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Overview of EFSA and European national authorities' scientific opinions on the risk assessment of plants developed through New Genomic Techniques

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Abstract

The European Commission requested EFSA to provide an overview on the risk assessment of plants developed through new genomic techniques (NGTs), taking into account its previous scientific opinions, its ongoing work on the topic as well as opinions published by competent authorities and national institutions since 2012, where available. In this report, NGTs are defined as techniques capable to change the genetic material of an organism and have emerged or developed since the adoption of the 2001 genetically modified organism (GMO) legislation. EFSA considered 16 scientific opinions issued by European member states ('MS opinions') as well as three EFSA GMO Panel scientific opinions on NGTs. A procurement to evaluate and summarise the MS opinions was conducted. Relevant information on the description of each NGT and information on the risk assessment of plants developed through one or a combination of the defined NGTs was extracted and summarised. The baseline for the types and nature of NGTs to be included in this report was defined based on the JRC, 2011 report on new plant breeding techniques as well as on the Explanatory Note on New Techniques in Agricultural Biotechnology from the European Commissioner for Health and Food Safety (EC-SAM, 2017) for some more recently developed NGTs, taking into account the NGT definition provided by the European Commission for this mandate. EFSA was not requested to develop new opinions on plants developed through specific NGTs, and thus, no critical appraisal of the reviewed scientific opinions was carried out.

Keywords: new genomic techniques, site-directed nuclease, oligonucleotide-directed mutagenesis, cisgenesis/intragenesis, RNA-dependent DNA methylation, agro-infiltration, base-editing, grafting, reverse breeding, genetically modified plants, risk assessment

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Summary

Following a request from the European Commission, EFSA was asked to provide an overview on the risk assessment of plants developed through new genomic techniques (NGTs). For this overview, EFSA was not requested to develop new opinions on plants developed through specific NGTs. Rather, EFSA was asked to take into account its previous scientific opinions, its ongoing work on the topic as well as opinions published by competent authorities and national institutions since 2012, where available. EFSA was not requested to carry out any critical appraisal of the reviewed scientific opinions.

The definition provided by the European Commission in the frame of this mandate is: NGTs are defined as techniques capable to change the genetic material of an organism and that have emerged or have been developed since the adoption of the GMO legislation in 2001.

Sixteen scientific opinions issued by European member states (provided to EFSA by the European Commission) as well as three EFSA GMO Panel scientific opinions on NGTs were evaluated and relevant information on the description/definition as well as on the risk/safety assessment of plants developed by these techniques was extracted and summarised. As regards to the scientific opinions issued by European member states, EFSA asked RIVM in the frame of a procurement to evaluate and summarise the relevant information in the opinions. A number of inclusion/exclusion criteria were defined in order to extract the relevant information on the NGTs from those MS opinions of a broader content.

Taking into account the definition for NGTs provided by the European Commission for this mandate, the JRC, 2011 report on new plant breeding techniques as well as the Explanatory Note on New Techniques in Agricultural Biotechnology from the European Commissioner for Health and Food Safety (EC-SAM, 2017) for some more recently developed NGTs, were considered as the baseline for the types and nature of NGTs to be included in this report. Based on these reports, a list of NGTs was defined and, when available, information on the description of the techniques and on aspects of the risk assessment of plants developed through one or a combination of these NGTs was included in this overview. The list of NGTs included: (1) cisgenesis and intragenesis; (2) zinc finger nuclease technology (defined more broadly as site-directed nuclease technology); (3) oligonucleotide-directed mutagenesis; (4) RNA-dependent DNA methylation; (5) grafting (on genetically modified rootstock); (6) reverse breeding; (7) agro-infiltration and (8) synthetic genomics. In addition, a recently developed NGT, base/prime-editing, is discussed in four MS opinions as well as in one EFSA GMO Panel opinion, and therefore, information on this technique is also included in this overview.

As regards the EFSA opinions, besides the three site-directed nuclease (SDN)-based NGTs (SDN-1, SDN-2 and SDN-3), information was also extracted on ODM, cisgenesis/intragenesis. In addition, some information on base/prime editing was extracted from the EFSA GMO Panel, 2020 scientific opinion on SDN-1/-2 and ODM. In line with the respective European Commission mandates, two of the EFSA GMO Panel scientific opinions on NGTs (EFSA GMO Panel, 2012a,b on cisgensis/intragenesis and SDN-3, respectively) focused on: addressing the risks for humans, animals and the environment by comparing plants developed via NGTs with plants obtained by conventional breeding methods and by currently applied genetic modification such as transgenesis; and evaluating the applicability of the EFSA GMO Panel GM plant risk assessment guidance documents to the assessment of plants developed by the considered NGTs. The GMO Panel scientific opinion on the safety assessment of plants developed using SDN-1, SDN-2 and ODM approaches (EFSA GMO Panel, 2020) addressed the risks in terms of impact on humans, animals and the environment by evaluating: (a) the applicability of the hazards identified and discussed in Section 4 of the EFSA opinion on SDN-3; (b) the validity/applicability of the conclusions of the EFSA opinion on SDN-3 in the context of the safety evaluation of plants developed by SDN-1, SDN-2 and ODM.

As regards the MS opinions, information was extracted related to all the NGTs defined in JRC, 2011 except for synthetic genomics as well as on newly developed NGTs such as those based on the CRISPR technology, including base editing. In addition, two MS opinions discussed how different NGTs can be used in various combinations. Some MS opinions were produced to serve as advice to a Ministry while others were published as a technical report or a scientific publication. It is noted that some MS opinions discussed the interpretation of the Directive 2001/18/EC in relation to SDNs and intermediate plants. Such information however is out of the scope of this European Commission mandate and it is thus not included in this report.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

On November 2019, the Council of the European Union requested the European Commission (EC), in light of the Court of Justice's judgment in Case C-528/16, to submit a study regarding the status of new genomic techniques (NGTs) under Union law and a proposal, if appropriate in view of the outcomes of the study.[1]

The Council's Decision, based on Article 241 of the Treaty on the Functioning of the European Union, requested the Commission to submit:

- a study, by 30 April 2021, regarding the status of new genomic techniques under Union law and in light of the Court of Justice's judgment;
- if appropriate, in view of the outcomes of the study, a proposal (accompanied by an impact assessment) or otherwise to inform the Council on other measures required as a follow-up to the study.

For this study, the Commission needs an analysis of the status of new genomic techniques that includes safety considerations. In this context, the Commission intends to include in the study relevant existing scientific opinions addressing the safety assessment of plants developed through NGTs.

EFSA published in 2012 two scientific opinions on NGTs: on the risk assessment of plants developed through cisgenesis and intragenesis (EFSA GMO Panel, 2012a) and on the risk assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function (SDN-3, EFSA GMO Panel, 2012b). In addition, a scientific opinion was recently published on plants developed using type 1 and type 2 Site-Directed Nucleases (SDN-1 and SDN-2) and oligonucleotide directed mutagenesis (ODM) (EFSA GMO Panel, 2020).

The EC mandated EFSA, in accordance with Article 29 of Regulation (EC) No178/2002, to provide an overview on the risk assessment of plants developed through NGTs. It was later on agreed by EFSA and EC that the mandate would be addressed in accordance with Article 31 of Regulation (EC) No178/2002 leading to an EFSA scientific output.[2]

EFSA was not requested to develop new opinions on plants developed through specific NGTs. Rather, EFSA was asked to consider 'its previous scientific opinions, its ongoing work on the topic as well as opinions published by competent authorities and national institutions since 2012,[3] where available'. EFSA was not requested to carry out any critical appraisal of the reviewed scientific opinions.

In the frame of this mandate the following definition was provided by the EC: NGTs are defined as techniques capable to change the genetic material of an organism and that have emerged or have been developed since the adoption of the GMO legislation in 2001.[4]

2. Data and methodologies

2.1. Data

To address the mandate's terms of reference (ToR), EFSA took into account the content of: (a) the 16 scientific opinions issued by the European competent authorities and national institutions (hereafter 'MS national bodies') on NGTs provided by European Commission to EFSA[5] (Appendix A); (b) the three EFSA scientific opinions on NGTs (EFSA GMO Panel, 2012a,b, 2020). For the MS opinions, the document number refers to the number of the respective scientific opinion as listed in Appendix A whereas the EFSA opinions are cited as 'EFSA GMO Panel_*'year of publication'* e.g. EFSA GMO Panel, 2012a for the EFSA opinion on cisgenesis and intragenesis.

[1] https://ec.europa.eu/food/plant/gmo/modern_biotech/new-genomic-techniques_en
[2] https://open.efsa.europa.eu/questions/EFSA-Q-2020-00103
[3] This timeline proposed in the EC mandate is to align with the first EFSA scientific opinions on NGTs dating from 2012.
[4] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC - Commission Declaration**.** *Official Journal L 106, 17/04/2001 P. 0001 - 0039*.
[5] The MS opinions provided to EFSA by the European Commission were collected following a request to the members of the 'Joint Working Group of the Standing Committee on Plants, Animals, Food and Feed Section Genetically Modified Food and Feed, Regulatory Committee for Directive 2001/18/EC and Regulatory Committee for Directive 2009/41/EC on new genomic techniques'.

2.2. Methodologies

In producing this scientific report, EFSA asked The Dutch National Institute for Public Health and the Environment (RIVM) in the frame of a procurement (ref number: PO/EFSA/GMO/2020/01) to prepare an overview summarising the relevant information in the 16 scientific opinions produced by the MS national bodies addressing the safety assessment of plants developed through NGTs.

The content in both the MS and EFSA opinions was evaluated and the relevant information was extracted and summarised and is presented in the below sections. As regards the MS opinions, the presented information in this document is based on the RIVM report. In order to extract the relevant information on the NGTs from the MS opinions of a broader content, a number of inclusion/exclusion criteria were defined (see Van der Vlugt, 2021, Section 2.2). Briefly, only information on definition and risk assessment-related issues for plants developed through the considered NGTs was extracted. Hence, elements such as those related to detection methods, risk management/policy, risk assessment considerations of organisms other than plants (e.g. gene drives in insects) or methods that fall outside the given definition of NGTs (e.g. RNAi approaches) were excluded. In some cases, a technique was mentioned in the MS opinions (see Appendix A), but no information based on the applied inclusion/exclusion criteria was actually extracted (see Van der Vlugt, 2021).

In order to extract the relevant information, firstly, a baseline for each NGT was defined based on the description of these techniques in a reference report from the Joint Research Centre (JRC) of the European Commission for the types and nature of new plant breeding techniques (JRC, 2011). For some more recently developed NGTs, considerations described in the Explanatory Note on New Techniques in Agricultural Biotechnology from the European Commissioner for Health and Food Safety (EC-SAM, 2017) were also taken into account. The baseline description of each NGT covered, information on the function and, if available, on the delivery method(s) to plant cells. What was then extracted from the MS and EFSA opinions was information on the description of each NGT additional to this baseline as well as information on the risk assessment aspects of plants developed through the defined NGTs. In order to prevent the repetition of similar information from the different documents, the extracted information is presented once with reference to all relevant opinions and page numbers.

3. Assessment

This chapter contains all the relevant information extracted from the 16 MS scientific opinions and the three EFSA GMO Panel scientific opinions on NGTs. Each NGT is described in a separate section and includes a subsection on the baseline description for each technique according to JRC, 2011 and, if applicable, and/or according to the EC-SAM, 2017 report; a subsection containing the extracted information on the description of each technique additional to the baseline; a subsection containing the extracted information on the risk assessment aspects of each NGT.

3.1. Cisgenesis and intragenesis

3.1.1. Baseline description of the cisgenesis and intragenesis techniques

In JRC, 2011 (p. 20), cisgenesis is described as a genetic modification technique by which DNA fragments only from the same species or from a cross-compatible species are inserted. In addition, the inserted genes, associated introns and regulatory elements are contiguous and unchanged.

In the case of intragenesis, the inserted DNA can be a new combination of DNA fragments from the same species or from a cross-compatible species. Intragenesis can also include the use of silencing approaches, e.g. RNA interference (RNAi). However, as described in the data and methodologies section, the RNAi technology is excluded from the scope of this report.

Cisgenic and intragenic plants are currently produced by the same transformation techniques as transgenic plants with *Agrobacterium*-mediated transformation being the most frequently applied method. Biolistic approaches are also applied in some cases.

Cisgenesis and intragenesis are mentioned in MS opinion 1, 8, 15 and 16 published in 2012 and 2019. Since no relevant information was extracted from opinion 8 and 16, these opinions are not described in the following subsections. In addition, cisgenesis and intragenesis are discussed in EFSA GMO Panel, 2012a.

3.1.2. Extracted information on the description of the cisgenesis and intragenesis techniques

3.1.2.1. Extracted information from EFSA GMO Panel, 2012a

In the EFSA opinion on the safety assessment of plants developed through cisgenesis and intragenesis (EFSA GMO Panel, 2012a), it is further described that for cisgenesis the inserted DNA fragments (gene including its introns and flanked by its native promoter and terminator) are in the normal sense orientation and can harbour one or more cisgenes and they do not contain any parts of transgenes or inserted exogenous sequences. Genes must be isolated, cloned or synthesised and transferred back into a recipient where they are stably integrated and expressed. In the case that any T-DNA border sequences remain in the resulting organism after transformation, it is referred as 'cisgenesis with T-DNA borders'.

In plants produced by intragenesis, the DNA fragments may be arranged in sense or antisense orientation compared to their orientation in the donor organism. Insertion via intragenesis may include the full or partial coding region of a gene frequently combined with another promoter and/or terminator from a gene of the same species or a crossable species.

3.1.2.2. Extracted information from the MS opinions

In Opinion 15, it is stated that the DNA used for genetic modification comes from the donor plant as a whole and it is not established from several fragments (p. 4).

Regarding intragenesis, it is reported that the genetic elements are rearranged *in vitro* and introduced into plants from within the sexual compatibility group (opinion 1, p. 18). Intragenesis allows designing traits using genetic elements from the crop itself (opinion 1, p. 20).

Opinion 1 provides a table (p. 21) describing the major characteristics of the transgene, intragene and cisgene. Here, the presence of *Agrobacterium* sequences used as transfer-DNA (T-DNA) is considered as a cisgene characteristic.

Opinion 1 (p. 131) includes text expressing reservations against the EFSA position that also genes from the tertiary gene pool (= from naturally not crossable organisms of the same species) should be regarded as cisgenes. According to the authors, this consideration invalidates the basic definition of a cisgene (= gene from a cross-compatible species).

3.1.3. Extracted information on the risk assessment aspects of the cisgenesis and intragenesis techniques

3.1.3.1. Extracted information from EFSA GMO Panel, 2012a

EFSA GMO Panel, 2012a addressed the risks in terms of impact on humans, animals and the environment by comparing plants developed by cisgenesis and intragenesis with plants obtained by conventional breeding methods and by transgenesis. An assessment of conventional breeding approaches served as the baseline. In addition, EFSA GMO Panel, 2012a included considerations as regards the applicability of the GMO Panel GM plant risk assessment guidance documents to the assessment of plants developed by cisgenesis and intragenesis.

When considering hazards related to plants developed by cisgenesis or intragenesis, the major considerations made by the GMO Panel included: the source of the DNA and the safety of the gene products; alterations to the host genome at the insertion site and elsewhere; the potential presence of non-plant sequences in the insert; the expression of the trait and its potential wider applications.

Source of the DNA and the safety of the gene products (EFSA GMO Panel, 2012a, section 4.1 p. 9-10); The GMO Panel considered that genes derived from the same gene pools as those used for conventional plant breeding can be used for cisgenesis and intragenesis. However, unlike with conventional breeding, cisgenesis and intragenesis do not introduce the range of other genes and sequences that can be associated with linkage drag and thus the introduction of the unwanted traits and hazards associated with these other genes/sequences can be avoided. As regards the hazards associated with the introduced genes, the risks arising from the use of a related plant-derived gene by cisgenesis are similar to those from conventional plant breeding. However, when a related plant-derived gene is used in intragenesis, some new combinations of genetic elements may arise that are not found in cisgenic and conventionally bred plants and these may present, as for transgenic plants, novel traits with novel hazards.

Alterations to the genome (EFSA GMO Panel, 2012a, section 4.2, p. 10-13); EFSA GMO Panel, 2012a states that during the development of cisgenic, intragenic and transgenic plants, alterations to the genome can be expected to occur over and above the introduction of the inserted gene(s), caused by various processes and mechanisms. Any undesirable changes occurring in the genome in conventional breeding or in the production of transgenic, cisgenic or intragenic plants might be removed by backcrossing. This will depend on the reproductive biology of the species and the commercial practices used for breeding.

Cisgenesis and intragenesis can be achieved by the same transformation techniques as transgenesis. The potential for 'random' changes to the genome caused by the insertion event is, however, not limited to these approaches and is in fact independent of the breeding methodology. Mutational processes such as insertions/deletions/rearrangements of endogenous genes and regulatory sequences not only occur in transgenesis, cisgenesis and intragenesis but they are also known to occur in conventional breeding approaches. New open reading frames (ORFs) will be created at random during conventional breeding, cisgenesis, intragenesis and transgenesis potentially giving rise to new proteins. However, with transgenesis, exogenous, non-host (and even non-plant) DNA is involved, possibly leading to the formation of sequence combinations and ORFs which would normally not occur with conventional breeding or cisgenesis. Similarly, with intragenesis, new combinations could arise in ORFs, due to reconfiguration of the host sequences.

Plant regeneration in tissue culture can be a major contributor to genetic and epigenetic changes (somaclonal variation) and can have a significant impact on phenotype. This can occur during the development of cisgenic, intragenic and transgenic plants but also in conventional breeding and plant propagation whenever tissue culture processes are used. Undesirable phenotypes can be discarded by the breeder or may be eliminated by backcrossing where possible.

Presence of non-plant sequences in the insert (EFSA GMO Panel, 2012a, section 4.3, p. 13-14); EFSA GMO Panel, 2012a discussed that as with transgenesis, in some cases, short sequences not originating from the donor plant (e.g. multiple cloning sites or T-DNA border sequence repeats (left border (LB) and right border (RB) in the case of *Agrobacterium*-mediated transformation)) might be inserted into the recipient plant together with the cisgene or intragene, as the result of the transformation method. Border sequences are short DNA sequence repeats up to 25 nucleotides. Evidence indicates that sequences similar to the T-DNA border sequences can be found in plants. Also, vectors specifically constructed for cisgenic/intragenic approaches can be employed which use DNA sequences originating from the same plant species or related species to insert the target genes to circumvent issues arising from the use of bacterial border sequences. In conclusion, any hazards related to T-DNA border sequences would not differ from those in conventional breeding.

EFSA GMO Panel, 2012a also discussed that other sequences which may be transferred to the plant include vector backbone sequences or selectable marker genes. By definition, cisgenic and intragenic plants must not contain vector backbone sequences of bacterial origin. Marker genes are not usually derived from the recipient species (breeders' gene pool) and in such cases cannot be present in cisgenic/intragenic plants. Marker genes can be eliminated from the final plant by several techniques.

Expression of the trait/modification of gene expression (EFSA GMO Panel, 2012a, section 4.4, p. 14-15); EFSA GMO Panel, 2012a states that the expression of novel proteins and metabolites or increased levels of endogenous proteins and metabolites might raise safety issues in both natural variants and plants generated by any breeding technology. As with transgene expression, numerous factors can influence the level of cisgene and intragene expression.

In cisgenesis, the transferred genes are derived from sexually crossable species (alleles of endogenous genes), and they will always be flanked by their native promoters. Although it might be expected that the use of a native promoter is more likely to result in an expression pattern similar to the donor plant this is not guaranteed. For example, the length of the cis regulatory elements transferred as part of the cisgene to the recipient plant will likely impact on the expression pattern.

Intragenesis offers considerably more options for modifying gene expression and trait development than cisgenesis since genes and their promoters and regulatory elements are interchangeable within the intragenes. Intragenesis can also include intragenes which target gene silencing, e.g. RNAi using within-species DNA sequences. Achieving gene silencing in plant breeding is, however, not confined to transgenic, cisgenic and intragenic approaches as, alleles of silenced genes have also been selected in conventional plant breeding.

Applicability of the guidance documents (EFSA GMO Panel, 2012a, section 5, p. 17-19); As stated in EFSA GMO Panel, 2012a, in order to assess the adequacy of the current EFSA guidance documents for the risk assessment of cisgenic and intragenic plants, the EFSA GMO Panel focused on the *Guidance*

for risk assessment of food and feed from genetically modified plants (EFSA GMO Panel, 2011a) and the *Guidance on the environmental risk assessment of genetically modified plants* (EFSA GMO Panel, 2010).

As regards the molecular characterisation (MC) data requirements described in EFSA GMO Panel, 2011a, it was considered that since cisgenes are derived from the breeders' gene pool and contain their own promoter and terminator, some MC elements should be reconsidered (e.g. ORFs analysis within the insert is not needed as no new internal junctions are created). In addition, some flexibility in the field trial design for protein expression analysis might be needed. Thus, an update of the existing risk assessment guidance should be considered to introduce additional flexibility.

As regards the food and feed data requirements described in EFSA GMO Panel, 2011a, it was concluded that based on the origin and the structure of the cisgenes remaining unchanged in the recipient plant, it can be expected that in cisgenic/intragenic plants, the gene products would be similar to those in the donor. Moreover, with regard to the exposure assessment of cisgene-encoded proteins and associated metabolites, sufficient information may already exist on the levels of safe consumption and specific toxicity testing might not be required. In summary, the GMO Panel considered that the general approach and all elements described in EFSA GMO Panel, 2011a for the risk assessment of food and feed from GM plants is, at the present time, sufficient for the evaluation of cisgenic/intragenic plants and derived food and feed. However, it can be envisaged that, on a case-by-case basis, lesser amounts of event-specific data are needed.

As regards the applicability of the guidance on the environmental risk assessment of GM plants (EFSA GMO Panel, 2010), EFSA GMO Panel, 2012a confirmed that all elements described in the guidance can apply to cisgenic/intragenic plants, and the relevance of applying specific elements of the guidance is defined on a case-by-case basis.

3.1.3.2. Extracted information from the MS opinions

In cisgenic plants, native promoters may lead to the constitutive expression of genes, which may be above the native expression level of a gene in its original format. The modified levels of expression can alter the environmental behaviour of the plant and render considerations concerning the exposure of potential consumers necessary (opinion 1, p. 27).

According to opinion 1 (p. 30), gene pyramiding (the combination of multiple genes into a single genotype) may result in gene interactions that might differ depending on the genetic background into which they have been introduced.

The random introduction of a cisgene in a genome (by e.g. *Agrobacterium-mediated* transformation) may result in a change of expression or interruption of an endogenous gene. The inserted cisgene could also merge with a gene that is already present in the plant's genome, and could, in theory, lead to the creation of new proteins (opinion 15, p. 5). Moreover, a drawback of *Agrobacterium*-mediated transformation is that – using a T-DNA-based transformation technology – cisgenic plants (like transgenic plants) can contain short non-coding bacterial border sequences. At least three to four nucleotides from the right T-DNA border sequence are transferred into the plant genome. Due to the imprecise nature of T-DNA integration (nicking of the left border T-DNA) at the left border site, non-T-DNA sequences from the vector backbone are frequently integrated into the plant genome (opinion 1, p. 74). Overall, opinion 1 (p. 107) argues that the molecular characterisation is a critical part of the risk assessment of cisgenic plants, as only a solid characterisation of the DNA sequence of the insert and the flanking sequences can actually demonstrate its cisgenic character.

Opinion 1 (p. 108) also mentions that to assess the possibility of unintended effects, a comprehensive molecular characterisation of cisgenic plants modified by an established genetic transformation technique like *Agrobacterium*-mediated transformation or particle bombardment is always necessary. Furthermore, substantial equivalence tests carried out to compare different phenotypic characteristics like composition and agronomic parameters can be applied to cisgenic plants. This is because, in general, also a cisgenic plant could differ substantially from its conventional comparator, as a novel gene derived from a cross-compatible species could disturb the plant's metabolism. Comparative tests can therefore be used to strengthen the conclusions of the molecular characterisation and to confirm the absence of any unanticipated effects caused by the genetic modification process.

Opinion 1 (p. 108) also discusses the possibility that cisgenic transformation results in plants that are not substantially different in terms of phenotypic characteristics, and thus posing similar risks for human and animal health as traditionally bred plants. This question can be answered with reliable certainty only if comprehensive comparative analyses between a cisgenic plant and its conventional counterpart – based on the state-of-the-art field designs using powerful statistical approaches – are conducted.

The toxicological and allergenicity risk assessment of GM plants should provide sufficient information to conclude whether or not the derived food and feed has the potential to harm humans and/or animals. Cisgenic plants as per strict definition express proteins that originate from cross-compatible species only; therefore, it is not clear how unambiguous the definition of cisgenesis is in terms of food safety. If the (distant) relative is also being used as a food source, the safety assessment of the newly introduced protein may benefit from the knowledge that it is already part of the human diet. The food safety assessment should take this into account and be conducted accordingly. On the other hand, if the wild relative may not form part of the human diet yet, and in that case, it would be prudent to assess the safety of the newly introduced sequences and protein(s). At any rate, it will be necessary to check for overexpression of newly expressed proteins possibly caused by gene interactions and/or epigenetic effects (opinion 1, p. 109).

Specific toxicity testing may not be required in cases where it is well documented that both the donor plant and the newly expressed proteins in cisgenic/intragenic plants have a sufficient history of safe consumption as food and feed also taking into account the intake levels. However, if the intake levels are outside of the 'known to be safe range', further safety assessment is needed (opinion 1, p. 131).

Furthermore, the possible occurrence of unintended effects in the new plant variety will not be different for cisgenic varieties compared to transgenic varieties (opinion 1, p. 109).

Opinion 1 (see p. 110) also discusses that a much higher risk of adverse effects becomes evident in the case of cultivation compared to imported/processed plant material. For such cases, additional points need to be considered as e.g. impacts of the specific cultivation, management and harvesting techniques. Much more attention has also to be paid to any potential for unwanted interactions with target and non-target organisms, gene transfer (horizontal and vertical) and negative effects on soil and biogeochemical processes.

In conclusion, it can be said that the current EU regulatory framework for genetically modified food and feed, as well as the respective EFSA Guidance Document (EFSA GMO Panel, 2011a) in general, will be applicable also for GM plants developed using cisgenic or intragenic techniques (opinion 1, p. 111).

Provided that the plant harbours only the cisgene and it is indeed cisgenic according to the definition, the data requirements for risk assessment may be reduced and only parts of the risk assessment may be implemented on a case-by-case basis thus enabling the simplification of the risk assessment process (opinion 1, p. 124).

Finally, the authors of opinion 1 (p. 131) agree with EFSA's position that there is no need for the open reading frame (ORF) searches within the insert as no new junctions with the plant genome are generated within the insert. However, insert junctions and flanking sequences have to be risk assessed in the same way as with transgenes.

As stated in opinion 15 (p. 5), cisgenic plants cannot acquire properties that are not present in the species itself or in crossable species. Merging of a cisgene with an endogenous gene may also arise under natural conditions or through conventional breeding. Given that the cisgene is controlled by its own regulatory signals, the level of expression will remain within the range of expression levels of traditionally bred plants. Therefore, cisgenic plants do not present a higher risk to humans and the environment than those of traditionally bred plants.

In the case of intragenesis, the regulatory elements of the inserted genes may be replaced by the regulatory signals of other genes as long as they originate from crossable relatives. The regulatory signals of a gene determine the extent, the location and timing of gene expression. Therefore, the gene's expression can be changed by replacing the regulatory signal (in particular the promoter) of a gene with other regulatory signals or by adding regulatory signals. As a consequence, the intragene may be expressed at different times, in different plant parts or in altered levels (opinion 15, p. 7). Moreover, chimeric genes and chimeric regulatory sequences can be created by DNA shuffling using sequences of different alleles or genes from crossable relatives. The creation of these genes or regulatory sequences makes it possible to obtain new proteins and/or to change expression profiles (opinion 15, p. 8).

3.2. SDN technology

3.2.1. Baseline description of the SDN techniques

The information on the SDN technology from EC-SAM, 2017 is used as a baseline for the description of the SDN technology. EC-SAM, 2017 defines site-directed nuclease (SDN) as: an enzyme (endonuclease) that creates site-specific double-strand breaks (DSBs) at defined sequences. SDN

typically recognises a specific DNA sequence and 'cleaves' DNA within such a sequence or nearby. The recognition of the DNA target is mediated by the protein molecule itself (in protein-directed SDNs) or by an associated guide RNA molecule (in RNA-directed SDNs). SDNs cut DNA at selected target sites producing double-stranded breaks (DSBs) while enabling the insertion of random (SDN-1), or non-random (SDN-2) mutations or even the insertion of large segments such as genes (SDN-3), in precise locations (p 58). On p. 58, it is also discussed that all three SDN approaches rely on natural cellular mechanisms for repairing such DNA cuts; SDN-1 relies on the non-homologous end joining (NHEJ) pathway and SDN-2 and SDN-3 on homology-directed repair (HDR).

EC-SAM, 2017 mentions two groups of nucleases: (1) the protein-directed SDNs such as ZFN and TALEN, proteins that consist of a DNA-binding domain which recognises a specific DNA sequence attached to a nuclease which cuts the DNA strand at the binding site; (2) the RNA-directed SDNs that are based on the CRISPR-Cas system. They consist of a protein module (Cas nuclease) bound to a guide RNA (gRNA), the sequence of which targets the nuclease to the complementary DNA sequence in the genome (p. 59-60). At p. 61, it is mentioned that the simultaneous or staggered introduction of changes at several locations in the genomes is possible by using several different RNA guides.

The EC-SAM, 2017 report (p. 60-61) also mentions that common techniques such as *Agrobacterium tumefaciens* mediated T-DNA transfer or biolistic bombardment are employed to deliver the DNA constructs encoding CRISPR-Cas9 into plant cells. During this process, CRISPR-Cas9 constructs are integrated into the plant genome but crossed out in the next generation.

The SDN technology is mentioned in 14 out of 16 MS opinions and it is also discussed in EFSA GMO Panel, 2012b (SDN-3) and EFSA GMO Panel, 2020 (discussing SDN-1 and SDN-2 together). Some MS opinions discussed together all types of SDN technology (SDN-1, SDN-2 and SDN-3) whereas others presented information on an individual type of SDN technology (see Appendix A). Furthermore, Opinion 1 addressed specifically ZFNs, whereas Opinion 4, 5, 13 and 14 dealt specifically with CRISPR-Cas (also see Appendix A). Since no information was extracted from opinions 9, 12 and 14, these opinions are not described in the following subsections.

The information related to the SDN technology in general is first presented in the Sections 3.2.2 (description) and 3.2.3 (aspects on risk assessment). Extracted information related to each specific SDN approach is presented in Sections 3.3–3.5.

3.2.2. Extracted information on the description of the SDN techniques

3.2.2.1. Extracted information from EFSA GMO Panel, 2012b, 2020

In EFSA GMO Panel, 2012b, the following information is presented on the description of each of the three SDN approaches:

- With SDN-1 technology, the SDNs are introduced (stably or transiently), generating random site-specific mutations (changes of single base pairs, short deletions and insertions) by non-homologous end-joining (NHEJ). In case of an insertion, the inserted material is derived from the organism's own genome i.e. it is not exogenous. The DNA ends (from the strand break) may also become joined to a completely unrelated site, which results in chromosome translocation.
- With SDN-2 technology, homologous repair DNA (donor DNA) is introduced together with the SDN complex to create specific nucleotide sequence changes by homologous recombination (HR). The SDN-2 technique can result in minor or more substantial changes to the nucleotide sequences of the target gene.
- With SDN-3 technology, a large stretch of donor DNA (up to several kilobases) is introduced together with the SDN complex to target DNA insertion into a predefined genomic locus. The predefined locus may or may not have extensive similarity to the DNA to be inserted. The insertion can take place either by HR or by NHEJ. The technique targets the delivery of transgenes (insertions).

Although the SDN-1, -2 and -3 approaches all target a specific locus in the genome and use a nuclease to induce breaks in DNA, the three SDN approaches trigger different repair outcomes, the intended changes range from point mutations to large insertions and deletions (p. 6).

The gene encoding the SDN that will recognise the target locus, and the donor DNA bearing the transgene flanked by DNA stretches with homology to this locus, can be delivered into the plant cell via various methods. The SDN-encoding gene can be expressed transiently or can be stably integrated into the host genome (p. 11). Once the targeted integration of the transgene has been achieved, the

introduced SDN gene and the donor DNA at non-targeted loci can be removed by segregation to generate plants containing only the targeted integration of the transgene and no exogenous DNA (p. 12).

EFSA GMO Panel, 2020 does not contain any additional information on the description of the SDN techniques *per se* compared to EC-SAM, 2017 and EFSA GMO Panel, 2012b. It is, however, mentioned that ZFNs, TALENs, meganucleases and the CRISPR-Cas system can all be used to achieve random (SDN-1) and predicted (SDN-2) mutations of a targeted genomic locus and precise insertion of a DNA sequence (SDN-3) (EFSA GMO Panel, 2020, Section 3.1.2, p. 7). As regards the methods for delivery of the 'SDN module' (molecular components necessary to achieve the genetic mutation), EFSA GMO Panel, 2020 (EFSA GMO Panel, 2020, Section 3.1.3, p. 7) discussed that site-directed mutagenesis by SDN methods can be achieved by the stable integration, the transient expression, or the 'DNA-free' delivery of the SDN module. In the case of stable integration and for sexually propagated crops, the SDN module can be removed by segregation. This step will probably not be performed for commonly asexually (vegetatively) propagated crops. It is also mentioned that multiple delivery systems for SDN components have been tested for different plant tissues, including PEG-fusion, electroporation and biolistics.

3.2.2.2. Extracted information from the MS opinions

In opinion 16, p. 6, the provided definitions for the different SDN technologies are concise:

- SDN-1 is a type of mutagenesis using site-specific nucleases; it creates a random mutation in a precise genome location;
- SDN-2 is a type of mutagenesis using site-specific nucleases allowing the introduction of a particular mutation into a specific location in the genome;
- SDN-3 is a type of mutagenesis using site-specific nucleases allowing the introduction of a desirable DNA fragment into a chosen location in the genome.

The cellular response, as to which of the repair mechanisms will be used to repair the DSB (Opinion 11, p. 16-17) depends on the phase of the cell cycle in which the DSB is induced, i.e. whether or not the cell is in a cell division phase. NHEJ can be used at any stage of the cell cycle, whereas repair of the DSB by HDR occurs only in the S and G2 phases of the cell cycle, almost exclusively in meiosis in the case of plants.

Opinion 3, p. 13–14 (similarly opinion 8, p. 26 and opinion 13, p. 4) presents an overview of the different CRISPR-Cas delivery techniques:

Stable transformation of gRNA-Cas9 gene cassettes: guide RNA (gRNA)-Cas9 gene cassettes including a selectable marker gene are transformed into plant cells and become stably integrated during a selection step. gRNA-Cas9 is expressed from transgenic DNA. Transformation methods mainly used are *Agrobacterium*-mediated gene transfer and microprojectile (particle) bombardment, or electroporation and polyethylene-mediated transformation for plant protoplasts. In crop species which can be propagated by sexual reproduction, the genome-edited progeny free of the CRISPR-Cas9 cassette including the marker gene can be selected in the next generation(s). In this case, transgenic events are present in intermediate products during the production process but are lacking in the final product.

Transient transformation of gRNA-Cas9 gene cassettes: gRNA-Cas9 gene cassettes are transformed into plant cells and CRISPR-Cas9 is expressed from these templates. The applied transformation methods are as those described above. The production process does not include a selection step for stable genomic integration of the gene cassette. A second strategy for the transient delivery of the gRNA-Cas9 gene cassette uses viral vectors. They may either be delivered via *Agrobacterium*-mediated gene transfer, via viral particles or isolated viral RNA.

Delivery of pre-assembled gRNA-Cas9 ribonucleoprotein complexes: Ribonucleoprotein complexes are delivered into plant cells to directly exert their function via e.g. PEG-mediated particle delivery. In the case of SDN-1 technology, this method does not involve exogenous DNA delivery into plant cells.

SDN techniques can be used to make more than one modification to the genome simultaneously (opinion 8, p. 22):

- At one locus:
  - SDN-2: using one 'repair template' a combination of several mutations/insertions/deletions at the same locus can be produced
  - SDN-3: allowing insertion of more than one transgene at just one locus.

- At multiple loci: SDN techniques can be applied simultaneously to multiple regions of the genome by introducing multiple nucleases and/or guide RNAs and multiple repair templates.

In Opinion 8 (p. 23), the authors debated the boundaries between the different SDN technologies and came to the following agreement:

- SDN-2 differs from SDN-1 because it makes use of a template allowing modification, by recombination, of the gene targeted by the nuclease-induced break.
- SDN-2 differs from SDN-3 because in the case of SDN-2, the gene to be modified is present in the plant, remains in its location in the genome and keeps its copy number.
- The boundary between SDN-2 and SDN-3 with regard to the type of modification is not necessarily clear. With SDN-2, the modified DNA sequence could lead, for example, to the formation of new RNA transcripts (even short, such as small RNA) or could contain new regulating sequences to control gene expression, as could SDN-3.

3.2.3. Extracted information on the risk assessment aspects of the SDN techniques

3.2.3.1. Extracted information from EFSA GMO Panel, 2012b, 2020

Information on the risk assessment aspects for SDN approaches in EFSA GMO Panel, 2020 is presented for the SDN-1 and the SDN-2 techniques. A summary of this information is included in the section covering SDN-1 (see Section 3.3 of the present document). In addition, information on the risk assessment aspects for SDN-3 approaches extracted from EFSA GMO Panel, 2012b is described in the SDN-3 specific section of the present report (see Section 3.5).

3.2.3.2. Extracted information from the MS opinions

Opinion 1 is focused on the use of ZFNs. It is mentioned on p. 54 that transient DNA-transfer methods for the delivery of ZFN-expression constructs into target cells may still lead to unwanted and hard to detect traces of exogenous DNA (resulting from the ZFN construct) in the mutated lines. Thus, even when using transient ZFN expression, crop plants can potentially be classified as transgenic or be subjected to extensive investigation to confirm that they do not possess any traces of exogenous DNA within their genome.

Furthermore, as regards the target site specificity of ZFNs, opinion 1 (p. 51) mentions: zinc finger proteins detect typical consensus sequences for binding. This implies that these DNA-binding proteins do also recognise sequences aberrant from the canonical consensus sequence albeit efficient binding will occur at a significantly lower frequency compared to their primary targets. Although the ZFN technology is a substantial improvement in respect to random insertion of exogenous genetic material, this process is still inherently unspecific to a certain degree and may introduce unintended effects due to binding and cleavage at non-target sites in the plant genome. This observation must be taken into account for regulation and risk assessment of organisms produced by this methodology. Target site sequence specificity may be enhanced by combining up to six zinc finger protein elements targeting 18 base pairs at each side of the restriction site.

In opinion 3 (p. 26), it is mentioned that recognition of the target site by a CRISPR-Cas9 complex is guided by two different signatures: the presence of a protospacer adjacent motif (PAM) and the complementarity of the spacer sequence in the sgRNA to the protospacer sequence. Next to nucleotide composition, structural features of the gRNA backbone influence CRISPR-Cas9 efficiency and thus also contribute to off-target activity. Opinion 3 presents a table (p. 104–108) listing studies that report the analysis of off-target activity in plant cells.

To limit off-target effects, several software applications to automate gRNA selection are available (opinion 3, p. 28). For plant species where the whole genome sequence is available, the main strategy is to design a very specific gRNA sequence and to check for the presence of off-target sequences in the genome to which the guide RNA sequence could bind non-specifically. Different software platforms have been developed to design gRNA sequences which will very specifically bind to the region containing the intended mutation site (opinion 6, p. 5). In opinion 8 (p. 48), it is however mentioned that to limit possible off-target mutations through choosing the gRNA sequence, the plant's genome sequence must be known. Given the range of natural genetic variability, the reference sequences will not necessarily match the sequence of the variety under consideration. It is therefore hard to identify possible off-target mutations. Verification by whole genome sequencing is difficult in crop plants because of the size and diversity of sequence repeats in these genomes.

Furthermore, the CRISPR-Cas9 specificity can be improved by increasing the number of nucleotides required to recognise the target site in the plant genome. This can be done using the Cas9 nickase or Cas9 FokI fusion protein strategies which both reduce the number of possible off-target sites (opinion 6, p. 5). Novel types of Cas proteins have also been discovered that could contribute to reducing 'off-site' targeting (opinion 4, p. 4). To this end, newly developed spCas9-HF or the Cas12a nuclease, both possessing higher specificity are mentioned in opinion 6, p. 6.

Experimental strategies to limit off-target effects include the application of paired nickases or RNA-guided FokI nucleases. The essence is that single-stranded DNA breaks (nicks) are introduced and by targeting two complementing paired nickases properly spaced to the same locus, a double-stranded break (DSB) is generated. At the same time, specificity is increased since two spacer sequences are needed for induction of a DSB (opinion 3, p. 28; opinion 6, p. 5-6).

The delivery method used for the CRISPR-Cas9 complex to the cells also greatly influences the frequency of off-target mutations due to the final concentration of the Cas9 and gRNA present in the cells (opinion 13, p. 9). Studies have shown that delivery of the CRISPR-Cas9 complex as ribonucleoprotein (RNP) reduces the number of off-target mutations since RNP complexes degrade much faster in the cell than DNA constructs (opinion 6, p. 6).

In opinion 6 (p. 5), it is mentioned that CRISPR-Cas9 induces relatively more off-target mutations than ODM and protein-based nucleases due to a less specific binding capacity. For CRISPR-Cas9, the recognition sequence is a 20-nucleotide sequence complementary to a 20-nucleotide genomic sequence located where the mutation is intended. Although a 20-nucleotide gRNA recognition sequence is long enough to occur only once in the vast majority of plant genomes, the specific binding is highest for the 8–12 nucleotides of the gRNA following the PAM sequence. This means that the gRNA can bind to sequences with mismatches between the gRNA and the plant genomic DNA in the last 8–12 nucleotides.

It is difficult to make a general estimate of the off-target mutation frequency induced by the CRISPR-Cas9 tool in plants (opinion 6, p. 6). In this opinion, a table is provided presenting studies where off-target mutations induced by CRISPR-Cas9 were identified. A surprising result of a study mentioned in this opinion on genome-edited rice plants was that most mutations were created by the tissue culture process which caused 102–248 single-nucleotide variations and 32–83 indels per mutated plant. This is also indicated in opinion 7 (p. 2): off-target mutations in plants are generally less frequent than the somatic mutations that can emerge from tissue cultures. Interestingly, in opinion 10, p. 31, it is stated that CRISPR-Cas9 studies on *Arabidopsis thaliana*, indicated 178 potential off-target sites but no insertions or deletions ('indels') in these genetic loci were detected. In opinion 11, p. 20, it is noted that there are fewer scientific studies on the number of off-target mutations induced by TALENs and ZFNs than for CRISPR-Cas9.

Off-target mutation frequencies can be estimated by whole-genome sequencing (WGS). In order to get maximum information from this method, the appropriate controls need to be included to discriminate between the mutagenesis effects induced by tissue culturing and CRISPR-Cas (opinion 6, p. 6).

When considering modifications through genome editing, it should not be overlooked that mutations also occur naturally, e.g. due to UV radiation or errors in DNA replication (opinion 10, p. 31).

Off-target effects can lead to unintended effects. Off-target activity caused by DSBs at loci with imperfect complementarity to the spacer sequence might lead, depending on the SDN technique, to either (i) induction of random mutations at off-target loci, (ii) deletion of genomic fragments, (iii) integration of cis-, intra-, or transgenes at unintended loci or (iv) a combination of those (opinion 3, p. 28-29).

Moreover, potential unintended effects through using transgenic CRISPR-Cas9 intermediate lines may be (i) retention of the transgene in resulting organisms and (ii) generation of background mutations due to the performed transformation process, which are passed on to resulting organisms. An unintended effect due to the use of viral vector systems is a viral contamination of progeny (opinion 3, p. 29).

The (random) unintended mutational load of CRISPR-Cas9 genome edited plants is much smaller in comparison to conventional mutation breeding methods, based on available data sets. Generally, for plant breeding applications, CRISPR-Cas9 specificity is important; however, since there is selection and backcrosses during plant breeding practices, off-target effects can be segregated away in the final product or can be determined as tolerable (analogous to classical mutation breeding) (opinion 3, p. 31; opinion 4, p. 4).

In opinion 8, p. 21–22, the following characteristics are described for off-target mutations due to SDN technology:

- They are found in sequences similar to the target sequences (one to five nucleotide differences depending on the technique). They are therefore computationally predictable in known genomes, or can be identified by sequencing.
- The biochemistry of off-target mutations is the same as that of natural variations. Since SDNs produce double-stranded breaks in DNA, physiological repair systems are called into play.
  - The fact that there is little variation in some regions of the genome is accounted for by the functional importance of these regions (negative selection pressure). Effects due to off-target mutations in these regions would therefore highly likely be associated with a phenotype that the breeder could choose whether to keep or not.
  - Some types of mutation at given sites (e.g. variations in the number of repetitions in repeated regions) are influenced by the mechanisms involved in producing these mutations but are not indicative of any particular risks. An SDN-induced break in these regions would therefore have the same consequences as a natural break followed by physiological repair.
- For SDN-2 and SDN-3, the consequence of an off-target mutation will probably be the same as for SDN-1, since the probability for the template DNA to recombine in the area of the DNA break is very small. This also applies to off-target mutations found in ODM, where sequence homology is necessary for the used oligonucleotides.

Furthermore, the following unintended effects are noted for the SDN technology, specifically if intermediate plants were carrying the effector (opinion 8, p. 46):

- Persistence of nuclease expression may result in a larger number of off-target modifications
- Persistence of gRNA alone does not seem to be associated with any specific risks;
- Persistence of a nuclease (such as Cas9) and gRNA together may result in a larger number of off-target modifications;
- Crossing of plants containing these effectors (e.g. a plant containing Cas9 with a plant containing gRNA) may result in genetic modifications in offspring;
- In the particular case of a sequence recognised by gRNA being homologous to a region in which a transgene encoding a nuclease and guide RNA is stably inserted, this could lead to a gene drive event. This is, however, considered highly unlikely.

3.3. SDN-1 technology

3.3.1. Baseline description of the SDN-1 technique

See Section 3.2.1 for the baseline description of the SDN-1 technique.

3.3.2. Extracted information on the description of the SDN-1 technique

3.3.2.1. Extracted information from EFSA GMO Panel, 2012b, 2020

See Section 3.2.2.1 for the extracted information on the description of the SDN-1 technique as defined in the two EFSA scientific opinions.

3.3.2.2. Extracted information from the MS opinions

In opinion 3, p. 16, the type(s) of mutations that can be obtained by SDN-1 is described in detail: SDN-1 can be exploited to induce for example gene knock-outs by frameshift mutations when targeted to coding regions. The DSB can also be targeted to non-coding regions, e.g. to impair or delete regulatory elements, thereby inducing a change in gene expression. In extension, two DSBs can be induced by the delivery of two Cas9-gRNA modules targeting different locations, resulting in the deletion of the region in-between. Finally, the induction of two DSBs has the potential to induce chromosomal re-arrangements (inversion, duplication or translocation events) which may be exploited for genome editing.

An advantage of SDN-1 (Opinion 3, p. 24) is that it only involves the delivery of the nuclease complex and may result in:

- targeted deletions using two DSBs that range from small deletions of, e.g. ~50bp in tomato to ~ 245 kbp in rice. The latter resulted in deletion of a diterpenoid synthetic gene cluster of 10 loci, exemplifying the potential to eliminate large genomic regions.
- multiplexing ability using CRISPR-Cas9 that has been shown e.g. in rice plants targeting up to seven and eight sites simultaneously with different gRNAs.

According to opinion 3 (p. 25), data sets describing the type of mutations generated by SDN-1 are reported mainly for *Arabidopsis*, rice and soybean. The most frequently detected mutations are insertions of a single adenosine or thymidine nucleotide, followed by small deletions of predominantly one nucleotide and deletions of < 10 nucleotides. Other detected mutations are nucleotide replacements and insertion of > 1 nucleotides, but to a lesser extent. There is the indication that dependent on the gRNA, the targeted locus or the experimental setting, the mutation spectrum may differ (Opinion 3, p. 25-26).

3.3.3. Extracted information on the risk assessment aspects of the SDN-1 technique

3.3.3.1. Extracted information from EFSA GMO Panel, 2020

The GMO Panel scientific opinion on the safety assessment of plants developed using SDN-1, SDN-2 and ODM approaches (EFSA GMO Panel, 2020) addressed the risks in terms of impact on humans, animals and the environment by evaluating: (a) the applicability of the hazards identified and discussed in Section 4 of the EFSA opinion addressing the safety assessment of plants developed using ZFN-3 and other SDNs with similar function (EFSA opinion on SDN-3) which in turn compared SDN-3 developed plants to those derived from transgenic and conventionally bred plants; (b) the validity/applicability of the conclusions of the EFSA opinion on SDN-3 in the context of the safety evaluation of plants developed by SDN-1, SDN-2 and ODM.

Although the elements described below refer to the SDN-1 technique, they are relevant also for SDN-2 and ODM approaches.

(a) *applicability of the hazards identified and discussed in Section* 4 *of the EFSA opinion on SDN-3 (EFSA GMO Panel, 2020, Section* 3.2.1*, p. 8);*

Overall, two possible scenarios were envisaged depending on whether or not any nucleic acid sequence intentionally deployed during the genome editing process (e.g. the full SDN module or part of it) is present in the plant genome; if present, the product would be risk assessed as a transgenic plant with regard to the genome-integrated exogenous DNA and as a gene-edited plant with respect to the target sequence(s) modified via SDN-1; if not present, the assessment will only focus on the modification(s) resulting from the SDN activity. Specific points discussed in section 4 of the EFSA opinion on SDN-3 (the source of the DNA and the safety of the gene products; alterations to the host genome at the insertion site and elsewhere) and their applicability for SDN-1, approaches were evaluated in EFSA GMO Panel, 2020 and they are summarised below:

– *source of the genes and the safety of the gene products (EFSA GMO Panel, 2020, Section* 3.2.2.1 *p. 8)*; EFSA GMO Panel, 2020 states that SDN-1 approaches differ from SDN-3 and transgenesis in that they do not aim to insert any DNA sequence but rather to modify an already existing endogenous sequence. 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 consider the history of safe use. For example, based on the available information about the newly brought function, two very different scenarios could be envisaged. On the one end, the new allele obtained by genome editing and the associated trait characterising the final product are already present in a consumed and/or cultivated variety of the same species. In this case, the risk assessment may focus on the knowledge of that variety (the history of safe use) and specific data on the edited gene and its product may not be needed. On the other end, the modified allele and associated trait present in the final product have never been described before. In this case, data on the new allele and the associated trait would be needed to perform the risk assessment. Several other scenarios can be envisaged between these two possibilities and consequently, a range of data requirements may apply depending on the specific case. Depending on the product under assessment, in some cases, only a subset of the data required for SDN-3 would be needed. It was concluded in EFSA GMO Panel, 2020 that the section 4.1 of the EFSA opinion on SDN-3 ('Source of genes and safety of gene products') is applicable only in part to plants developed by SDN-1 approaches.

– *alteration at the insertion site (EFSA GMO Panel, 2020, section 3.2.2.2.1 p. 9);* EFSA GMO Panel, 2020 discussed that the successful application of genome editing approaches such as SDN-1 (or SDN-2, ODM and prime/base editing) results in a sequence modification which is targeted to a specific predetermined genomic locus where no exogenous DNA is inserted. Thus, the safety considerations described in section 4.2.1 of the EFSA opinion on SDN-3 (e.g. the optimisation of the newly created junctions between the plant DNA and the inserted DNA) are all irrelevant for plants obtained using SDN-1 approaches. Based on the above, EFSA GMO Panel, 2020 concluded 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 approaches.
– *alteration elsewhere in the plant genome (EFSA GMO Panel, 2020, Section* 3.2.2.2*.2 p. 9-10)*; EFSA GMO Panel, 2020 discussed that besides the modification(s) at predetermined plant genomic regions induced by SDN-1 approaches, other changes elsewhere can also be introduced as a result of off-target activity of these applications. These unintended mutations outside the original target sequence can be either predictable (for SDN-1 and SDN-2) or not (for some base editing systems). Published experimental evidence since the EFSA opinion on SDN-3 shows that considerable efforts have been made to improve the specificity and efficiency and reduce the off-target effects of SDN-based technologies, particularly for the CRISPR-Cas system. Recently published experimental evidence describing the type and number of off-target mutations generated by the application of SDN-based methods such as SDN-1 has confirmed that the off-target mutations potentially induced by SDNs are of the same type as those mutations used in conventional breeding, including spontaneous mutations and those produced by physical and chemical mutagenesis. Moreover, these publications also confirmed that the number of off-target mutations generated by SDN-based methods is lower than the number of mutations observed in conventional breeding due to spontaneous or induced mutations. Thus, the GMO Panel considered that the analysis of potential off–targets would be of very limited value for the risk assessment. In addition, despite the availability of some biochemical and bioinformatic tools to identify potential off–target mutations, the limited availability and/or completeness of plant genomic sequences and their intraspecies and – intravarietal – variability would not always allow for a reliable prediction of potential off-target mutations. EFSA GMO Panel, 2020 also mentions that the EFSA opinion on SDN-3 stated that backcrossing steps which follow the transformation process would likely remove off-target mutations from the genome of the final product; this aspect is still considered applicable to plants generated via SDN-1.

It is also discussed that when plant transformation is applied to introduce the SDN module, plasmid DNA or other exogenous DNA can be unintentionally integrated in the plant genome. Furthermore, exogenous DNA whose sequence may be known *a priori* can be integrated as a result of the application of some methods to achieve SDN-1 modifications (e.g. transient expression and DNA-free methods). If the final product is not intended to retain any such exogenous DNA, the applicant should assess the potential presence of a DNA sequence derived from the methods used to generate the SDN modification (e.g. plasmids or vectors). It was also noted that that the assessment of the unintentional integration of exogenous DNA is already part of the molecular characterisation in the risk assessment of GM plants, under EU Regulations, and thus, this is not to be considered a new requirement for risk-assessing genome-edited plants. Based on the above, it was concluded in EFSA GMO Panel, 2020 that that the Section 4.2.2 of the EFSA opinion on SDN-3 ('alteration elsewhere in the genome') is applicable to plants developed by SDN-1 approaches.

(b) *the validity/applicability of the conclusions of the EFSA opinion on SDN-3 for the safety evaluation of plants developed by SDN-1:*

– *conclusions associated with the risk assessment of a transgene, intragene or cisgene insertion in SDN-3 plants (EFSA GMO Panel, 2020, Section* 3.3*, point 1 and 3, p. 10);* EFSA GMO Panel, 2020 concluded that they are not applicable to plants via SDN-1 approaches since these methods aim at modifying an endogenous DNA sequence without the insertion of any transgene, intragene or cisgene. It is noted though that in case the SDN module is stably introduced in the plant genome as a transgene, the produced plant should be considered as a transgenic plant and the presence of the transgene will be risk assessed according to all the provisions laid down in the EU regulation of GMOs.
– *conclusions associated to potential off-target mutations* (EFSA GMO Panel, 2020 *Section* 3.3*, point 2, p. 10);* it is stated that as the SDN-1 technique uses the same molecular mechanisms to generate DSB as SDN-3 the conclusions for SDN-3 are also applicable to SDN-1.

– *conclusions associated to the applicability of the GMO Panel GM plant risk assessment guidances (EFSA GMO Panel, 2020, Section* 3.3*, point 4, p. 10-11);* EFSA GMO Panel, 2020 concluded that the existing guidance documents for the food and feed (EFSA GMO Panel, 2011a) and the environmental risk assessment (EFSA GMO Panel, 2010) are sufficient but are only partially applicable to the risk assessment of plants generated via SDN-1 approaches. Indeed, in the absence of any transgenes, intragenes or cisgenes, the amount of experimental data needed for the risk assessment will mainly depend on the modified trait introduced and even less experimental data would be needed for plants produced via SDN-1 compared to plants generated via SDN-3.

3.3.3.2. Extracted information from the MS opinions

Opinion 4, p. 5 discusses the following on the risk assessment aspects related specifically to SDN-1: off-target changes induced by applications of SDN-1 by the CRISPR-Cas9 system are of the same type as those changes produced by conventional breeding techniques, therefore not raising additional safety concerns. Moreover, unintended mutations can be segregated away during the selection and breeding process.

As regards information requirements related to the risk assessment of SDN-1, opinion 5 describes a specific field trial with SDN-1 genome edited maize; it is stated at p. 2/9 that it is necessary to provide a scheme indicating the targeted gene and the position(s) of the matching sequence(s) between the gRNA(s) and the gene. In case of a large deletion, generated by SDN-1, it should be explained whether two gRNAs were used.

Regarding the minimisation of off-target effects and proper design of gRNAs, according to the authors, it is worth describing which stringency criteria were applied in the bioinformatic analysis and with which results. For instance, it would be interesting to know whether and which possible secondary targets were identified by the bioinformatic analysis. In case such sites are identified, targeted sequencing in the final GM events could be performed. Discussing possible off-targets is of relevance to describe the aim and justification of the field trial (probably more than for identifying possible hazards and risks) (opinion 5, p. 2/9).

Also, in this particular case of the field trial with SDN-1 genome edited maize (opinion 5, p. 3/9), a description of the mutation and of the likely expressed peptides resulting from the genetic modification is needed: in two out of the three mutations introduced, single-nucleotide insertions have been obtained. The consequences of such insertion depend on their location in the mutated gene. New polypeptides are likely to result from frameshift mutations. As these changes are intentionally introduced into the GM plants, the authors are of the opinion that they could be described in detail. The same holds true for the third intended modification. In case new peptides are produced, bioinformatic analysis could provide insight into their potential allergenicity and toxicity, as it is done for the newly expressed polypeptides in transgenic GMOs.

It is stated by the authors of opinion 5 (p. 3/9) that in line with Part B of Directive 2001/18/EC,

- an estimation of the copy number is needed. In line with this, it is worthwhile to ask for information on the number of homologous genes in maize;
- there is no need to analyse which maize genes have been interrupted due to the insertion of a new DNA construct. In line with field trial evaluations, it is considered that information on which off-target mutations have occurred (through targeted sequence analysis), should not be asked in the early stages of development.

3.4. SDN-2 technology

3.4.1. Baseline description of the SDN-2 technique

See Section 3.2.1 for the baseline description of the SDN-2 technique.

3.4.2. Extracted information on the description of the SDN-2 technique

3.4.2.1. Extracted information from EFSA GMO Panel, 2012b, 2020

See Section 3.2.2.1 for the extracted information on the description of the SDN-2 technique as defined in the EFSA scientific opinions.

3.4.2.2. Extracted information from the MS opinions

In Opinion 8, p. 16, it is mentioned that in the case of the SDN-2 technology, a DNA template is introduced into the cell together with the site-directed nucleases, enabling the nature of the modification to be defined. The template itself is not incorporated into the genome.

Regarding the introduction of a (single- or double-strand) DNA molecule into the cell to be modified, in Opinion 10, p. 9, it is mentioned that the introduced DNA can be several thousand nucleotides long and is homologous (i.e. identical) to the flanking sequences of the induced double-strand break site, differing from the endogenous DNA sequence only by one to a few positions at the site of the double-strand break. On p. 30, it is stated that with SDN-2, no exogenous DNA fragments are integrated.

Opinion 11, p. 10 discusses the aspect of integration in more detail: small DNA fragments are introduced into the organism together with the site-specific nuclease system. The introduced DNA serves as a repair matrix and is homologous to the regions flanking the double-stranded break site produced by the nuclease differing from the target sequence by one or a few nucleotides. The sequence change is then introduced into the genome when repairing the double-strand break by a cellular DNA repair mechanism using the imported DNA via homology-directed repair (HDR). Using SDN-2, mutations from one or a few pairs of nucleotides or a small insertion or deletion can be deliberately introduced into the genome.

3.4.3. Extracted information on the risk assessment aspects of the SDN-2 technique

3.4.3.1. Extracted information from EFSA GMO Panel, 2020

As stated above in the section summarising the risk assessment aspects for the SDN-1 technology (Section 3.3.3.1), the elements described for the SDN-1 technique are applicable also for SDN-2 approaches.

3.4.3.2. Extracted information from the MS opinions

For SDN-2, the same applies regarding the unintended mutational load as for SDN-1 (opinion 3, p. 32). The repair template, however, may be integrated as a whole at the locus with the targeted DSB for example by the NHEJ repair pathway, as well as at other sites in the genome.

3.5. SDN-3 technology

3.5.1. Baseline description of the SDN-3 technique

See Section 3.2.1 for the baseline description of the SDN-3 technique.

3.5.2. Extracted information on the description of the SDN-3 technique

3.5.2.1. Extracted information from EFSA GMO Panel, 2012b

See Section 3.2.2.1 for the extracted information on the description of the SDN-3 technique from the EFSA scientific opinion on the safety assessment of plants developed using zinc finger nuclease 3 and other site-directed nucleases with similar function.

3.5.2.2. Extracted information from the MS opinions

There was no additional information specifically on SDN-3 technology compared to what has already been reported in the above general section on SDN technology (Section 3.2.2.2).

3.5.3. Extracted information on the risk assessment aspects of the SDN-3 technique

3.5.3.1. Extracted information from EFSA GMO Panel, 2012b

The EFSA GMO Panel scientific opinion on the safety assessment of plants developed using Zinc Finger Nuclease 3 and other site-directed nucleases with similar function (EFSA GMO Panel, 2012b) addressed the risks in terms of impact on humans, animals and the environment by comparing SDN-3 developed plants with plants obtained by conventional breeding methods and by currently used genetic modification methods as defined by Directive 2001/18/EC, focusing on transgenesis. An assessment of conventional breeding approaches served as the baseline. In addition, EFSA GMO Panel, 2012b included

considerations as regards the applicability of the GMO Panel GM plant risk assessment guidance documents to the assessment of plants developed by the ZFN-3 (SDN-3) technique.

When considering hazards related to plants developed by SDN-3 approaches compared with transgenic and conventionally bred plants the major considerations made by the GMO Panel included: the source of the DNA and the safety of the gene products; alterations to the host genome at the insertion site and elsewhere (also including the potential presence of non-plant sequences in the insert and the expression of the trait/modification of gene expression and its potential wider applications).

Source of the DNA and the safety of the gene products (EFSA GMO Panel, 2012b section 4.1 p. 18)

The GMO Panel considered that the SDN-3 technique can be applied to integrate DNA to a particular genomic locus. With respect to the genes introduced, the SDN-3 technique does not differ from the other genetic modification techniques used and can be used to introduce transgenes, intragenes or cisgenes. As described in EFSA GMO Panel, 2012b, when a plant-derived gene from a breeders' pool is introduced by cisgenesis, hazards are similar to those of conventionally bred plants. When however a similar plant-derived gene is used in intragenesis, new combinations of genetic elements may arise and these may present novel traits with novel hazards. Hazards specific for transgenic plants can be identified as the transgenes and their products may originate from any source including non-plant sources.

Alterations to the genome (EFSA GMO Panel, 2012b Section 4.2 p. 18-20)

EFSA GMO Panel, 2012b states that as DNA is introduced into an exact, predefined location in the plant genome via SDN-3, the hazards arising from random integration are minimised. Genomic alterations additional to the targeted DNA integration may occur which can be caused by various processes and mechanisms (e.g. DSB repair mechanisms). Undesirable changes occurring in the genome in any breeding approach (conventional, transgenic cisgenic or intragenic breeding) can in most cases be removed by backcrossing and/or selection. Some issues associated with the existence of potential hazards (somaclonal variation, creation of novel ORFs, modification of gene expression, *presence of non-plant sequences in the insert* etc.) already described in the EFSA GMO Panel, 2012a scientific opinion on the safety assessment of cisgenic and intragenic plants are also considered relevant for SDN-3 developed plants. Specific points on the SDN-3 technique are also discussed (Section 4.2.1 – alterations at the insertion site and Section 4.2.2 – alterations elsewhere in the plant genome):

- *alterations at the insertion site (EFSA GMO Panel, 2012b section 4.2.1 p. 19 and section 4.3 p. 20);* it is described that 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. However, unlike transgenesis where DNA integration occurs essentially randomly, the SDN-3 technique enables a targeted insertion to a predefined genomic site. Therefore, the SDN-3 technique can optimise the genomic environment for gene expression and hazards that might arise from insertions at random loci (e.g. disruption of endogenous genes and/or regulatory elements) can be reduced. The integration can occur via homologous recombination (HR) or non-homologous end joining (NHEJ) and this can impact on changes induced at the insertion site: when DNA is inserted by SDN-3 via HR the genome alterations at the insertion site will be predetermined by the design of the donor DNA. In the case of targeted NHEJ, genomic changes due to DNA repair will be similar to those observed in currently developed transgenic plants.
- *alterations elsewhere in the plant genome (EFSA GMO Panel, 2012b, section 4.2.2, p. 19-20 and section 4.3, p. 21);* EFSA GMO Panel, 2012b discusses that such changes can occur as a result of a tissue culture step (somaclonal variation) that may be employed during the SDN-3 process or as a result of the off-target activity of the SDN. Alterations due to tissue culturing are not specific to SDN-3 and they can occur with other breeding processes (this issue is discussed in detail in EFSA GMO Panel, 2012a). As regards changes occurring due to off-target activity of the SDN, it is stated that this will depend on the specificity of the SDN and the presence of sequences similar to the SDN recognition site in the recipient genome. Several methods have been developed to reduce the frequency of off-target modifications and the

> SDN-3 technique can be tested *in vitro* and optimised for off-target activity (p.20). Both physical and chemical mutageneses cause a considerable number of mutations throughout the genome. SDNs would induce fewer unintended changes than most conventional mutagenesis techniques, and where they do occur the changes would be of the same types (small deletions/insertions/substitutions or larger rearrangements) as those produced by conventional breeding techniques. Such changes also occur naturally after repair or spontaneous mutations

Applicability of the current guidance documents (EFSA GMO Panel, 2012b, section 5, p. 21)

As stated in EFSA GMO Panel, 2012b, in order to assess the adequacy of the current EFSA guidance documents for the risk assessment of plants developed using the SDN-3 technique, the EFSA GMO Panel has focused on the 'Guidance for risk assessment of food and feed from genetically modified plants' (EFSA GMO Panel, 2011a) and the 'Guidance on the environmental risk assessment of genetically modified plants' (EFSA GMO Panel, 2010).

Similar to the applicability/adequacy assessment of the above-mentioned guidance documents for the evaluation of cisgenic/intragenic plants and derived food and feed, it is stated in EFSA GMO Panel, 2012b that the general approach and all elements in these guidance documents are applicable for the evaluation of food and feed products derived from plants developed by the SDN-3 approach and for performing an environmental risk assessment and do not need to be developed further. However, in line with the conclusions discussed in EFSA GMO Panel, 2012a, it can be envisaged that on a case-by-case basis (e.g. where the SDN-3 technique is used for cisgenesis), lesser amounts of event-specific data are needed for the risk assessment. Moreover, there will be cases where for plants developed following a successful SDN-3 strategy, the potential for unintended effects e.g. positional and off-target effects, is significantly reduced compared with transgenesis, and with conventional breeding. There is therefore a need for flexibility in the data requirements for risk assessments.

3.5.3.2. Extracted information from the MS opinions

There was no additional information specifically on SDN-3 technology compared to what has already been reported in the above general section on the SDN technology (Section 3.2.3.2).

3.6. Base editing

3.6.1. Baseline description of the base editing technique

In EC-SAM, 2017, alternative variants of CRISPR-Cas9 are described: 1) A Cas9 nickase (p. 64), which cuts only one of the DNA strands favouring HDR and preventing NHEJ; 2) a nuclease-deficient Cas9, termed dCas9 (p. 66–67) which maintains the ability to bind both the gRNA and targeted DNA, without cleaving it. Both dCas9 and Cas 9 nickase have been thus used as a sequence-specific RNA-guided DNA-binding platform for the development of new tools for engineering the genome. EFSA notes that no new NGTs are specifically mentioned in EC-SAM, 2017.

One newly developed NGT based on dCas9 or Cas9 nickase enzymes, called 'Base editing', is mentioned in opinions 6, 10, 11 and 16 (published in 2018 and 2019). In addition, base editing is briefly discussed in EFSA GMO Panel, 2020. The extracted information is presented below.

3.6.2. Extracted information on the description of the base editing technique

3.6.2.1. Extracted information from EFSA GMO Panel, 2020

EFSA GMO Panel, 2020 (Section 3.1.2, p. 7) mentions that other recently emerged techniques such as base editing and prime editing can be used to generate specific nucleotide changes in a targeted sequence without deploying any template DNA and without inducing DSB in the target locus. In addition, it is stated that the GMO Panel considers that the genetic modifications obtained using base editing and prime editing are comparable to those created by the SDN-2 technology. EFSA GMO Panel, 2020 (Section 3.2.2.2.1, p. 9) also mentions that the application of prime and base editing does not induce DSB in the plant genome at any stage during the process; rather, they induce in general a single strand break at the target site.

3.6.2.2. Extracted information from the MS opinions

In opinion 6 (p. 4), base editing is mentioned: two base editing systems based on the CRISPR-Cas9 tool have recently been developed which can alter a particular nucleotide in a DNA sequence without

the use of a DNA repair template. One system can change cytosine (C·G) to thymine (T·A) and the other adenine (A·T) to guanine (G·C). These systems have been shown to work effectively in important crop plants, such as tomato, canola, corn, rice and wheat.

In opinion 16, p. 13, base editing is described as the newest type of site-specific mutagenesis, which makes it possible to intentionally change one base pair without introducing double-strand DNA breaks. This type of mutagenesis is based on enzymes called deaminases that modify cytosine or adenine bases in single-strand DNA – i.e. remove amino functional groups. In this way, it is possible to programme cytosine–guanine pair replacement to a thymine–adenine pair, and adenine–thymine to guanine–cytosine. After merging the deaminase domain with catalytically inactive dCas9, it is possible to direct it to the DNA base pair that is being modified. Base editing could be used to introduce many point mutations at the same time.

An advantage of base editing over the SDN-1 or SDN-2 technology is that it allows for targeted conversions of one particular nucleotide of the genome into another. As this approach does not involve the use of exogenous DNA, such as in the case of the SDN-2 technique, it cannot be accidentally incorporated into the plant genome. Moreover, no DSB is induced in the genome (opinion 11, p. 11).

Since base editing is based on the same principles as nuclease technology, it is in principle also possible to alter several genes simultaneously or successively by deamination. However, there are currently no examples of this (opinion 11, p. 31).

In opinion 10, p. 19, it is mentioned that in addition to modifications at the DNA level, RNA modifications through base editing are also possible. This involves coupling an adenosine deaminase acting on RNA (ADAR) protein to a deactivated dCas13 enzyme. Here again, an adenosine is replaced by an inosine which acts like a guanine during splicing and translation of the mRNA.

3.6.3. Extracted information on the risk assessment aspects of the base editing technique

3.6.3.1. Extracted information from EFSA GMO Panel, 2020

With respect to the 'alterations elsewhere in the genome', besides the considerations made for SDN-1, SDN-2 and ODM approaches due to the off-target activity associated with these applications and their processes, EFSA GMO Panel, 2020 (Section 3.2.2.2.2 p. 9) also mentions that some base editing systems have been shown to present Cas9–independent off–target effects linked to the base editor activity itself. For these reasons, base editing off-target activity might result in unintended mutations outside the original target sequence that can be unpredictable. However recently, considerable efforts have been made towards improving the efficiency and specificity of SDN-based technologies, particularly for the CRISPR-Cas system, including base editing.

3.6.3.2. Extracted information from the MS opinions

In opinion 11 (p. 19), it is mentioned that base editing using an inactive Cas9 protein with combined cytosine deaminase may result in off-target effects in a window of five nucleotides surrounding the nucleotide of interest. This also may happen in the case of off-target binding of Cas9. As this technique has so far rarely been used in plants, it is not possible to make a statement about the absolute off-target rate at present. However, data from *Escherichia coli* indicate that additional off-target effects are possible. The deaminase (activation-induced deaminase, AID) analysed in this *E. coli* study has shown an increased rate of cytosine deamination genome-wide, irrespective of the fusion proteins (zinc fingers and TALEN) used for target sequence detection.

In opinion 16, p. 13, it is mentioned that base editing makes it possible to intentionally change one base pair without introducing double-strand DNA breaks. It is therefore considered a safer alternative, as it has a lower chance of toxic exposure and side mutations.

3.7. Oligonucleotide-directed mutagenesis (ODM)

3.7.1. Baseline description of the ODM technique

In JRC, 2011 (p. 21), ODM is described as a technique based on the use of single-stranded DNA oligonucleotides for the induction of targeted mutations in the plant genome. The genetic changes that can be obtained by ODM include the introduction of a new mutation (replacement of one or a few base pairs, short deletion or insertion) or the reversal of an existing mutation (EC-SAM, 2017, p. 57). The chemically synthesised oligonucleotides usually employed are approximately 20–100 nucleotides long

and they can also consist of a mix of DNA and RNA bases. The oligonucleotide targets the homologous sequence in the genome and creates one or more mismatches corresponding to the non-complementary nucleotides. The cell's own gene repair mechanisms recognise these mismatches and induce their correction. The oligonucleotides are expected to be degraded in the cell, but the induced mutations will be stably inherited.

Oligonucleotides can be delivered to plant tissues by commonly used methods such as particle bombardment and electroporation or PEG-mediated transfection of protoplasts.

ODM is mentioned in MS opinions 1, 6, 8, 10, 11, 12, 14, 15 and 16 published between 2012 and 2020. Since no relevant information was extracted from opinions 8 and 15, these opinions are not described in the following subsections. In addition, ODM is discussed in EFSA GMO Panel, 2020.

3.7.2. Extracted information on the description of the ODM technique

3.7.2.1. Extracted information from EFSA GMO Panel, 2020

EFSA GMO Panel, 2020 further specifies that the application of ODM methods is set apart from SDN-based techniques and it results in predicted mutations (as with SDN-2) of a targeted genomic locus without the insertion of exogenous DNA at the genomic locus (EFSA GMO Panel, 2020, section 3.1.1 p. 6–7). Furthermore, it is mentioned in Section 3.1.2 (EFSA GMO Panel, 2020, p. 7) that 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. As regards the methods for delivery, EFSA GMO Panel, 2020 states that for ODM, the chemically synthesised oligonucleotide is directly delivered to the plant cell without the need of any stable or transient expression system (EFSA GMO Panel, 2020, section 3.1.3 p. 8).

3.7.2.2. Extracted information from the MS opinions

In opinion 1 (p. 38), it is mentioned that the oligonucleotides induce site-specific nucleotide modifications such as substitutions, deletions and insertions. As a consequence, the (coding) DNA sequence may be changed enabling to create knock-outs or resulting in altered regulation of gene(s) expression.

The possibility of introducing insertions by ODM is also mentioned in opinion 11, p. 11 and opinion 16, p. 14, whereas it is stated in opinion 11 (p. 19) that almost exclusively base substitutions occur with ODM.

In opinion 6 (p. 7), ODM is described as a technique that allows genes to be deactivated, activated or modified in a targeted manner (the report does not sufficiently describe what is meant by 'modified'). The integration of exogenous DNA is not possible using ODM.

Opinion 10 (p. 7) states that the oligonucleotides are complementary to the specific DNA sequence to be modified in the cell genome and serve as a matrix for the targeted insertion of mutations which affects only one to a few nucleotides. The cellular mechanisms leading to the mutations are not fully understood; it is assumed, however, that DNA repair enzymes play an important role.

Besides the use of single-stranded DNA and DNA-RNA chimeras as oligonucleotides, oligonucleotides containing modified nucleobases and/or modified ribose can also be used to increase binding to the target sequence. These are known as locked nucleic acids (LNA). Nucleobases linked by peptide bonds (peptide nucleic acids (PNA) are also suitable as oligonucleotides to direct the mutagenesis (opinion 11, p. 11).

In opinion 14, p.4, the authors state that they have concluded that an oligonucleotide used in the 'oligo-directed mutagenesis' gene editing technique is not to be regarded as a recombinant nucleic acid, because the nucleotide sequence of this short stretch of DNA is the same as that of the genome of the organism to be mutated. Reference to another COGEM opinion[6] (opinion 14, p. 9) is given.

[6] COGEM (2010), advisory opinion on the status of oligonucleotides in the context of directed mutagenesis. CGM/100701-03, https://cogem.net/publicatie/de-status-van-oligonucleotiden-in-de-context-van-gerichte-mutagenese/

3.7.3. Extracted information on the risk assessment aspects of the ODM technique

3.7.3.1. Extracted information from EFSA GMO Panel, 2020

As already described above (Section 3.3.3.1), the GMO Panel scientific opinion on the safety assessment of plants developed using SDN-1, SDN-2 and ODM approaches (EFSA GMO Panel, 2020) addressed the risks in terms of impact on humans, animals and the environment by evaluating: the applicability of the hazards identified and discussed in section 4 of the EFSA opinion addressing the safety assessment of plants developed using ZFN-3 and other SDNs with similar function which in turn compared SDN-3 developed plants to those derived from transgenic and conventionally bred plants; the validity/applicability of the conclusions of the EFSA opinion on SDN-3 in the context of the safety evaluation of plants developed by SDN-1, SDN-2 and ODM.

As with SDN-2, the risk assessment aspects summarised above for the SDN-1 approach (Section 3.3.3.1) are applicable also for ODM approaches**.**

However, as regards the risk assessment aspects related to alterations at the insertion site (EFSA GMO Panel, 2020, section 3.2.2.2.1, p. 9), it is stated that unlike SDN-1 and SDN-2 approaches, the ODM approach is not designed to induce DSBs, and in this respect, it is different from SDN-3. Moreover, with respect to the alterations elsewhere in the genome (EFSA GMO Panel, 2020, section 3.2.2.2.2, p. 9-10) and more specifically in relation to the off-target activity, it is mentioned that while there is an increasing number of publications containing data on off-target effects for SDN-based technologies, the GMO Panel noticed that information on the off-target mechanism and frequency for ODM is quite limited. In addition as regards the validity/applicability of the conclusions of the EFSA opinion on SDN-3 in the context of the safety evaluation of plants developed by SDN-1, SDN-2 and ODM, EFSA GMO Panel, 2020 (section 3.3, point 2, p. 10) further specifies that in the case of ODM, although very limited information on the mechanisms and frequency of off-target effect is available in the literature, it is reasonable to assume that the same conclusions also apply because this technology is based on sequence-specific site recognition, as are SDN-based methods.

3.7.3.2. Extracted information from the MS opinions

Unintended modification of other sites (off-target effects) is possible by ODM. It is however extremely difficult to prove that these changes have occurred from the oligonucleotide or naturally (spontaneously) during the breeding process (opinion 1, p. 41; opinion 6, p. 5).

Opinion 1 (p. 126) presents a general view on risk assessment of the ODM technique: aiming at the modification of endogenous genes; no exogenous DNA sequences are foreseen to be stably introduced when applying ODM. The original gene pool remains unaffected. The data requirements for risk assessment might be specified case-by-case and, if applicable, reduced. It may be envisaged to adapt the risk assessment of plants derived from ODM with respect to the specific investigated mutation and the properties of the conferred trait(s). The definition of the risk assessment data requirements could be governed by the specific trait and its characteristics. Food safety aspects have to be evaluated, in particular if the expression of proteins is increased due to the modification. The characteristics of the modified protein have to be considered and are also important for evaluating potential environmental risks.

The occurrence of off-target effects by using ODM is influenced by a number of factors (opinion 10, p. 31), e.g. other genome regions that are very similar to the target region, presence of DNA methylation, modifications of histones and accessibility of chromatin and reaction conditions (during the experiment). Bioinformatic tools are becoming increasingly effective in predicting possible off-target effects (opinion 10 p. 31).

Opinion 11 (p. 21) states that there are no published data available on the off-target rate, but due to the technique used (no DNA breakage) and the DNA repair mechanism (mismatch repair), it should be less than 1% in relation to the sequences to which the oligonucleotide can attach at all.

In opinion 11, p. 30, it is stated that, in the case of techniques with several successive sequence changes in a gene, the treated plants must undergo a cell culture passage after each change, and this may lead to the proliferation of undesirable somaclonal mutations. In addition, opinion 12 (p. 2) mentions that the likelihood of unintended effects increases with the number of mutations produced. Two examples of 'unintended effects' are given: one refers to proteins that acquire modified characteristics to exhibit similarities with an allergen. The second is about modification(s) in a promoter region, introducing the risk that the protein's spatiotemporal expression is modified. It is therefore

possible that a new protein or metabolite may be found in the edible parts of the plant, and it may not be known to what extent this could be toxic or allergenic. It is impossible to define a guideline for the number of base pairs that can be modified in order to predict an increased health risk.

3.8. RNA-dependent DNA methylation (RdDM)

3.8.1. Baseline description of the RdDM technique

In JRC, 2011, RdDM is described as a technique that enables modification of the gene expression due to epigenetics. More specifically, RdDM induces the transcriptional gene silencing of a target gene via methylation of the promoter sequence. To this purpose, genes encoding RNAs which are homologous to the promoter region are delivered to the plant cells. Once transcribed, these genes give rise to double-stranded (ds) RNAs which, after processing by specific enzymes (naturally present in plant cells), induce methylation of the target promoter sequence thereby inhibiting the transcription of the target gene.

In plants, methylation patterns are meiotically stable and will be inherited by the following generation. As such, the progeny of the transgenic plant will include plant lines which, due to segregation in the breeding population, do not contain the inserted genes but retain the desired trait. The methylated status can continue for a number of generations. This progeny does not contain foreign DNA sequences and no changes are made in their genomic sequence.

The genes encoding the dsRNAs are delivered to plant cells by standard transformation methods (i.e. biolistic transformation and transformation with *Agrobacterium tumefaciens*).

RdDM is mentioned in MS opinions 2, 8 and 16 published in 2013, 2017 and 2019, respectively.

3.8.2. Extracted information on the description of the RdDM technique from the MS opinions

In opinion 2, it is explained that RdDM is different from RNAi technology in the way that RdDM takes place in the nucleus of the cell by a mechanism called transcriptional gene silencing (TGS), whereas RNAi takes place in the cytoplasm by a different mechanism called post-transcriptional gene silencing (PTGS). Both mechanisms can be used for the artificial silencing of target genes (p. 18).

Active transcription of the target gene is a requirement for the successful application of RdDM. Maintenance of DNA methylation might be determined by the distribution of the cytosines, the structure of the transcribed region or the type of plant tissue (p. 18). Passage to the progeny only occurs if methylation took place in germline cells (p. 21).

The small RNAs that initiate RdDM are generally 24 nt in length (p. 18).

Opinion 2 mentions the induction of the RdDM process by transient methods as well, e.g. using a transient viral vector producing and transmitting small RNAs to the nucleus of the plant cell. This method could be attractive as no changes or mutations are introduced into the DNA sequence of the organism (p. 20).

It should be noted that DNA methylation can also be achieved by other approaches: epigenetic modifications can be achieved through expression or transient/stable transfer of a transgene or specific proteins (e.g. through agro-infiltration, CRISPR with a fusion protein having methyltransferase activity (Cas9-MT), or modification induced by a transient viral infection (opinion 8, p. 16).

In opinion 16 (p. 6), RNA-directed DNA methylation is stated as directed DNA cytosine base-methylation; its location is determined by the gRNA. It can work through RNA interference that naturally exists in plants, or with artificial tools where a dCas9 protein carries the DNA modification domain to the desired location. References related to CRISPR-based epigenetics are given.

In opinion 8 (p. 26), three techniques for obtaining dsRNA are listed:

- Transient transgenesis: Introduction of a fragment that will not be integrated in the genome and will not replicate independently. This leads to the transient presence of the transgene in an intermediate organism. Induced methylation can be stable over several generations.
- Transgenesis using autonomous replicons: Delivery of a DNA or RNA fragment that replicates autonomously. This is the case, e.g. for (DNA or RNA) virus sequences that can replicate, known as VIGS (virus-induced gene silencing).
- Integrated transgenesis and negative segregation: Integration of a DNA fragment in the genome (transgenesis). The transgene can be eliminated by crossing or excision.

Opinion 16, p. 15 mentions next to RdDM also RNA-directed histone modification as a technique to regulate the gene expression without changing the DNA sequence.

3.8.3. Extracted information on the risk assessment aspects of the RdDM technique from the MS opinions

Opinion 2 (chapter 3.6, p. 18-33) elaborates on aspects of risk assessment. Most extracted parts of the text are integrally relevant and challenging to summarise without risking losing important information discussed in this document. For clarity, these parts are cited in a separate subsection below. The conclusions (chapter 7, p. 54-55) are also cited in another separate subsection below.

Information extracted from chapter 3.6 of opinion 2

It is indicated that it is relevant to note that there is evidence that RdDM is also capable of acting *in trans* in plants: silencing of one allele is mediated in heterozygous combinations by an already silenced allele (p. 22).

On page 25, it is stated that templates for dsRNA are intended to be inserted into the plant genome; the resulting plant by definition constitutes a GMO plant and the risk assessment procedures should be according to current guidance documents and regulation regarding GMOs.

Molecular characterisation

Regarding molecular characterisation, the requirement for providing sequence information of the transgenic insert from the final product is not applicable for plants obtained by RdDM. However, when stable insertion of templates for dsRNA into the plant genome is foreseen, these plants are transgenic and should be checked according to the EFSA guideline (EFSA GMO Panel, 2011a). Hereby, sequence data are essential to provide evidence that the insertion locus is free from deleterious mutations in the final product (p. 26). The required information on molecular characterisation (as adapted from the EFSA guidelines) is stated as follows (p. 27):

- Description of the methods used for the genetic modification
- Source and characterisation of nucleic acid used for transformation
- Nature and source of the vector(s) used including nucleotide sequences intended for insertion
- General description of the trait(s) and characteristics which have been introduced or modified
- Information on the sequences actually inserted/deleted or altered. The copy number of intact and fragmented inserts should be established.
- Information on the expression of the inserted/modified sequence; including potential alterations to the flanking regions to exclude:
  - the formation of potential fusion proteins
  - the unintended expression of a host promoter in close proximity of the insert locus and
  - the expression of a host gene as a read-through product from the insert derived promoter
- Genetic stability of the inserted/modified sequence and phenotypic stability of the GM plant.

The final commercial product is not supposed to contain any process-related aberrations in the plant genomic sequence (DNA mutations, deletions, insertions or other sequence rearrangements), and should not contain any transgenic inserts, fragments thereof or transformation vector backbone sequences. The absence of the vector, insert and additional superfluous DNA sequences in the final product must be demonstrated (Southern blot analysis). The insertion locus and the associated flanking regions identified during the pre-commercial phase of the plant development process should be checked for sequence rearrangements in the commercialised product by DNA sequencing (p. 27).

Unintended effects: Information about intended and unintended changes in the plant genome methylation pattern should be provided (p. 27).

To avoid unintended effects and assess a potential impact on non-target organisms, a thorough in silico analysis of the involved RNA sequences is recommended. Upon transformation of plant cells with gene cassettes coding for dsRNA secondary siRNAs with an altered target sequence specificity may be formed. The newly generated siRNAs may affect non-target genes or non-target organisms (p. 27).

On p. 27, it is stated that the following unintended or side effects were reported in the literature:

- Spreading of DNA methylation beyond flanking regions of the targeted nucleotide sequence;
- Promoter DNA methylation does not necessarily induce transcriptional inactivation.

It is mentioned on p. 28 that the risk assessment of crop plants produced by RdDM needs to include comprehensive comparative analyses using suitable conventional counterparts with a well-established history of safe use, based on state-of-the-art field designs and the use of powerful statistical analyses.

Toxicity and allergenicity are part of the risk assessment. Regarding these aspects, many considerations are provided at p. 29:

In the mammalian system, dsRNA molecules longer than 30-bp function as potent triggers of the innate immune system and activate phosphokinase R in the sequence independent interferon pathway which shuts down cellular protein synthesis as an antiviral defence strategy. It is unclear, whether transgene encoded dsRNA molecules – usually with a fragment length between 200 and 400bp – may exert similar effects upon ingestion by animal or human consumers under certain circumstances.

The peer-reviewed literature currently lacks studies which assess the safety of consuming endogenous longer dsRNAs, siRNAs or miRNAs in human food or animal feed. Neither the overall amounts of small RNA molecules nor the presence of benign small RNAs in conventional plants are sufficient as evidence that all novel small RNAs will be safe in the food chain or environment.

Small RNAs exert their function via sequence-specific interactions with their target molecules. These sequence-determined activities cannot be considered as 'generally recognised as safe' (GRAS) in general terms. Recent evidence is indicating that plant-derived small RNAs survive the passage through the mammalian gastro-intestinal tract, pass the gut barrier and have an impact on the regulation of gene expression of mammalian liver enzymes.

It remains to be nearer determined (p. 30) whether the naturally existing small RNAs present in plant- and animal-derived foods that make up the human diet could play an active (patho)physiological role in humans by influencing the expression of endogenous genes. The same applies to GM plants based on the expression of non-coding RNA.

For RdDM plants, food safety and risk assessment seem to be mainly associated with two important issues. The first being the question of whether unintended effects in relation to the applied stable or transient transgenic approach have occurred and whether these are relevant for the food safety of the plant, and the second being the question whether the synthetic RNA molecules could have negative effects on animal and human health.

In conclusion, potential risks arising from the application of RdDM on plants either caused by the transgenic approach itself or the introduced synthetic nucleotides need to be assessed by conducting *in vivo* animal studies that identify potential unforeseen consequences. Additionally, thorough studies of gene expression (*in vitro* and *in vivo*) to identify the genes whose expression might be affected by specific miRNA techniques are needed (p. 30).

Environmental effects

For the assessment of potential adverse effects on the environment associated with the application of transient silencing systems (non-coding RNA), the following points need to be addressed (p. 30):

- Persistence of small RNA molecules,
- Effects on soil microbes or related viruses,
- Higher susceptibility to plant diseases,
- Alterations of siRNA and effects on host transcriptome,
- Non-target effects on organisms ingesting plants (animals, humans),
- Unintended effects on molecular and cellular interactions.

For plants produced by RdDM that are to be put on the market, it would be necessary to improve the present monitoring and surveillance systems (p. 31).

Information extracted from chapter 7 of opinion 2

In the concluding chapter 7, the authors summarise the most important aspects relevant for the risk assessment according to their view (p. 54):

- Plant-derived small RNAs are mobile
- Spreading of the methylation cannot be predicted
- Distribution of the silencing signal to non-target tissue
- Gene silencing is not tissue-specific
- The phenotypic stability of the silencing signal is unclear (amplification/fading of the signal is possible)

- Variation in silencing effects seems to depend on the generation number
- To date, it has not been shown that RdDM has transgenerational stability
- Promoter DNA methylation does not necessarily induce transcriptional inactivation
- RNA silencing is environment dependent
- Uncertainties concerning the fate and effects of ingested RNA molecules
- The method is in the face of intense development
- Scarce database

Furthermore, the authors list a number of risk assessment aspects that should be considered based on the current EFSA guidance (EFSA GMO Panel, 2011a, see RIVM report, section 3.8.2.2).

3.9. Grafting

3.9.1. Baseline description of the grafting technique

In JRC, 2011, grafting, and more specifically grafting on GM rootstock, is defined as a method whereby the above-ground vegetative component of one plant (also known as the scion) is attached to a rooted lower component (also known as the rootstock) of another plant to produce a chimeric organism with improved cultivation characteristics.

Transgenesis, cisgenesis and a range of other techniques can be used to transform the rootstock and/or scion. When a non-GM scion is grafted onto a GM rootstock, leaves, stems, flowers, seeds and fruits would not carry the genetic modification with respect to changes in genomic DNA sequences. The rootstock can be improved by genetic modification regarding improved rooting capacity or resistance to soil-borne diseases, resulting in an increase in yield of harvestable components such as fruits.

In grafted plants, small RNAs (e.g. expressed by RdDM) can also move through the graft so that the silencing signal can affect gene expression in the scion (p. 21).

Grafting is mentioned in MS opinions 2, 8 and 16 published in 2013 and 2019, respectively. Since no information was extracted from opinion 8 and 16, these opinions are not described in the following paragraphs.

3.9.2. Extracted information on the description of the grafting technique from the MS opinions

Grafting is a common technique that has been used for centuries in plant breeding. In grafting, the scion (tissues or parts) of a plant is grafted onto the rootstock of another plant. Particularly in fruit growing and viticulture, grafting has been used for many centuries (opinion 2, p. 42).

In the course of development of genetically engineered crops, grafting combined with transgenesis is applied as a novel biotechnology application. A range of molecular techniques can be used to transform the rootstock and/or scion, this process is often referred to as 'transgrafting'. If a GM-scion is grafted onto a non-GM rootstock, then stems, leaves, flowers, seeds and fruits will be transgenic. But the reverse process is of major interest using new plant breeding techniques: to graft a non-GM scion onto a GM rootstock. The rootstock is used for modifying or introducing traits that can lead to improved characteristics of the rootstock and of the whole plant and consequently of the 'end product' (opinion 2, p. 42).

Furthermore, it is stated on p. 42 of opinion 2 that RNA molecules (mRNAs, miRNAs, siRNAs), plastid DNA, peptides, proteins, hormones and metabolites resulting from the genetic modification in the GM rootstock can pass the graft junction. As a result, fruits may harbour novel traits without detectable changes to their DNA sequence. Nevertheless, it is possible that heritable changes induced by epigenetic modification of genomic DNA may occur as a result of the movement of spatial translocation of molecules produced due to the expression of the transgene in the rootstock (opinion 2, p. 45).

One of the challenges in this respect is to relate these moveable elements directly to the genetic modification in the rootstock and to differentiate them from naturally occurring plant molecules and metabolites in the non-GM scion (opinion 2, p. 42).

On p. 43 of opinion 2, a number of research studies demonstrating the detection of transgenic molecules (e.g. proteins) in the untransformed scion grafted on transgenic rootstocks are listed. On the following page 44, research studies are presented in which the detection of transgenic molecules could not be established.

3.9.3. Extracted information on the risk assessment aspects of the grafting technique from the MS opinions

Opinion 2 contains extended information on aspects of the risk assessment. The extracted information from p. 47-50 is cited below:

Molecular characterisation

The major safety issue is the unintended transfer of macromolecules produced by the transgene to tissues of the non-transgenic part of the plant which would lead to unintended aberrations in gene, protein and trait expression.

The transgenic part of a grafted plant has to be thoroughly assessed for adverse effects on human and animal health and the environment according to Directive 2001/18/EC and Regulation (EC) No 1829/2003 and a special focus has to be put on the risk assessment of the remaining, non-transgenic sections of the plant. Several aspects require special attention:

Chromosomal DNA changes in non-transgenic parts of the grafted plant

So far, the crossing of the graft junction has only been demonstrated for plastid DNA. No movement of chromosomal DNA has been evident. Concerning risk assessment, this circumstance puts a special focus on the kind of transformation protocol, which has been applied, and the localisation of the transgenic insert(s). Detailed information from the applicant concerning the biolistic transformation protocol and whether plastid DNA was involved or targeted is required. *Agrobacterium*-mediated transformation, which guides the transgenic inserts to the nucleus of the plant cell, appears to provide no additional hazard for interference with the non-transgenic part of the grafted plant. Long distance transfers of transgenic organelle DNA is unlikely, however, the borders between the transgenic and non-transgenic part of the plant have to be clearly defined and non-transgenic tissue has to be checked for the presence of transgenic organelle DNA via Southern blot or PCR approaches.

mRNA translocation

Long distance translocation of mRNAs has been demonstrated to be a highly regulated and selective process in plants. Although transport and targeting appear to depend on specific sequences present in the 5' and 3' untranslated regions, the possibility of an unintended transfer of transgene-derived mRNAs remains. Therefore, close monitoring for the presence of transgene-specific mRNAs in the non-GM part of the grafted plant RT-PCR or Northern blots is recommended.

Effects of translocation of small non-coding RNAs

Small non-coding RNAs are mobile in plants and do not restrict their gene silencing effects on single cells, where these RNAs are generated. The usually induced silencing effect is transported over long distances in the plant and the possibility of an unintended effect on gene expression also in non-transgenic parts of the grafted plant remains. According to the authors, in general, all dsRNA- and siRNA-related risks already identified for RdDM applications in plant breeding are relevant for the risk assessment of grafting.

Protein translocation

Protein translocation has been demonstrated to take place over long distances and appears to be tightly regulated. As the presence of transgenic proteins in the non-transgenic part of the organism cannot be excluded, tight monitoring for transgenic proteins in non-GM tissue is recommended.

Adventitious shoots from the callus (i.e. the border region between the transgenic and non-transgenic part of the plant) or from the GM rootstock have to be closely monitored as for instance the fruits resulting from this process are transgenic.

Substantial equivalence/comparative assessment

Regarding substantial equivalence (p. 48), potential risks arise from the presence of small RNA in food products (e.g. fruits) derived from non-GM scions but originating from the GM rootstock. Against this background comparative assessments between these non-GM scions and conventionally produced scions (not using GM rootstocks) and food products thereof are indicated.

Regarding current EU standards concerning the use of conventional counterparts in the comparative analysis as part of the risk assessment of GM plants, the grafting technique (use of GM

rootstocks) has not been evaluated. This means that a conventional counterpart has not been defined for this new breeding technique.

Per definition, the conventional counterpart should be a conventional plant with a history of safe use and a genetic background as close as possible to the GM plant. In this respect, the comparative assessment can be carried out by comparing the GM rootstock with its conventional counterpart (isogenic non-GM rootstock) for compositional and phenotypical equivalence.

The compositional and agronomic assessment should be in accordance with current EFSA requirements (EFSA GMO Panel, 2011a,b), so that the results can indicate whether there are differences (intended or unintended) or a lack of equivalence between the GM rootstock and the conventional counterpart.

Toxicological and allergenicity risk assessment

In the case of rootstocks developed by transgenic methods either using traditional transformation processes or new plant breeding techniques, synthetic RNA molecules or newly expressed proteins could pass the graft junction and be present in products derived from untransformed scions.

Besides comparative tests, additional studies must be performed to ensure that transgenic proteins, RNA and other mobile metabolites, as well as unintended effects due to the presence of these compounds in scion tissues that remained undetected during molecular characterisation and equivalence testing, do not have the potential for having adverse effects on humans and animals.

There are still considerable uncertainties with respect to the safety of small non-coding RNA, especially dsRNA, and the potential occurrence of unintended effects cannot be ruled out. Thus, the evaluation of the safety of plants produced by (trans-)grafting techniques should include toxicological testing of the whole food and/or feed derived from the grafted scion.

Environmental risk assessment

The main issue for the ERA of plants produced by transgrafting (grafting non-GM scions on GM rootstocks) concerns the fact that a GM rootstock is released into the environment with possible hazards and consequences as follows:

- Mobile metabolites may cause an unintended effect on e.g. phytoplasms, nematodes, psyllids and aphids
- Suckers and adventitious shoots may be a source of unintended effects, in particular, if they emerge from the transgenic rootstock.

According to the authors, particular risks as the transfer of genetic material to organisms e.g. leaf-eating insects may arise from unwanted but mostly uncontrollable suckers that are not removed regularly. The formation of root bridges should be noted in relation to a GM rootstock-to-microorganisms (and other soil organisms) gene transfer.

As regards the transmission of mobile transgenic elements (small RNA, proteins, peptides) into non-GM scion tissues, it is noteworthy that siRNAs have been shown to exert toxic effects on target insects and are used as pesticides by direct feeding or via application in liposomes.

Moreover, nematodes have the capability to directly take up dsRNA from the environment or via ingestion of dsRNA expressing bacteria, and it should be assessed whether beneficial nematodes in close proximity may suffer from exposure to modified plants expressing silencing siRNAs.

The authors of opinion 2 conclude on p. 57:

Grafting has a history of safe use as it is state of the art for the breeding of grapevine and fruit trees. New risks may be associated if GMOs are involved. The following potential risks may arise from grafting of a non-GM scion onto a GM rootstock:

- Metabolites resulting from transgenesis, termed 'transgenic metabolites' in opinion 2 (proteins, hormones, siRNAs etc.) can be transported from the transgenic rootstock to the upper stem where they accumulate and cause an effect.
- These mobile metabolites may have unanticipated effects on, e.g. phytoplasms, nematodes, psyllids, aphids, including their potential transfer.
- There is currently no sufficient database concerning potential effects on humans and animals consuming products of these plants.
- Adventitious shoots may be a source of unintended effects, in particular if they emerge from the transgenic rootstock.

When produced through standard transformation methods, the risk assessment of the GM rootstock should be fully performed according to the current EFSA Guidance (EFSA GMO Panel, 2011a). This should include the analysis of the products that are to be consumed by humans and/or animals. Special attention should be paid to the environmental risk assessment (EFSA GMO Panel, 2010), in particular for perennials like grapevine or trees.

There is no conclusive information available to the risks emerging from particular transgenic metabolites. Due to the great variation in potential effects of the molecules produced by the transgene that may affect the whole plant and derived products, a case-by-case evaluation will be necessary. On the same basis, effects on the environment have to be assessed, and possibilities for identification and quantification will have to be evaluated. Generally, the regulatory framework concerning the relevant plants and their products should be reconsidered.

3.10. Reverse breeding

3.10.1. Baseline description of the reverse breeding technique

According to JRC, 2011 (p. 21), reverse breeding is a method in which the order of events leading to the production of a hybrid plant variety is reversed. It facilitates the production of homozygous parental lines that, once hybridised, reconstitute the genetic composition of an elite heterozygous plant, without the need for back-crossing and selection.

The method of reverse breeding includes the following steps:

- Selection of an elite heterozygous line that has to be reproduced;
- Suppression of meiotic recombination in the elite heterozygous line through the silencing of genes such as *dmc1* and *spo11.* This is normally achieved by plant transformation with transgenes encoding RNA interference (RNAi) sequences; however, also different approaches can be used, as mentioned in Section 3.12 of this report;
- Production of haploid microspores (immature pollen grains) from flowers of the resulting transgenic elite heterozygous line;
- Use of double haploid (DH) technology to double the genome of the haploid microspores and to obtain homozygous cells;
- Culture of the microspores in order to obtain homozygous diploid plants;
- Selection of plant pairs (called parental lines) that do not contain the transgene and whose hybridisation would reconstitute the elite heterozygous line.

The reverse breeding technique makes use of transgenesis to suppress meiotic recombination. In subsequent steps, only non-transgenic plants are selected. Therefore, the offspring of the selected parental lines would genetically reproduce the elite heterozygous plant and would not carry any additional genomic change.

Reverse breeding is mentioned in MS opinions 2 and 15 published in 2013 and 2019, respectively.

3.10.2. Extracted information on the description of the reverse breeding technique from the MS opinions

Opinion 2 (p. 34) states that creation of chromosome substitution lines is possible. A chromosome substitution line contains one or more chromosomes from one parent in the genetic background of the other parent. Chromosomes can be shuffled in all possible combinations when a single chromosome from one inbred is transferred into the background of a different inbred parent.

Generally, the method of reverse genetics is applicable to all species with a chromosome number of 12 or less, and for which a doubled haploid technique, preferably microspore culture, is available. More than 290 varieties (non-GMO) have already been released by making use of the DH technique (opinion 2, p. 35).

Transgenes are only used in intermediate breeding steps when producing homozygous parental lines from heterozygous plants by ruling out meiotic recombination (opinion 2, p. 35). This essential step, the suppression of recombination, is achieved by introducing an RNAi construct that suppresses the action of the genetic recombination in the heterozygous plant line. The knockdown in the expression of an essential gene can be achieved by targeting genes using RNAi but also by other methods. In crops in which stable transformation is difficult or impossible to achieve other techniques like VIGS can be used. Alternatively, target genes may be silenced by silencing molecules delivered by graft transmission. This genetically engineered parent is then crossed with a non-GM identical

heterozygous parent line. Half of the progeny will no longer contain this RNAi-producing transgene and the resulting achiasmatic gametes (gametes where crossovers did not occur) are then selected and regenerated into doubled haploid plants. Upon selection and crossing, these plants can be used as hybrids like the primary hybrid. Consequently, these products no longer contain transgenes (opinion 2, p. 35-36).

The hybrid resulting from reverse breeding is genetically identical to the initial hybrid. As neither a transgene nor a product of the transgene is present in these plants, they are not recognisable or detectable (opinion 2, p. 36).

3.10.3. Extracted information on the risk assessment aspects of the reverse breeding technique from the MS opinions

As for RdDM and grafting, opinion 2 (p. 37–39) provides detailed information regarding aspects of risk assessment for reverse breeding:

Molecular characterisation

The intermediate plant is clearly transgenic, produced by standard transformation methods using standard vectors. Thus, according to the authors, this plant should be thoroughly analysed. The molecular analysis of the intermediate plant provides an indication of potential unintended effects in the negative segregant that results from the transformation process.

Genetic stability is not a relevant element of the risk assessment of plants resulting from reverse breeding. The suppression of meiosis in the primary transformants is sufficient to achieve the desired effect. The genetic trait needs to be available during a defined time point in the breeding scheme but is not desired at later stages of product development and during commercialisation.

Substantial equivalence

Based on the present perspective, there is no reason to exclude tests for substantial equivalence for the risk assessment for plants produced by reverse breeding. Reverse breeding includes a step at which suppression of crossover formation in a parental plant (an F1 elite plant) is implemented. Based on current scientific knowledge (one experimental paper), opinion 2 concluded that this step uses genetic transformation mechanisms similar to those known from standard GM plants with the possibility to create negative effects due to unintended genetic and epigenetic variations.

As discussed in Opinion 2, in cases where appropriate comparators are not available (e.g. where significant compositional changes have been targeted), it is suggested that the EFSA GMO Panel guidelines should be followed to carry out a comprehensive safety/nutritional assessment on the GM plant *per se* (EFSA GMO Panel, 2011a). However, the F1 elite plants can be used as conventional counterpart as defined by EFSA, and thus, the problem that a conventional counterpart is not available and the approach of substantial equivalence cannot be employed as mentioned by EFSA Guidance (EFSA GMO Panel, 2011a,b) does not exist for reverse breeding.

Field tests and comparative analysis should be comprehensively and thoroughly performed in order to enable a reliable estimation of any potential difference in composition or phenotypic characteristics. Additionally, the comparative data should be checked against field trial information derived from the cultivation and selection process of doubled haploid plants.

Toxicological and allergenicity risk assessment

Possible unintended effects in relation to reverse breeding and negative segregants, in general, were taken into consideration by an expert Panel during a workshop hosted by Food Standards Australia New Zealand in 2012.[7] The Panel noted that no firm conclusions could be reached on how transgene-free end products are produced or on the reliability of the process overall due to the lack of sufficient technical details.

It was further concluded by this expert Panel that, even though there should not be any particular hazards associated with the GM component of the technique, it would be helpful to develop some criteria for distinguishing techniques such as reverse breeding from those where the final food-producing lines are clearly GM and also for ensuring that a complete barrier/genetic separation exists between the early GM breeding lines and the non-GM food-producing lines.

[7] http://www.foodstandards.gov.au/publications/Pages/New-plant-breeding-techniques-workshop-report.aspx

Based on these observations and considering uncertainties with respect to unintended effects, the current EU regulation is applicable and the risk assessment procedures should be according to current GMO guidance (EFSA GMO Panel, 2011a). It is however clear that some elements of the risk assessment such as expression analysis and assessment of newly expressed proteins will not be applicable.

Environmental risk assessment

According to current knowledge, it is not expected that final products (plants) obtained by reverse breeding should have an increased environmental risk potential compared to conventionally bred plants. Because the methods to suppress meiotic recombination may vary greatly, it cannot be excluded that the ERA should include elements of the relevant EFSA Guidance (EFSA GMO Panel, 2010) when assessing the intermediate plants.

From the concluding chapter 7 of opinion 2 (p. 56), the following information is extracted:

To date, only one report is available which describes reverse breeding in *A. thaliana* by the floral dip method. The following assessment is based on this publication.

- All potential risks associated with *Agrobacterium*-mediated transformation need to be considered.
- Using RNAi, the unintended effects due to short RNAs have to be excluded by *in silico* analyses.
- Reverse breeding includes the production of double haploids by *in vitro* methods which are prone to somaclonal variation; however, the DH technology is state of the art in modern traditional plant breeding. In addition, plant breeders apply rigorous selection during the breeding process. The variety registration includes testing of Value for Cultivation and Use (VCU) over several years and in different environments. These procedures should widely minimise risks associated with the traditional breeding process.

Furthermore, it is stated on p. 55 that negative segregants constitute the final product of reverse breeding, but they are not to be used as comparators because they lack history of safe use and unintended effects due to the genetic modification cannot be excluded.

The intermediate product of reverse breeding (the GM plant) should be thoroughly risk assessed for its molecular characteristics based on current EFSA Guidance (EFSA GMO Panel, 2011a). In particular, this includes:

- Description of the methods used for the genetic modification.
- Source and characterisation of nucleic acid used for transformation.
- Nature and source of vector(s) used including nucleotide sequences intended for insertion.
- Information on the sequences actually inserted/deleted or altered. The copy number of intact and fragmented inserts should be established.
- Information on the expression of the inserted/modified sequence including the flanking regions to exclude unintended effects related to the transformation process.

Concerning the environmental risk assessment, it is important to perform *in silico* analyses (in particular gene homologies) to largely exclude potential interactions with other organisms.

As the negative segregant could potentially contain unintended modifications due to the transformation process, their presence should be largely excluded by the analysis of the stably transformed plant. The negative segregant itself should not contain the insert and its absence has to be verified (opinion 2, p. 55).

In opinion 15, p. 4, it is stated that reverse breeding makes use of genetic modification techniques, but the genome sequence of the final product is not modified (no inserted DNA present). The finally produced plants have no new properties, and their risks are therefore similar to those of traditionally bred plants.

3.11. Agroinfiltration

3.11.1. Baseline description of the agroinfiltration technique

According to JRC, 2011, agro-infiltration is a technique applied to plant tissues, mostly leaves, and these tissues are infiltrated with a liquid suspension of *Agrobacterium* sp. containing the desired gene (s) to be expressed in the plant. The genes are locally and transiently expressed at high levels.

The technique is often used in a research context, but also as a production platform for high-value recombinant proteins. In all cases, the plant of interest is the agro-infiltrated plant and not the progeny.

Depending on the tissue and the type of gene constructs infiltrated, three types of agro-infiltration can be distinguished:

- 'Agro-infiltration sensu stricto': non-germline tissue (typically leaf tissue) is infiltrated with non-replicative constructs in order to obtain localised expression in the infiltrated area.
- 'Agro-inoculation or agro-infection': non-germline tissue (typically leaf tissue) is infiltrated with a construct containing the foreign gene in a full-length virus vector in order to obtain expression in the entire plant.
- 'Floral dip': Germline tissue (typically flowers) is immersed into a suspension of *Agrobacterium* carrying a DNA construct in order to obtain transformation of some embryos that can be selected at the germination stage. The aim is to obtain stably transformed plants. The resulting plants are GMO that do not differ from GM plants obtained by other transformation methods.

Agro-infiltration is mentioned in opinion 1, 8, 15 and 16 published between 2012 and 2019. Since no information was extracted from opinions 8 and 16, these opinions are not described in the following paragraphs.

3.11.2. Extracted information on the description of the agroinfiltration technique from the MS opinions

In opinion 1 (p. 56), it is mentioned that the genes introduced by agro-infiltration are usually not incorporated into the plant genome, but are rather temporarily active as free DNA molecules in the plant cell resulting in a rapid transcription into RNA molecules: mRNA in case of genes which are expressed into proteins, or dsRNA when RNAi constructs are used to modify endogenous gene expression. The presence and expression of the introduced genes are transient (gene expression can persist up to 12 days post-inoculation) and the gene effects would fade away in time.

Agro-inoculation (Agro-infection) is frequently applied when using VIGS-vectors (opinion 1, p. 57). VIGS is a post-transcriptional gene silencing mechanism (PTGS) to transiently suppress the endogenous expression of a target gene by infecting plants with a recombinant virus vector carrying a host-derived sequence. Infection and systemic spreading of the virus cause the targeted degradation of the gene transcripts. VIGS-vectors are composed of a modified viral genome and include a fragment from the host plant gene to be silenced.

Besides the inoculation of leaves, agrodrench has been described as a method of inoculation when using VIGS in very young seedlings (opinion 1, p. 58).

3.11.3. Extracted information on the risk assessment aspects of the agroinfiltration technique from the MS opinions

A consequence of agroinfiltration may be that the used *Agrobacterium* moves from the site of infiltration throughout the whole plant and hence to the parts used for further propagation, causing infection and possibly stable transformation (opinion 1, p. 58). Several studies have shown that *Agrobacterium* can move internally through the xylem vessels in grape, and natural pathogenic agrobacteria were able to move systemically inside the plant beyond the site of inoculation for a number of plant species (i.e. tomato, rose, grapevine). It cannot be excluded that DNA introduced in the plant tissue by this technique can be incorporated into the nuclear DNA, but it is assumed that this is a very rare event.

Opinion 15 (p. 4) mentions that agro-inoculation is a technique that makes use of genetic modification approaches, but the genome sequence of the finally produced plants is not modified (no inserted DNA present). These plants have no new properties, and their risks are therefore similar to those of traditionally bred plants.

3.12. Combinations of techniques

Two MS opinions (opinions 1 and 2) discuss how several NGTs can be used in various combinations.

Opinion 1 (p. 63-65) refers to the following combinations:

Cisgenesis and ODM: ODM-induced alterations of a cisgene (p. 63), including its regulatory elements may lead to enhanced expression of the modified gene. If deemed as a hazard as determined by an appropriate analysis, this should be considered during the risk assessment. Depending on the trait, the environmental but also food and feed safety may be affected.

Cisgenesis and SDN-1 or SDN-2 (p. 64) may be used for similar genetic alterations as when combining ODM and cisgenesis. Using SDN-3, the targeted integration of DNA stretches of several kbp in length using homologous repair templates can be achieved. Targeted integration of the gene of interest into the genome would minimise risks associated with the potential gene integration into another genomic location. All the SDN techniques could potentially cause detrimental off-target mutations.

Furthermore, opinion 2 (p. 52-53) extends on the previous combinations with the following:

As with cisgenesis, intragenesis may also be used in combination with ODM and SDN technologies.

The SDN-1/-2 and ODM techniques may be used in combination with agroinfiltration; agroinfiltration serves as the delivery method to introduce the effectors in the plant cell. The SDN-1, -2 and ODM modules would act only transiently, whereas the genetic changes introduced by the SDN and ODM techniques will persist.

Agroinfiltration can also be combined with RdDM. Agroinfiltration can be used to introduce the effector molecules into plants, which in turn would result in gene silencing by RdDM. In contrast to RdDM induced by stable transformation, this combination is of particular interest in the context of applying new plant breeding techniques as no modification to the plant genome *per se* occurs.

The effector molecules needed for reverse breeding are introduced usually by stable transformation with an RNAi construct. Similar effects could also be achieved by other gene silencing methods. Methods that can be potentially used to induce gene silencing such as SDN, ODM or RdDM might be suitable, as well as cisgenesis or intragenesis that could interact with the target gene by perturbing its function. However, currently, there is no reason to anticipate that one of the new plant breeding methods would be the method of choice for reverse breeding in the near future. The safety issues related to the use of negative segregants would not be relevant if the meiotic recombination was silenced by transient delivery methods.

Grafting (on GM rootstock) is currently combined with standard GM techniques. Any NGT that allows producing a desired effect in a scion could potentially be used in combination with grafting and would be a matter of case-by-case proof of concept. Grafting has been mentioned as a suitable method to suppress meiotic recombination for reverse breeding (as stated in opinion 2, p. 53).

In opinion 2, p. 52, it is stated that it is generally conceivable that all new plant breeding techniques could be combined. However, some combinations appear more likely than others. As there is substantial progress concerning research and development in the new plant breeding techniques, new possibilities for combinations may arise.

4. Conclusions

The present scientific report was produced to address a mandate of the European Commission to EFSA to provide an overview of the opinions of the MS national bodies and EFSA on the risk assessment of plants developed through new genomic techniques (NGTs). In line with the terms of reference of the European Commission mandate, no critical appraisal of the content of the extracted information was carried out.

Taking into account the definition for NGTs provided by the European Commission for this mandate, the JRC, 2011 report on new plant breeding techniques as well as the Explanatory Note on New Techniques in Agricultural Biotechnology from the European Commissioner for Health and Food Safety (EC-SAM, 2017) for some more recently developed NGTs, were considered as the baseline for the types and nature of NGTs to be included in this report. Based on JRC, 2011, eight NGTs were considered: site-directed nuclease technology (SDN-1, SDN-2 and SDN-3), oligonucleotide-directed mutagenesis (ODM), cisgenesis and intragenesis, RNA-dependent DNA methylation (RdDM), grafting (on GM rootstock), reverse breeding, agro-infiltration and synthetic genomics. In addition, some newly developed CRISPR-Cas-based techniques applied in SDN approaches as well as additional CRISPR-Cas-based methods such as base editing were considered.

The information included in this report is on the description of the defined list of NGT techniques and on aspects of the risk assessment for plants developed through one or a combination of these NGTs. No information on synthetic genomics was included in any of the opinions, and therefore, no

information on this technique is presented in this report. In contrast, 14 out of the 16 MS opinions as well as two of the three EFSA opinions discussed the SDN approaches (SDN-1, SDN-2 and SDN-3).

As regards the EFSA opinions, besides the three SDN-based NGTs, information was also extracted on ODM, cisgenesis/intragenesis. In addition, some information on base/prime editing was extracted from the EFSA GMO Panel, 2020 scientific opinion on SDN-1/-2 and ODM. In line with the respective European Commission mandates, two of the EFSA GMO Panel scientific opinions on NGTs (EFSA GMO Panel, 2012a,b on cisgensis/intragenesis and SDN-3, respectively) focused on (a) addressing the risks for humans, animals and the environment by comparing plants developed via NGTs with plants obtained by conventional breeding methods and by currently applied genetic modification methods, such as transgenesis and (b) evaluating the applicability of the EFSA GMO Panel GM plant risk assessment guidance documents to the assessment of plants developed by the considered NGTs. The GMO Panel scientific opinion on the safety assessment of plants developed using SDN-1, SDN-2 and ODM approaches (EFSA GMO Panel, 2020) addressed the risks in terms of impact on humans, animals and the environment by evaluating: (a) the applicability of the hazards identified and discussed in section 4 of the EFSA opinion on SDN-3; (b) the validity/applicability of the conclusions of the EFSA opinion on SDN-3 in the context of the safety evaluation of plants developed by SDN-1, SDN-2 and ODM.

As regards the MS opinions, information was extracted related to all the NGTs defined in JRC, 2011 except for synthetic genomics as well as on newly developed NGTs such as those based on the CRISPR technology, including base editing. Fourteen out of the 16 opinions discussed the SDN technology. In addition, two MS opinions discussed how different NGTs can be used in various combinations (e.g. cisgenesis and ODM or SDN-1/-2). Some MS opinions were produced to serve as advice to a Ministry while others were published as a technical report or a scientific publication. It is noted that some MS opinions discussed the interpretation of the Directive 2001/18/EC in relation to SDNs and intermediate plants. Such information, however, is out of the scope of this European Commission mandate and it is thus not included in this report.

5. Documentation as provided to/by EFSA

- Letter from the European Commission to the EFSA Executive Director dated 09 January 2020, requesting EFSA to produce an overview on the risk assessment of plants developed through new genomic techniques.
- Letter from the EFSA Executive Director to the European Commission dated 11 February 2020, acknowledging the reception of the mandate.
- Letter from the EFSA Executive Director to the European Commission dated 29 June 2020, proposing to address the mandate in accordance with Article 31 of Regulation (EC) No 178/2002.
- Letter from the European Commission to the EFSA Executive Director dated 13 July 2020, agreeing with the EFSA's proposal to address the mandate in accordance with Article 31 of Regulation (EC) No 178/2002.

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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 recognise 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.
Effector	molecules (proteins or nucleic acids (RNA or DNA)) used to obtain the desired modification in the plant.

Epigenetics	the molecular mechanisms (e.g. DNA methylation) controlling expression of a genetically encoded trait. DNA methylation is reversible, and, although it can be inherited between generations, whether it is retained will depend on the environment.
Exogenous DNA	DNA originating outside the plant which can be introduced naturally or by technological intervention.
Homology-directed repair	abbreviated as HDR, a molecular mechanism which allows the repair of DNA double-strand breaks using a homologous sequence of DNA as template.
Intended effect	effect that is designed to occur and which fulfil the original objectives of the genetic modification.
Intermediate plant	plant developed during the genome editing process that does not represent the final product
Negative segregant	plants that are negative segregants lack the transgenic event and can be produced, e.g. by self-fertilisation of hemizygous GM plants, or from crosses between hemizygous GM plants and non-GM plants.
New genomic technique	techniques capable to change genetic material of an organism and that has emerged or has been developed since the adoption of the GMO legislation in 2001.
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 effect	DNA modifications occurring at an unintentional location in the genome.
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.
Resulting plant	plant that results after having gone through all the steps of the particular technique.
Unintended effect	effect that is considered to be consistent (non-transient) difference between the GM plant and its appropriate comparator, which goes beyond the primary intended effect(s) of introducing the transgene(s).

Abbreviations

Cas	CRISPR-associated
COGEM	commissie genetische modificatie
CRISPR	clustered regularly interspaced short palindromic repeats system
DH	double haploid
DNA	deoxyribonucleic acid
DSB	double stranded break
DsRNA	double stranded RNA
GM	genetically modified
GMO	genetically modified organism
gRNA	guide RNA
HDR	homology-directed repair
HR	homologous recombination
JRC	Joint Research Centre
MS	Member State
NGT	new genomic technique
NHEJ	non-homologous end-joining
ODM	oligo-directed mutagenesis
PAM	protospacer adjacent motif
PEG	polyethylene glycol
PTGS	post-transcriptional gene silencing
RA	Risk assessment
RdDM	RNA-dependent DNA methylation
RIVM	Rijksinstituut voor volksgezondheid en milieu

RNA	ribonucleic acid
RNAi	RNA interference
SDN	site-directed nuclease
TALEN	transcription activator like effector nuclease
TILLING	targeting induced local lesions in genome
TGS	transcriptional gene silencing
VIGS	virus-induced gene silencing
WGS	whole-genome sequencing
ZFN	zinc finger nuclease

Appendix A – List of MS opinions

Table A.1: Overview of the 16 scientific opinions issued by the MS national bodies and used in this report. The 'opinion number' is used in the underlying report as a reference. For all opinions, it is indicated what type of NGT is mentioned by a 'yes' in the table. The use of a specific SDN is indicated if applicable. For more details, see Appendix A in Van der Vlugt (2021)

Opinion Number	Identifier given by EFSA	Title of report	Author	New Genomic Technique									
				SDN-1	SDN-2	SDN-3	ODM	Cisgenesis intragenesis	RdDM	Grafting	Reverse breeding	Agro-infiltration	Base editing
1	AT_2012	Cisgenesis - A report on the practical consequences of the application of novel techniques in plant breeding	Bundesministerium für Gesundheit	ZFN	ZFN	ZFN	yes	yes	–	–	–	yes	–
2	AT_2013	New plant breeding techniques	Bundesministerium für Gesundheit	–	–	–	–	–	yes	yes	yes	–	–
3	AT_2017	RNAi-based techniques, accelerated breeding and CRISPR-Cas: basics and application in plant breeding	Bundesministerium für Gesundheit	yes	yes	yes	–	–	–	–	–	–	–
4	BE_2016	Advice of the Biosafety and Biotechnology Unit (SBB) concerning genome editing in plants using the CRISPR/Cas system	Wetenschappelijk Instituut Volksgezondheid WIV-ISP	CRISPR	–	–	–	–	–	–	–	–	–

Opinion Number	Identifier given by EFSA	Title of report	Author	New Genomic Technique									
				SDN-1	SDN-2	SDN-3	ODM	Cisgenesis intragenesis	RdDM	Grafting	Reverse breeding	Agro-infiltration	Base editing
5	BE_2019	Advice of the Belgian Biosafety Advisory Council on notification B/BE/19/V1 (maize) from VIB under Directive 2001/18/EC	Biosafety Advisory Council	CRISPR	–	–	–	–	–	–	–	–	–
6	DK_2019	Induced genetic variation in crop plants by random or targeted mutagenesis: convergence and differences	Dept of Molecular Biology and Genetics, Aarhus University, Denmark	yes	yes	–	yes	–	–	–	–	–	yes
7	ES_2019	National Biosafety Commission Report on the site directed mutagenesis ("gene editing")	Ministerio Para La Transición Ecolológica	yes	yes	yes	–	–	–	–	–	–	–
8	FR_2017	Scientific opinion on new plant breeding techniques	Haut Conseil des Biotechnologies	yes	yes	yes	yes	yes	yes	yes	–	yes	–
9	8_1_DE_2019	'Genetic engineering - public hearing	Federal Agency for Nature Conservtion (BfN)	yes	yes	yes	–	–	–	–	–	–	–
10	9_2_DE_2019	Genome editing	Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit	yes	yes	yes	yes	–	–	–	–	–	yes
11	10_3_DE_2018	Scientific report on new techniques in plant breeding and animal breeding and their uses in food and agriculture	Federal Office of Consumer Protection and Food Safety (bvl)	yes	yes	yes	yes	–	–	–	–	–	yes

Opinion Number	Identifier given by EFSA	Title of report	Author	New Genomic Technique									
				SDN-1	SDN-2	SDN-3	ODM	Cisgenesis intragenesis	RdDM	Grafting	Reverse breeding	Agro-infiltration	Base editing
12	11_DK_2020	Comments from DTU on studies of the risk arising from the new mutagenesis techniques	National Food Institute at the Technical University of Denmark (DTU Food)	yes	yes	yes	yes	–	–	–	–	–	–
13	14_NL_2014	CRISPR-Cas advisory report - Revolution from the lab	Commissie Genetische Modificatie (COGEM)	CRISPR	CRISPR	CRISPR	–	–	–	–	–	–	–
14	15_NL_2017	Advisory opinion on CRISPR-Cas and directed mutagenesis in plants	Commissie Genetische Modificatie (COGEM)	CRISPR	CRISPR	–	yes	–	–	–	–	–	–
15	16_NL_2019	Advisory opinion on the Dutch proposal for the exemption of certain GM plants	Commissie Genetische Modificatie (COGEM)	–	–	–	yes	yes	–	–	yes	yes	–
16	17_LT_2019	Analysis of new gene modification techniques or methods	UAB Caszyme, Vilnius	yes	yes	yes	yes	yes	yes	yes	–	yes	yes