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Overview of sixteen scientific opinions on genetically modified plants obtained by new genomic techniques

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Abstract

RIVM was asked by EFSA in the frame of a procurement (PO/EFSA/GMO/2020/01) to produce an overview of 16 scientific opinions published by European competent authorities and national institutions since 2012 on genetically modified plants obtained by new genomic techniques (NGTs). The 16 opinions were provided by the European Commission in the frame of a mandate asking EFSA to provide an overview on the risk assessment of plants developed through NGTs based on its own previous and current work and on work carried out at national level. 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. Based on this definition for NGTs provided by EC, the following NGTs as described in a report of the Joint Research Council on new plant breeding techniques, published in 2011, were considered: (1) Zinc finger nuclease technology, (defined more broadly as site-directed nuclease technology); (2) oligonucleotide directed mutagenesis; (3) cisgenesis and intragenesis; (4) RNA-dependent DNA methylation; (5) grafting (on genetically modified rootstock); (6) reverse breeding; (7) agro-infiltration and (8) synthetic genomics. The current report presents the information on the defined NGTs method description and on the risk assessment of plants developed through these NGTs, as extracted from the 16 opinions. In order to extract the relevant information a baseline description for each NGT was set and inclusion and exclusion criteria for information extraction were defined. Most (14 out of 16) opinions discuss the SDN technology, whereas no opinion contains information on synthetic genomics. A new NGT, base-editing, is described in four opinions and therefore information on this technique is also presented. As specified by the procurement, no critical appraisal on the information extracted from the scientific opinions was performed.

Key words:

new genomic technique, site-directed nuclease, oligonucleotide directed mutagenesis, cisgenesis/intragenesis, RNA-dependent DNA methylation, agro-infiltration, base-editing, grafting, reverse breeding

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Summary

RIVM was asked by EFSA to provide an overview of 16 scientific opinions issued by European competent authorities and national institutions since 2012 on new genomic techniques (NGTs). This overview is aimed at supporting the EC mandate to EFSA to provide an overview on the risk assessment of plants developed through NGTs based on its own previous and current work and on work carried out at national level in Europe.

NGTs are defined as techniques capable to change genetic material of an organism and that have emerged or have been developed since the adoption of the GMO legislation in 2001. Taking into account this definition on NGTs provided by EC and the report of the Joint Research Council (JRC) on new plant breeding techniques published in 2011, the following NGTs are considered in this study: (1) Zinc finger nuclease technology, redefined according to recent insights as site-directed nuclease (SDN) technology; (2) oligonucleotide directed mutagenesis (ODM); (3) cisgenesis and intragenesis; (4) RNA-dependent DNA methylation (RdDM); (5) grafting (on genetically modified rootstock); (6) reverse breeding; (7) agro-infiltration and (8) synthetic genomics.

In the present report each NGT is first described based on the information in the JRC report and in two EFSA opinions from 2012 as well as information in the Explanatory Note on New Techniques in Agricultural Biotechnology from the European Commissioner for Health and Food Safety (EC-SAM, 2017) for more recent SDN techniques such as CRISPR-Cas. The information on the description of the NGTs from these reports set the baseline for the information extraction from the 16 scientific opinions.

In order to extract the relevant information from the 16 scientific opinions a number of inclusion and exclusion criteria were defined. Using these criteria as a reference, information on the description of the NGT additional to the baseline and on the risk assessment of plants developed through the defined NGTs was identified and extracted.

The 16 opinions that were evaluated are listed in a table (Appendix A) and the summarized extracted information is presented in Chapter 3. Fourteen out of the 16 opinions discuss the site-directed nuclease technology. In contrast, no information on Synthetic genomics is reported in any of the opinions, and therefore no information on this technique is presented in the current report. Information on a newly developed CRISPR-based technique, called Base-editing was included in four opinions and therefore it was added to the list of NGTs.

In line with the mandate's terms of reference and as specified by the procurement, no critical appraisal on the information extracted from the 16 scientific opinions was performed.

The information in this report, i.e. the extracted information from the 16 scientific opinions, supports EFSA's work in providing an overview on the risk assessment of plants developed through NGTs as requested by the EC.

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1. Introduction

1.1. Background and Terms of Reference

1.1.1. Background to provide context to this report

In 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. In the frame of this study, NGTs are defined as techniques capable to change genetic material of an organism and that have emerged or have been developed since the adoption of the GMO legislation in 2001.

The study by the EC needs to address a number of different aspects on the topic including an overview on the risk assessment of plants developed through NGTs taking into account the previous and ongoing work by EFSA as well as work produced by EU national bodies[1].

In order to support with the delivery of the above described study on NGTs, EFSA has been mandated by the EC to provide an overview on the risk assessment of plants developed through NGTs[2]. EFSA is asked, besides its own previous and current work on NGTs to also take into account opinions published by European competent authorities and national institutions since 2012 on this topic.

In preparing this overview EFSA has asked RIVM in the frame of a procurement to prepare an overview of the existing scientific opinions produced by the European competent authorities and national institutions addressing the safety assessment of plants developed through NGTs and report this overview in a technical report. In total 16 scientific opinions issued by the MS national bodies and provided by EFSA to RIVM were reviewed and summarised.

The call underlying this RIVM study was based on EFSA's 2020-2022 draft Work Programme for grants and operational procurements as presented in Annex IX of the draft Programming Document 2020 – 2022, available on the EFSA's website[3].

The work presented in this report was procured under the contract PO/EFSA/GMO/2020/01 entitled 'Support for preparatory work of the mandate for a scientific opinion on genetically modified plants developed through new genomic techniques'.

1.1.2. Terms of Reference as provided by EFSA to RIVM

The overall objective of this study, as mentioned above, is specified by EFSA in the following two tasks to RIVM:

Task 1: The information relevant for the risk assessment of plants developed through NGTs taking into account the definition for NGTs is extracted from 16 scientific opinions issued by the MS national bodies.

To this end the content of these opinions is evaluated and all the relevant information related to the NGT definition and risk assessment of plants developed through NGTs is extracted. For those opinions that have a broader content, only definition and risk assessment related issues for plants should be considered. 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 technology) should be excluded.

Task 2: The extracted information is summarized and presented in a technical report together with the description of the criteria applied to extract the relevant information.

[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] https://www.efsa.europa.eu/sites/default/files/event/mb-82/mb191218-a2.pdf

The summarized information from the 16 MS opinions is categorized as per each NGT in the technical report, as well as the methodology followed for the extraction of the relevant information.

1.2. Interpretation of the Terms of Reference by RIVM

In order to facilitate a common understanding on the types and definitions of NGTs, the information in the report of the Joint Research Council on new plant breeding techniques, published in 2011[4] (named JRC 2011 from here on) is taken as a baseline. In this report the following NGTs are described:

- Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)
- Oligonucleotide directed mutagenesis (ODM)
- Cisgenesis and intragenesis
- RNA-dependent DNA methylation (RdDM)
- Grafting (on GM rootstock)
- Reverse breeding
- Agro-infiltration
- Synthetic genomics

EFSA published in 2012 an opinion on ZFN-3 and other Site-Directed Nucleases (SDNs) of a similar function such as TALEN and Meganucleases (named EFSA 2012a from here on)[5] and described the different SDN approaches (SDN-1, SDN-2 and SDN-3). Thus, the term "SDN" is used from here on to describe the ZFN technology including the use of other SDNs.

Another scientific opinion was published by EFSA in 2012 on cisgenesis and intragenesis (named EFSA 2012b from here on)[6]. Information in both of these opinions was also considered in determining the baseline for describing the NGTs.

Since the publication of the JRC report in 2011, new techniques have been developed in particular with regards to genome editing, such as CRISPR-Cas[7], Base editing and Prime editing. CRISPR-Cas and Base editing are described in some of the 16 opinions and thus information on these techniques is also included in this report.

Considerations as regards the type and nature of these newly developed NGTs as described in the Explanatory Note on New Techniques in Agricultural Biotechnology from the European Commissioner for Health and Food Safety, published in 2017 (named EC-SAM 2017 from here on)[8] are also taken into account in determining the baseline.

It is also noted that, in line with the EC mandate sent to EFSA, no critical appraisal of the information in the MS opinions was carried out.

[4] JRC 2011: https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/new-plant-breeding-techniques-state-art-and-prospects-commercial-development: European Commission, Joint Research Centre, New Plant Breeding Techniques, State of the art and prospects for commercial development, 2011.

[5] EFSA 2012a: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2012.2943: EFSA Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function. EFSA Journal 2012;10(10):2943.

[6] EFSA 2012b: https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2012.2561: EFSA Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. EFSA Journal 2012;10(2):2561.

[7] CRISPR: clustered regularly interspaced short palindromic repeats system; Cas: CRISPR-associated.

[8] EC-SAM 2017: https://op.europa.eu/nl/publication-detail/-/publication/103eb49f-4047-11e7-a9b0-01aa75ed71a1/language-en/format-PDF/source-118057563: New techniques in agricultural biotechnology: CEU. SAM_ADV, Directorate-General for Research and Innovation, European Commission, 28 April 2017.

2. Data and Methodologies

2.1. Data

Sixteen scientific opinions issued by the MS national bodies on NGTs and provided by EFSA to RIVM represent the data used in this study. The opinions are listed in Appendix A. The authors, title and a short description of the focus of the opinion is given in this Table. The NGTs mentioned in each of the respective opinion are indicated. In this report the opinion number refers to the number of the respective MS opinion as listed in this Table (e.g. opinion 1).

2.2. Methodologies

As a first step, a baseline for each NGT was defined based on the description in JRC 2011, also taking into account EFSA 2012a and EFSA 2012b as well as EC-SAM 2017 for some of the more recently developed NGTs.

The baseline description of each NGT in this report covers, a description of the function and, if available, information on the method(s) of delivery to plant cells. Information additional to this baseline was extracted from the 16 MS opinions together with all information on the risk assessment of plants developed through the defined list of NGTs.

In order to extract the relevant information on the NGTs from the opinions, the following inclusion and exclusion criteria were defined in close collaboration with EFSA:

Inclusion criterion 1: Information on the description of the NGTs: 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, Synthetic genomics and Base-editing.

Inclusion criterion 2: Information on aspects of risk assessment of plants developed through one or a combination of techniques mentioned in inclusion criterion 1.

Exclusion criterion 1: The newly developed techniques gene drive and RNAi technology are not taken into account. The gene drive technology is excluded as it is mainly applied in organisms other than plants (see exclusion criterion 3) and the RNAi technology is excluded as it does not result in the modification of genetic material *per se*.

Exclusion criterion 2: Elements other than risk assessment considerations such as on risk management, detection methods and labeling.

Exclusion criteria 3: Information on risk assessment aspects related to organisms other than plants.

The information extraction was carried out by reading the full text of the 16 MS opinions, taking into account the above described inclusion and exclusion criteria. Relevant text that met the inclusion criteria was marked, extracted and cited in Chapter 3 of this report. For clarity, cited text was slightly adapted when needed (e.g. to align the terminology). In order to prevent the repetition of similar information from the different MS opinions, the extracted information is presented once with reference to all relevant opinions and page numbers. Although in some cases a technique was mentioned in an opinion (as indicated in Appendix A), no information based on the above-mentioned inclusion criteria was actually extracted and reported in Chapter 3.

3. Results - Extracted information per NGT

This chapter presents all the information extracted from the 16 MS opinions. Each section starts with the baseline description according to the JRC 2011 and, if applicable, the EFSA 2012a&b opinions and the EC-SAM 2017 report. The description of the technique according to the set baseline is followed by

two subsections presenting the extracted information on the description of the technique and on the risk assessment aspects, respectively.

3.1. SDN technology

The information on the SDN technology from EFSA 2012a and EC-SAM 2017 is used as a baseline for the description of the SDN technology. In EFSA 2012a the following information is presented on the different 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 recognize 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 but no other exogenous DNA (p. 12).

The EC-SAM 2017 report mentions two groups of nucleases: (1) the protein-directed SDNs such as ZFN and TALEN, proteins that consist of a DNA binding domain which recognizes 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) which is bound to a guide RNA (gRNA), the sequence of which targets the nuclease to the complementary DNA sequence in the genome (pp. 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 SDN technology is mentioned in 14 out of 16 MS opinions. Some 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 opinion 9, 12 and 14, these opinions are not described in the following paragraphs.

The information related to the SDN technology in general, is first presented in the sections 3.1.1 (description) and 3.1.2 (aspects of risk assessment). Extracted information dedicated to each specific SDN approach is presented in the sections 3.2 to 3.4.

3.1.1. Extracted information on the description

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, pp. 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, pp 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 above. The production process does not include a selection step for stable genomic integration of the gene cassette. A second strategy for 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 ribonucleo-protein complexes: ribonucleoprotein complexes are delivered into plant cells to directly exert their function via e.g. PEG mediated particle delivery. In 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 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.

3.1.2. Extracted information on aspects of risk assessment

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 recognize 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 which 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 recognize 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 in 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 to 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 to 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 to 248 single nucleotide variations and 32 to 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 by means of 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, to (ii) deletion of genomic fragments, (iii) integration of cis-, intra-, or transgenes at unintended loci or (iv) a combination of those (opinion 3, pp. 28-29).

Moreover, potential unintended effects by means of 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 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 datasets. 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, pp. 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 (1 to 5 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 (for example, 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 (for example, a plant containing Cas9 with a plant containing gRNA) may result in genetic modifications in offspring;
- In the particular case of a sequence recognized 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.

3.2. SDN-1 technology

See 3.1 for the baseline description.

3.2.1. Extracted information on the description

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, for example 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, for example ~50 bp in tomato to ~245 kbp in rice. The latter resulted in deletion of a diterpenoid synthetic gene cluster of ten loci, exemplifying the potential to eliminate large genomic regions.
- multiplexing ability using CRISPR-Cas9 that has been shown for example in rice plants targeting up to 7 and 8 sites simultaneously with different gRNAs.

According to opinion 3 (p. 25), datasets 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).

3.2.2. Extracted information on aspects of risk assessment

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 minimization 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.

3.3. SDN-2 technology

See 3.1 for the baseline description.

3.3.1. Extracted information on the description

In opinion 8, p. 16, it is mentioned that in 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.

3.3.2. Extracted information on aspects of risk assessment

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.

3.4. SDN-3 technology

See 3.1 for the baseline description.

3.4.1. Extracted information on the description

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 3.1).

3.4.2. Extracted information on aspects of risk assessment

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 3.1).

3.5. Base editing

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 Cas9 nickase have been thus used as a sequence-specific RNA-guided DNA-binding platform for the development of new tools for engineering the genome. It is noted that no new tools 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). The extracted information is presented below.

3.5.1. Extracted information on the description

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 – that is, remove amino functional groups. In this way, it is possible to program 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 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 by means of 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.

3.5.2. Extracted information on aspects of risk assessment

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 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.

3.6. Oligonucleotide directed mutagenesis (ODM)

In JRC 2011 (p. 20) 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 (SAM-EC, 2017, p. 57). The chemically synthesized oligonucleotides usually employed are approximately 20 to 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 are believed to 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 particle bombardment and electroporation or PEG-mediated transfection of protoplasts.

ODM is mentioned in MS opinion 1, 6, 8, 10, 11, 12, 14, 15 and 16 published between 2012 and 2020. Since no information was extracted from opinion 8 and 15, these opinions are not described in the following paragraphs.

3.6.1. Extracted information on the description

In opinion 1 (p. 38) it is mentioned that the oligonucleotides induce site-specific nucleotide substitutions, deletions and insertions. As a consequence, the (coding) DNA sequence may be changed resulting in knock-outs or 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 on page 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'). 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[9] (opinion 14, p. 4), that is not part of this RIVM study, is given.

3.6.2. Extracted information on aspects of risk assessment

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 individual change, and this may lead to the proliferation of undesirable soma-clonal 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.

3.7. Cisgenesis and intragenesis

In JRC 2011 (p. 20) cisgenesis is described as a technique of genetic modification that only makes use of the insertions of DNA fragments from the same species or from a cross-compatible species. 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), by introducing inverted DNA repeats. RNAi technology is however excluded from the scope of this report (see exclusion criterion 1 in 2.2 of this report).

[9] 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/

Cisgenic and intragenic plants are produced by the same transformation techniques as transgenic plants. *Agrobacterium*-mediated transformation is most frequently used and biolistic approaches are also applied in some cases.

In EFSA 2012b it is further detailed that for cisgenesis the inserted DNA fragments are in the normal sense orientation and they do not contain any parts of transgenes or inserted exogenous sequences. 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 organisms generated by intragenesis the DNA fragments may be arranged in sense or antisense orientation compared to the orientation in the donor organism. Insertion may also involve full or partial coding regions of a gene.

Cisgenesis and intragenesis are mentioned in MS opinion 1, 8, 15 and 16 published in 2012 and 2019. Since no information was extracted from opinion 8 and 16, these opinions are not described in the following paragraphs.

3.7.1. Extracted information on the description

In opinion 1 (p. 19) the inserted cisgene is more precisely described as a gene including its introns and flanked by its native promoter and terminator in the normal sense orientation. Cisgenic plants can harbor one or more cisgenes, but they do not contain any parts of transgenes or inserted exogenous sequences. Genes must be isolated, cloned or synthesized and transferred back into a recipient where they are stably integrated and expressed. In addition, in Opinion 15 it is stated that the DNA (used for genetic modification) as a whole comes from the donor plant 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. Also silencing techniques through RNAi may be employed (p. 18). Intragenesis allows designing traits using genetic elements from the crop itself (opinion 1, p. 20).

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

In a last chapter of opinion 1 (p. 131) the authors express some 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. From their point of view this position invalidates the basic definition of a cisgene (= gene from a cross-compatible species).

3.7.2. Extracted information on aspects of risk assessment

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 altered levels of expression can alter the environmental behavior of the plant and also render considerations concerning 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 of an endogenous gene, or this gene could be interrupted. 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 3-4 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 for 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 characterization 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 characterization and to confirm the absence of any unanticipated effects caused by the genetic modification process.

Opinion 1 (p. 108) discusses that it is also possible that cisgenic transformation results in plants that are not substantially different in 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 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 obviously 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 case a newly developed plant is planned to be cultivated in an area (e.g. European Union, country, region) instead of when plant material is only imported and processed. 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 2011a[10]) in general will be applicable also for plants genetically modified by 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 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 by 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. Creation of these genes or regulatory sequences makes it possible to obtain new proteins and/or to change expression profiles (opinion 15, p. 8).

3.8. RNA-dependent DNA methylation (RdDM)

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 opinion 2, 8 and 16 published in 2013, 2017 and 2019 respectively.

[10] EFSA 2011a: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2011.2150 : Guidance of the GMO Panel for risk assessment of food and feed from genetically modified plants. EFSA Journal 9(5) (2150): 1-37.

3.8.1. Extracted information on the description

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, for example, for (DNA or RNA) virus sequences that are able to 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.

3.8.2. Extracted information on aspects of risk assessment

Opinion 2 (chapter 3.6, pp. 18 to 33) elaborates on aspects of risk assessment. Most extracted parts of the text are integrally relevant and challenging to summarize without risking to lose important information discussed in this document. For clarity, these parts are cited in subsection 3.8.2.1. The conclusions (chapter 7, pp. 54-55) are cited in section 3.8.2.2 of this report.

3.8.2.1. 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 characterization, 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 2011a[10]). 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 characterization 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 commercialized 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 altered target sequence specificity may be formed. The newly generated siRNAs may have an effect on 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 400 bp – 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).

3.8.2.2. Information extracted from chapter 7 of opinion 2

In the concluding chapter 7 the authors present their most important issues relevant for risk assessment (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 seem 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, it is mentioned (pp. 54-55) that based on current EFSA Guidance (EFSA 2011a[10]), the following elements of the risk assessments of GM plants and derived food and feed should be considered:

- Characteristics of the recipient plant
- Genetic modification and its functional consequences
  - Description of the methods used for the genetic modification
  - Source and characterization of nucleic acid used for transformation
  - *In silico* analysis has to be performed to assess potential unintended effects of the involved RNA sequences
  - In case of stable transformation: Nature and source of vector(s) used including nucleotide sequences intended for insertion. Absence of superfluous DNA sequences in the final product must be demonstrated.
  - Information on the sequences actually inserted/altered
  - Information on the expression of the inserted/modified sequence (*in vitro* and *in vivo*) including the flanking regions to characterise potential spreading of DNA methylation beyond the targeted nucleotide sequence
  - Characterization of the alteration in and stability of the methylation pattern of the targeted DNA (regulatory) region
- Agronomic and phenotypic characteristics of the plant, including compositional characteristics; general description of the trait(s) and characteristics which have been introduced or modified
  - Phenotypic stability of the plant
  - Equivalence tests/comprehensive comparative analyses between crops produced by RdDM and conventional counterparts
- Potential toxicity and allergenicity of gene products and the whole plant and its derived products
  - pathophysiological role in humans or animals by influencing the expression of endogenous genes
  - *in vivo* animal studies that identify potential unforeseen consequences
- Dietary impact and potential for nutritional impact (if applicable; case by case decision)
- Environmental risk assessment

- Analysis of potential alterations related to persistence and invasiveness; persistence of small RNA molecules
- Impacts of specific cultivation, management and harvesting techniques
- Higher susceptibility to plant diseases
- Interaction of the GM plant with target organisms or non-target organisms; effects on soil microbes or related viruses and nematodes; non-target effects on organisms ingesting plants
- Effects on biogeochemical processes
- Effects on human and animal health
- Unintended effects on molecular and cellular interactions

3.9. Grafting

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.

3.9.1. Extracted information on the description

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 which 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 are able to pass the graft junction. As a result, fruits may harbor 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 (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.

3.9.2. Extracted information on aspects of risk assessment

Opinion 2 contains extended information on aspects of risk assessment. The extracted information from pp. 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 localization 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 2011a[10], EFSA 2011b[11]), 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. According to the authors, the requirements, thus, are not different from those present in EFSA Guidelines (EFSA 2011a[10]).

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.

[11] EFSA 2011b: Guidance of the GMO Panel on selection of comparators for the risk assessment of genetically modified plants and derived food and feed. The EFSA Journal 9(5):2149: 1-21.

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 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 2011a[10]). 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 2010[12]), 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

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 hybridized, 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

[12] EFSA 2010: Guidance of the GMO Panel on the environmental risk assessment of genetically modified plants. The EFSA Journal 8(11):1879: 1-111.

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 hybridization 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.

3.10.1. Extracted information on the description

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 cross overs 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, pp. 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 recognizable or detectable (opinion 2, pp. 36).

3.10.2. Extracted information on aspects of risk assessment

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 analyzed. The molecular analysis of the intermediate plant provides an indication of potential unintended effects in the negative segregant that results from the transformation process (EFSA 2011b[11]).

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 timepoint in the breeding scheme but is not desired at later stages of product development and during commercialization.

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 2011a[10]). 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 2011a[10]; EFSA 2011b[11]) 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[13]. 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.

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 2011a[10]). 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 2010[12]) when assessing the intermediate plants.

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

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 minimize 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 2011a[10]). In particular, this includes:

- Description of the methods used for the genetic modification.
- Source and characterization 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. Agro-infiltration

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.

3.11.1. Extracted information on the description

In opinion 1 (p. 56) it is mentioned that the genes that are introduced in the host cells 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 and the gene effects would fade away in time. Gene expression can persist up to 12 days post-inoculation.

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).

3.11.2. Extracted information on aspects of risk assessment

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* is able to 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.

In opinion 15 (p. 4) it is mentioned that agro-inoculation is a technique making 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. Combination of Techniques

Two opinions (opinion 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 considered 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

RIVM has been asked by EFSA to evaluate 16 scientific opinions issued by European member states on new genomic techniques (NGTs). The evaluation of these MS opinions was carried out as part of an EC mandate requesting EFSA to provide an overview on the risk assessment of plants developed through NGTs. In line with the terms of reference of the EC mandate no critical appraisal of the content of the extracted information has been carried out.

Taking into account the definition for NGTs provided by EC that should be considered in the mandate, the JRC 2011 report was taken as the baseline for the types of NGTs to be included in this report: 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. Considerations as regards the type and nature of some more recently developed NGTs 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. In addition, information on a newly developed CRISPR-based technology called base-editing was reported in four MS opinions and was thus included in the list of NGTs (see inclusion criterion 1).

Based on the inclusion and exclusion criteria defined to extract the relevant information from the MS opinions covering the NGTs mentioned above, the information included in this report is on the description of the defined list of NGT techniques and on aspects of the risk assessment of plants developed through one or a combination of these NGTs. Consequently, information on other genetic modification techniques, risk management, detection methods, labeling and risk assessment aspects related to organisms other than plants are not included in this report.

The 16 opinions that have been used for data extraction are listed in Appendix A. The NGTs that are discussed, or briefly mentioned in these opinions are also indicated in Appendix A. 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 opinions discussed the SDN technology. Furthermore, the opinions differed in their scope (see Appendix A); some opinions were produced to serve as advice to a Ministry while others were published as a technical report or a scientific publication.

Many opinions discussed the interpretation of the Directive 2001/18/EC in relation to SDNs and intermediate plants. Such information was not subject to the inclusion criteria and is not presented in chapter 3 of this report. Nevertheless, the main arguments presented on this topic were summarised and they are presented in Appendix B.

The information in this report, i.e. the extracted information from the 16 scientific opinions, supports EFSA's work in providing an overview on the risk assessment of plants developed through NGTs as requested by the EC.

Glossary

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.
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, for example, 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.
Off target effect	DNA modifications occurring at an unintentional location in the genome.
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
EC	European Commission
EFSA	European Food Safety Authority
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
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 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.

Opinion Number	Identifier given by EFSA	Title of report	Author	New Genomic Technique											Focus of the report
				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	-		This opinion provides an overview on the application of the indicated techniques and the potential consequences concerning detection, traceability, labelling and risk assessment. The opinion provides recommendations related to the GMO/non GMO status of plants developed through NGTs.
2	AT_2013	New plant breeding techniques	Bundesministerium für Gesundheit	-	-	-	-	-	yes	yes	yes	-	-		This opinion supplements the NGTs from the previous opinion. It describes applications, detection, traceability, risk assessment, drivers and constraints of the three NGTs. Besides these techniques it shortly notes possible combinations of NGTs. Scientific literature reporting experimental data are reviewed and presented in the Annex.
3	AT_2017	RNAi-based techniques, accelated breeding and CRISPR-Cas: basics and application in plant breeding	Bundesministerium für Gesundheit	yes	yes	yes	-	-	-	-	-	-	-		Besides SDN technology, this opinion also describes accelarated breeding (a strategy that uses a GMO to accelarate individual breeding cycles) and RNAi technology. Potential applications, safety considerations, detection and identification are addressed.
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	-	-	-	-	-	-	-	-	-	-	This opinion presents a reasoning why plants generated by a transient presence of SDN-1 (CRISPR/Cas9) should be considered for exclusion from the Belgian Decree on GMOs.
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	-	-	-	-	-	-	-	-	-	-	This document provides an advice to a field trial under consideration: GM maize containing three knock-out mutations resulting in an impaired DNA-repair mechanism. The mutations were obtained by SDN-1. The advice elaborates on the information needed for the risk assessment.
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		This document is a scientific publication on the convergence and differences between the targeted mutagenesis techniques (as indicated) and conventional mutagenesis.
7	ES_2019	National Biosafety Commission Report on the site directed mutagenesis ("gene editing")	Ministerio Para La Transición Ecológica	yes	yes	yes	-	-	-	-	-	-	-		This opinion reports on risks associated to SDN as gene editing technology and asks for clarification of NGT in light of 2001/18.
8	FR_2017	Scientific opinion on new plant breeding techniques	Haut Conseil des Biotechnologies	yes	yes	yes	yes	yes	yes	yes	-	yes	-		This opinion reports on risks to health and the environment associated with plants and products obtained by NPBTs, management measures, traceability and proposals for intermediate options between the provisions of the EU catalogue and those of Directive 2001/18/EC. It should be noted that the text refers to Appendix 7 (fact sheets on NGTs), which is missing in this report.
9	8_1_DE_2019	Genetic engineering - public hearing	Federal Agency for Nature Conservtion (BfN)	yes	yes	yes	-	-	-	-	-	-	-		This document reports a public hearing on the opportunities of new breeding methods, regulation according to the precautionary principle and promoting organic farming.
10	9_2_DE_2019	Genome editing	Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit	yes	yes	yes	yes	-	-	-	-	-	yes		This opinion focuses on new biomolecular engineering techniques, options for their application, potential risks and options for detection of their application.
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		This opinion describes the specific characteristics of NGTs, detection and identification of the genetic modification, the genome-edited organism and the technique used, and the state of play of the use and development of NGTs.
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	-	-	-	-	-	-		This opinion presents comments on the specified NGTs, important to understand when discussing risks and making decisions.
13	14_NL_2014	CRISPR-Cas advisory report - Revolution from the lab	Commissie Genetische Modificatie (COGEM)	CRISPR	CRISPR	CRISPR	-	-	-	-	-	-	-		This document is an advice to the Dutch Ministry on the technical aspects of CRISPR/Cas9, possibilities for medical and agricultural purposes, the possible risks and the question whether these applications are subject to GMO regulations.
14	15_NL_2017	Advisory opinion on CRISPR-Cas and directed mutagenesis in plants	Commissie Genetische Modificatie (COGEM)	CRISPR	CRISPR	-	yes	-	-	-	-	-	-		This document is an advice to the Dutch Ministry on the exemption from the GMO regulation on the application of CRISPR/Cas9 for directed mutagenesis in plants
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	-		This document is an advice to the Dutch Ministry on revision of the criteria of Annex IB to Directive 2001/18/EC to facilitate exemption of certain GM plants obtained by NGTs and having a corresponding safety profile with traditionally bred plants.
16	17_LT_2019	Analysis of new gene modification techniques or methods	UAB Caszyme, Vilnius	yes	yes	yes	yes	yes	yes	yes	-	yes	yes		This review describes health impacts of NGTs and presents examples of NGTs in Lithuania and Europe. It also provides recommendations for NGT regulation in Lithuania. PM: in this review NGT is referred as NGMM (new gene modification methods).

Appendix B - Extracted information regarding argumentations on the GMO status of plants obtained by NGTs

Opinion 4 argues that genome editing in plants using the CRISPR/Cas9 system in a way as described in this opinion (SDN-1 applied by the transient presence of exogenous DNA used in the intermediate step (T-DNA cassette encoding the components of the CRISPR/Cas9 system)) can be considered as a form of mutagenesis. Intermediate plants containing an exogenous T-DNA cassette encoding the components of the CRISPR/Cas9 system have been developed using a recombinant nucleic acid technique. They should therefore be considered as GMOs. When the T-DNA cassette encoding the components of the CRISPR/Cas9 system has been effectively segregated away, the resulting plants should not be considered as GMOs in the meaning of the GMO regulatory framework.

In opinion 6 it is argued that SDN-1, SDN-2, ODM and base editing can be grouped as techniques for 'precision breeding'. These targeted mutagenesis techniques provide more precision and fewer off-target mutations than the conventional mutagenesis techniques that are exempted from the Directive 2001/18/EC.

Opinion 7 (p. 4) states that prior to gene editing techniques, the introduction of novel traits into organisms was mainly based on conventional mutagenesis or transgenesis. Due to randomness, undesirable alterations in the genome can occur, such as interruption of genes and/or regulatory elements, or the creation of new open reading frames. The use of gene editing techniques offers the possibility of reducing the likelihood of these unwanted adverse effects and together with a rapid subsequent selection the desired genetic alteration is achieved while discarding all the others that may have been generated. Regarding plants, gene editing can give rise to varieties that are not genetically differentiated from those that carry the same modification generated spontaneously and yet, the regulatory requirements for each of these varieties would be different.

It is argued that SDN-1 and SDN-2 applications (using a oligonucleotide of 20 nucleotides in size or less) do not fall within the scope of the Directive, if the nuclease complex is delivered as RNA and / or protein.

Several of the new mutagenesis techniques use an intermediate step, where the plant (including plant cells) will contain recombinant DNA and is a GMO covered by the legislation. By using techniques such as back-crossings and cleavage, it is possible to get rid of the inserted recombined DNA while retaining the created mutation(s). One must be able to show that the resulting plant does not contain any 'residues' of inserted DNA which would enable the plant to be regarded as a GMO covered by legislation. Inserted new DNA does not necessarily pose a health risk, but the plant will necessarily still be a GMO. Criteria to declare that the plants are free from foreign DNA are lacking for offspring from a GM plant (cells) considered to be non-GMO (opinion 11, p. 3)

Opinion 14, p. : In Germany and the United Kingdom, and Finland and Sweden, the evaluating authorities are of the opinion that field trials of crops developed using oligo-directed mutagenesis and CRISPR technology, respectively, do not fall under the GMO regulations, and it can be inferred that the question of whether a recombinant nucleic acid has been used was considered to be of minor importance in these EU Member States. The Swedish Board of Agriculture considers that field trials with CRISPR-Cas9 mutated *Arabidopsis* plants are not subject to authorisation requirements under the GMO regulations because the plants contain no foreign DNA. The German Federal Office of Consumer Protection and Food Safety (BVL) states that directed mutagenesis is similar to classical mutagenesis. This shows that the legal status of directed mutagenesis using gene editing techniques is assessed differently even within the EU.

In opinion 15 (pp. 4-6) it is argued that also final plants obtained by reverse breeding and agro-inoculation should be exempted from the GMO regulation as the genomic DNA is not modified and no inserted DNA sequences are included. In the case of cisgenesis, plants are modified with DNA that is of the same species or of a crossable relative.

The authors of opinion 17 (p. 30) conclude that the regulation of NGTs, such as epigenome editing or methods in which the transgene used is not transferred to the offspring, remains unclear.