature reviewed in this paper supports this conclusion. In general, if the current GMO regulation is applied to genome-edited organisms, it will have a significant impact on the competitiveness of biotechnology applications in EU agriculture and biomedicine. EU-wide initiatives, such as the Farm to Fork Strategy, will be much more difficult to achieve. In global terms, EU-wide enforcement of GMO regulation on genome-edited crops may have a significant impact on achieving the UN Sustainable Development goals. While the EFSA opinion on SDN-1 and SDN-2 organisms provide a temporary solution for authorizing genome-edited organisms for food and feed in the EU, it still relies on the old GMO regulatory framework and guidance documents developed for organisms produced using established methods of genetic modification. It remains to be seen how many, if any, applications will follow. However, it is clear that even with the provisions of flexibility for data requirements included in the EFSA opinion, the risk assessment procedure as implemented in the EU will remain rather cumbersome. Therefore, it is hoped that the Council of the European Union will utilize the study on new genomic techniques to update the EU GMO legislation to better reflect the realities of modern agricultural biotechnology and the needs of the society.

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