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Detection of food and feed plant products obtained by targeted mutagenesis and cisgenesis

European Network of GMO Laboratories (ENGL)

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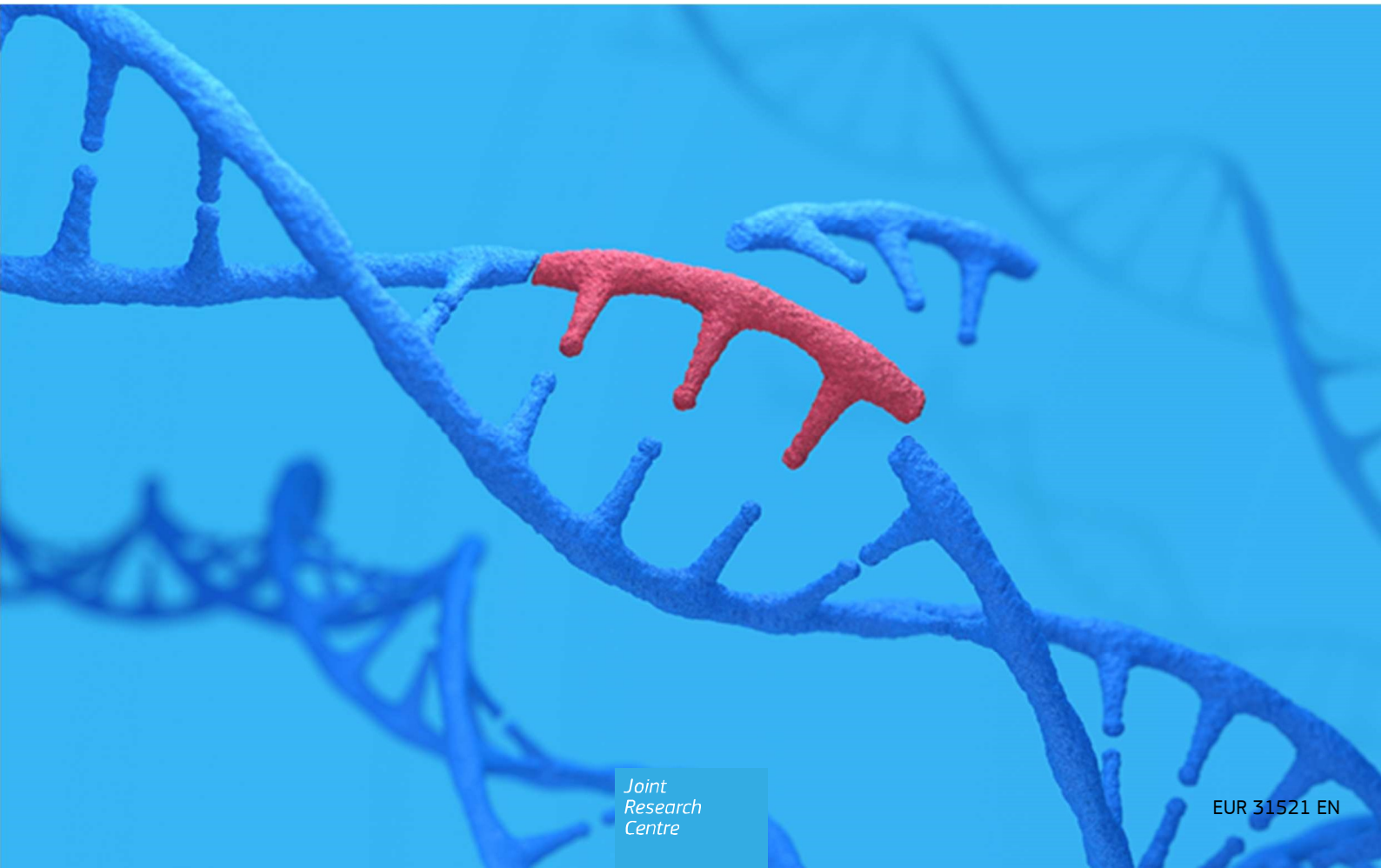

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Contents

Abstract

The current EU legislation on GMOs and GM food and feed requires analytical testing to support traceability of these products on the market. The European Network of GMO Laboratories has reviewed the implications of the analytical requirements when they are applied to plant products developed with the use of new genomic techniques, i.e. targeted mutagenesis and cisgenesis. This review concluded that analytical testing to support traceability is not considered feasible for all products obtained by targeted mutagenesis and cisgenesis, both due to technical restrictions and because of implementation issues.

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The ENGL Working Group (WG) on New Mutagenesis Techniques was established based on a mandate adopted at the 43rd meeting of the ENGL (European Network of GMO Laboratories) Steering Committee on 09 June 2021.

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Executive summary

The European Network of GMO Laboratories (ENGL) has re-examined and updated its report, originally published in 2019, on the challenges for the detection of food and feed plant products obtained by new mutagenesis techniques. In this report, the term "new mutagenesis techniques" is replaced by the more specific term "targeted mutagenesis" and the scope is extended to also cover cisgenesis. The current report therefore replaces the previous version and reaffirms and substantiates the conclusions made earlier. This review has been written in the context of the current GMO legislation and the associated requirements for analytical method validation and analytical enforcement of the legislation for traceability and labelling purposes. The focus of this report is on plants, developed using these new genomic techniques, which do not contain any inserted recombinant DNA, and on their derived products.

The **market authorisation** procedure for conventional GMOs requires the successful validation of one or more event-specific detection methods. When applied to plant products obtained by targeted mutagenesis or cisgenesis, several challenges were identified. Depending on the size of the modification and the nucleotide sequence of the surrounding region, it may not always be feasible to design a robust and quantitative method targeting the mutation GM event. The major challenge, however, is to demonstrate the event-specificity of the method, which is part of the minimum performance requirements for analytical methods of GMO testing. established by the ENGL.. The ENGL recently provided recommendations for applicants for the assessment of the event-specificity of such methods (ENGL, 2023). In case of non-unique sequences, it remains that any conclusion on the event-specificity of a method may not be court-proof. The reasons for this are that

i.) plant genomes are known to be highly variable and prone to mutations of the same type as those that may be induced by targeted mutagenesis or cisgenesis;

ii.) the available knowledge about the occurrence of such sequence variations in large sequence databases such as GenBank (www.ncbi.nlm.nih.gov/genbank/) is inherently incomplete as not all individuals of a plant species have been sequenced or new mutations have not been identified;

iii.) products derived from targeted mutagenesis or cisgenesis could in principle originate from conventional breeding, which, almost by default, makes it challenging to distinguish them from existing variants. It is therefore only possible to infer the absence of a similar mutation in other plants in the breeders' gene pool within the constraints of the knowledge available at that time. This creates an uncertainty that may lead to jurisdictional disputes.

Plant products obtained by targeted mutagenesis or cisgenesis for which no detailed information on the sequence alterations is available will be difficult to detect by analytical techniques. The analytical support provided by the enforcement laboratories to the competent authorities responsible for **control of the market** will therefore be less effective than for conventional GMOs. The screening methods that are routinely used to detect known common genetic elements present in most conventional GMOs cannot be applied or developed for such plant products. These products are also expected to contain multiple modifications that may segregate in subsequent generations and each of these will require a separate detection method to be validated and applied for enforcement purposes. Alternative technologies, such as next generation sequencing (NGS), are able to detect multiple targets, but the results are generally not quantitative and analysis of compound food or feed by NGS is challenging. The analytical enforcement system will be under resource and time pressure when many more analyses will need to be performed for the increasing number of such plant products.

Due to the inherent variability of plant genomes, it may be difficult to identify genomic mutations resulting from targeted mutagenesis, even if more costly and laborious sequencing-based methods would be integrated in the workflow of the control laboratories. A short mutation or larger deletion, substitution or insertion may be detected through sequence analysis, but this does not necessarily confirm that it was generated by targeted mutagenesis or cisgenesis.

In conclusion, limitations have been identified for the development and validation of robust, event-specific detection methods for different types of genomic modifications in plants resulting from targeted mutagenesis or cisgenesis. It is stressed that products that have identical DNA sequences but have been developed either naturally or by conventional breeding or by using new genomic techniques cannot be distinguished by analytical methods. For an effective market control of such products, and especially for unknown products entering the European Union (EU), analytical detection will need to be complemented by other enforcement measures. It is furthermore predicted that the current analytical enforcement system will suffer from an increased workload if food, feed and seed samples have to be analysed with individual methods for all known mutation events.

Scope

In 2019, the ENGL reviewed the possibilities and challenges for the detection of food and feed plant products obtained by new mutagenesis techniques (ENGL, 2019). The review was based on theoretical considerations as experimental evidence was not available at the time. Since then, the scientific literature has evolved and experimental evidence has been collected, which need to be reviewed in light of the conclusions made in the 2019 report.

The main task of the working group (WG) on new mutagenesis techniques was to review existing literature and recent experimental evidence on detection of plant products obtained by targeted mutagenesis or cisgenesis and to verify if the conclusions of the 2019 ENGL report are confirmed.

The regulatory framework to consider included Regulations (EC) No 1829/2003, Regulation (EU) 2017/625 and Directive 2001/18/EC. However, the WG also considered the implications of regulatory requirements in other parts of the world where such products may not be regulated nor labelled. The analytical questions concerned the screening, detection, identification and quantification of such plant products and derived food and feed. Specifically, the recommendations in the revised ENGL guidelines on minimum performance requirements for analytical methods of GMO testing were considered as well as the recent scientific literature on the topic. Also, the experimental evidence collected in recent years on detection methodologies for products containing genetic modifications was reviewed in light of their implementation in GMO analysis. Reflection was given to the differences between detection and identification in light of the occurrence of similar mutations obtained by targeted mutagenesis, conventional mutagenesis techniques used in plant breeding and spontaneous mutations that occur in nature.

1 Terminology used in this document

The term **conventional GMOs** will be used throughout this report to refer to GM plants obtained by recombinant DNA technology, i.e. using established genomic techniques, which are characterised by the presence of randomly introduced DNA sequences from sexually non-crossable species, i.e. foreign DNA.

Established Genomic Techniques (EGTs) - genomic techniques developed prior to 2001, when the existing GMO legislation was adopted (EC, 2021).

New Genomic Techniques (NGTs) - an umbrella term used to describe a variety of techniques that can alter the genetic material of an organism and that have emerged or have been developed since 2001, when the existing GMO legislation was adopted (EC, 2021).

Targeted mutagenesis - an umbrella term used to describe newer techniques of mutagenesis that induce mutation(s) in selected target locations of the genome without insertion of genetic material. The process usually results in a 'knock-out', i.e. the disruption of the functioning of a gene responsible for an unwanted effect, or in modifications of the expressed protein or of regulatory elements of a gene (EC, 2021). The outcome includes nucleotide substitutions, deletions and insertions of one to several nucleotides. Long deletions can be obtained by targeting two sequences in close vicinity, e.g. at two sites within a single gene. This is mostly achieved with the aid of the cell's natural DNA recombination/repair system activated following a double-strand DNA break created at a defined location by a site-directed nuclease (SDN), and, where applicable, with a repair template sequence consisting of an added nucleic acid molecule (Broothaerts et al., 2021). These techniques require the presence of the SDN in the recipient host cell, either following stable integration of recombinant DNA into the plant genome, by transient expression or through delivery of the proteins and/or RNA into the cell (Broothaerts et al., 2021).

This document refers only to plants, but also other organisms can be modified by targeted mutagenesis. When recombinant DNA has been integrated, it can be segregated out in subsequent generations, resulting in mutated plants that no longer contain any recombinant DNA (Gong et al., 2021; Liang et al., 2017). In the frame of this report, plants obtained with targeted mutagenesis techniques that contain inserted recombinant DNA or remaining inserts of the transformation vectors are disregarded, as these will be analogous to conventional GMOs.

Early but limited success of targeted mutagenesis was first achieved with protein-directed SDNs such as meganucleases, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The techniques of genome editing have advanced rapidly following the development of RNA-directed SDNs based on the bacterial CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system and CRISPR-associated (Cas) nucleases (SAM, 2017; Broothaerts et al., 2021). Editing of single nucleotides or short sequences can also be achieved using a specific set of techniques referred to as 'base editing' or prime editing, which modify DNA at specific sites without involving DNA double-strand breaks or using DNA templates (Abdullah et al., 2020; Molla et al., 2021).

'Short' DNA alterations, as mentioned in this report, are referring to changes in one or a few base pairs, while **'long' DNA alterations** refer to alterations of several dozen base pairs. However, there is ambiguity for alterations that lie between the concepts of 'short' and 'long' sequence alterations. When talking about the specificity of detection, the criterion to be assessed is not the sequence length itself, but whether or not a given DNA sequence is unique or occurs already in the plant species, or potentially could occur, and whether or not it can be unequivocally attributed to the application of targeted mutagenesis or cisgenesis.

Cisgenesis - a genetic modification involving genetic material obtained from the breeders' gene pool and transferred to the host using various delivery strategies; the incorporated sequences contain an exact copy of a sequence already present in the breeders' gene pool (e.g. promoter, coding sequence or regulatory sequence) (EFSA GMO Panel, 2022).

Intragenesis - a genetic modification involving genetic material obtained from the breeders' gene pool and transferred to the host using various delivery strategies; the incorporated sequences contain a re-arranged copy of sequences already present in the breeders' gene pool (EFSA GMO Panel, 2022).

In previous risk assessment reports (e.g. EFSA GMO Panel, 2011), cisgenesis referred to the (random) insertion of complete genes from a sexually compatible organism. Such cisgenes included all introns and were flanked by the native promoters and terminators in the normal sense orientation. Intragenesis referred to the insertion of a re-arranged gene composed of sequences from different genes derived from a sexually compatible species. Using **established genomic techniques** these insertions were introduced randomly in the genome. With the

development of NGTs that allow site-directed insertions of any sequence, the definition of cisgenesis and intragenesis was updated to include also the insertion and the substitution of partial gene sequences, consisting e.g. of only regulatory sequences or part of a coding sequence. In this document and unless clearly specified, the term cisgenesis is used as a more general term (that may also cover intragenesis).

Transformation (or GM) event refers to the altered sequence in the genome characterised by the insertion of a transgene. Such events may be the result of the use of established genomic techniques (leading to random insertion of the transgene) or targeted mutagenesis (leading to site-specific insertion of the transgene).

By analogy to the term 'transformation event' used in the GMO legislation (e.g. Regulation (EC) No 1829/2003), here, the term '**genome-edited event**' is proposed for the purpose of this report, which refers to the altered DNA sequence at a specific site in the genome as a result of targeted mutagenesis or cisgenesis (although the term 'genome editing' has a broader sense in ISO 5058-1, 2021). A prerequisite is that no recombinant DNA (e.g. from the transformation vector or other unintended integrations) is present in the genome of the modified plant. Furthermore, as genome editing may create several intended DNA alterations in the genome simultaneously, each of these events, when segregating independently, would require a specific detection and identification method (see Chapter 3).

The term '**detection**' means the 'finding' of a specific target sequence, i.e. detection *sensu stricto*, without necessarily being specific for the genome-edited event.

The term **'identification'** refers to the possibility to unequivocally allocate the detected sequence to a specific GM event; this is equally applicable to conventional GMOs and to plants obtained by targeted mutagenesis or cisgenesis.

The term **'quantification'** means the process of generating measured quantity values for a specific GM event.

GMO screening means using methods that target genetic elements common to various GMOs, with the aim to detect the presence of many GMOs in a sample with a minimal set of PCR assays.

2 Introduction

In the European Union (EU) the authorisation system for the introduction of Genetically Modified Organisms (GMOs) in the agro-food chain is governed by stringent legislation to ensure:

- the safety of food and feed for health and the environment;
- consumers' choice between GM, organic and conventionally-produced food;
- the functioning of the internal market, i.e. once authorised, GM products can be placed on the market anywhere in the EU[1].

The EU policy on GMOs is comprehensive as it addresses the development of GMOs, the stepwise release into the environment, the general cultivation and seed production, authorisation for food/feed use, marketing, labelling and enforcement.

The EU Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF), hosted by the Joint Research Centre (JRC) of the European Commission (EC), is legally mandated to assess and validate the GMO detection methods submitted by the applicants (GMO producers) as part of the authorisation dossier of GMOs[2]. For this task, the EURL GMFF is assisted by a network of National Reference Laboratories (NRLs) and official laboratories, known as the European Network of GMO Laboratories (ENGL). The ENGL has issued a guidance document explaining the minimum performance requirements for analytical methods of GMO testing (MPR) (ENGL, 2015) and an amendment including recommendations for methods targeting organisms obtained by new genomic techniques (ENGL, 2023). Since the labelling and traceability provisions in the legislation[2,3,4] are based on the GMO presence in the food or feed product, one of the requirements refers to the accurate quantification of the 'GMO fraction' in such products. GMOs or GM food and feed products that do not meet the requirements of the legislation should not be placed on the market (see Text box 1).

To ensure compliance with the food and feed legislation on GMOs, official enforcement controls the implementation of the labelling requirements and prevent infringement of the legislation due to the presence of unauthorised GMOs on the market. Under the 'Official Control Regulation'[5], Member States have appointed official laboratories to perform analyses of food, feed and seed products in their national markets. This is performed by applying – where available – first-line screening methods to detect commonly used genetic elements in known and unknown GMOs and, thereafter, the identification and quantification methods validated for authorised GMOs.

During the past years, several new plant breeding techniques, more recently called New Genomic Techniques (NGTs), have been employed to create diversity for exploitation in plant breeding (reviewed in SAM, 2017; Broothaerts et al., 2021). These include targeted mutagenesis techniques which are more generally known as "genome editing". Instead of the random mutation of many DNA sequences at the same time (as in conventional mutation breeding techniques) or the random insertion of new genes (as in conventional GMOs), targeted mutagenesis allows the site-specific alteration, i.e. mutation, of the DNA sequence.

[1] In line with Directive (EU) 2015/412 (*Off. J. Eur. Union* L68:1-8) Member States may, however, restrict or prohibit the cultivation of an authorised GMO on all or part of their territory.

[2] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Off. J. Eur. Union* L268:1-23.

[3] Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. *Off. J. Eur. Union* L268:24-28.

[4] Commission Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired. *Off. J. Eur. Union* L166: 9-15.

[5] Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products (Official Controls Regulation). *Off. J. Eur. Union* L95:1-142.

Box 1. Different authorisation statuses of GMOs under Directive 2001/18/EC[6] and Regulation (EC) No 1829/2003[2]

Authorised for placing on the market

GMOs and GM food and feed can be placed on the market only after an authorisation is granted. Authorisation so far mostly concerns the import of GMOs and products thereof and their use as food and feed. Few authorisations have been submitted for cultivation of GM plants and currently only one GMO event is authorised for cultivation (maize MON810).

Authorised GMOs can be present on the market in food and feed material. Validated identification and quantification methods and reference materials are available for these GMOs. According to Directive 2001/18/EC, Regulation (EC) No 1829/2003 and (EC) No 1830/2003, the presence of such authorised GMOs in food and feed shall be indicated on the label of the product. Labelling requirements do not apply for GMOs intended for food, feed or direct processing when the presence of GMOs is no higher than 0.9% per ingredient and provided that these traces are adventitious or technically unavoidable.

Unauthorised for placing on the market

- GMOs that have been authorised for any other purpose than for placing on the market, under Part B of the Directive 2001/18/EC. The authorisation for these purposes (e.g. experimental uses and field trials) is granted and applied at national level.

- GMOs that have not been authorised for placing on the market, as or in products, under Part C of Directive 2001/18/EC or Regulation (EC) No 1829/2003.

- Pending authorisation: a valid application for authorisation in the EU has been submitted under Directive 2001/18/EC or Regulation (EC) No 1829/2003.

- Authorisation expired: a GMO of which the authorisation has expired and no renewal application has been submitted.

GMOs in these categories are not allowed on the EU market and a zero-tolerance policy applies.

For feed use only, and under the conditions of Commission Regulation (EU) No 619/2011, GMOs in the last two categories shall be considered compliant when their presence is below the Minimum Required Performance Limit (MRPL) of 0.1% related to mass fraction. Results at or above the MRPL shall be notified to the Commission and other Member States through the Rapid Alert System for Food and Feed in accordance with Article 50 of Regulation (EC) No 178/2002. For pending authorisations, the requirements are that the GM material: a) must be authorised for commercialisation in a third country, b) a valid application submitted to the EU has been pending for more than three months, c) no adverse effects have been identified by EFSA when present below the MRPL, d) a validated quantification method and certified reference materials are available. For expired authorisations, certified reference materials have still to be available.

Applying genome editing including targeted mutagenesis to modify plant genomes can result in single nucleotide variants (SNVs), sequence substitutions, and insertions or deletions (InDels) of various sizes (Figure 1). These DNA alterations may be present either in a homozygous or heterozygous state in the genome or as bi-allelic alterations, i.e. two alleles of a gene of interest in a diploid plant may contain a different mutation (Clasen et al., 2016; Biswas et al., 2022). Likewise, several genes of a gene family or all those involved in a metabolic pathway may be altered simultaneously (Haun et al., 2014; Demorest et al., 2016; Najera et al., 2019). New Genomic Techniques also allow introduction of new sequences site-specifically, which can be copies of endogenous sequences (cisgenesis) or sequences from other species (transgenesis[7]). In cisgenic plants sequences derived from a sexually compatible plant have been inserted to complement or substitute existing sequences (Figure 1).

[6] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. *Off. J. Eur. Comm.* L 106:1-38.

[7] Targeted transgenesis is beyond the scope of this report, as there are no new analytical issues compared to the random transgene insertions in conventional GMOs.

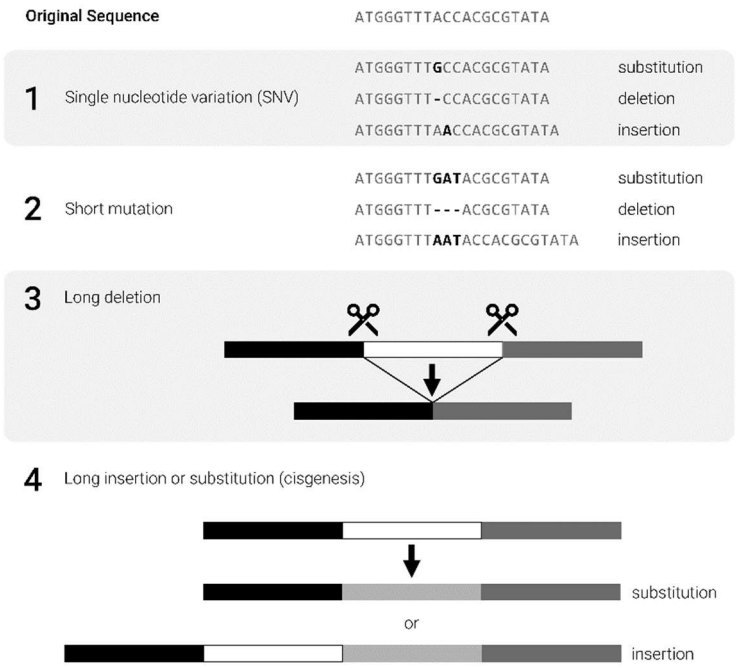

Figure 1. Different outcomes of targeted mutagenesis (1-3) and cisgenesis (4).

1 and 2: a single nucleotide or short sequence (in this example, 3 nucleotides) is replaced, deleted or inserted; 3: targeted mutagenesis applied to two genomic sites in close vicinity may result in the deletion of the intervening sequence; 4: a cisgenic sequence from the breeders' gene pool is inserted or substitutes the existing sequence.

In 2011, upon request of the Directorate-General for Health and Food Safety (DG SANTE), the JRC reviewed the state-of-the-art of some of the emerging new plant breeding techniques. This report defined their level of development and adoption by the breeding sector and the prospects for a future commercialisation of plants created by these techniques. Additionally, with support of several ENGL experts, the challenges for the detection of organisms developed through these techniques were evaluated (Lusser et al., 2011, 2012). The topic has since been discussed further during meetings of the ENGL and in dedicated working groups. In the past years, additional innovative techniques for genome editing, with wider potential and easier applicability, have rapidly advanced plant biology research and the development of applications for plant breeding (SAM, 2017; Khatodia et al., 2016; Basu et al., 2023; Wang and Doudna, 2023).

On 25th July 2018, the European Court of Justice (Case C-528/16) ruled that only organisms obtained by means of techniques/methods of mutagenesis that have conventionally been used in a number of applications and have a long safety record are excluded from the scope of Directive 2001/18/EC[8]. As a consequence of this ruling, organisms obtained by targeted mutagenesis techniques, i.e. genome editing (including cisgenesis), in contrast to conventional mutagenesis techniques, are considered not exempted from the GMO legislation. In October 2018, the JRC received a mandate from DG SANTE to elaborate, together with the ENGL, on the implications of this ruling for the detection of such organisms. The first version of this report was published in 2019 (ENGL, 2019). In 2021, the EC, on request of the Council of the European Union (the Council), published a study on the status of NGTs under EU law, which concluded i.a. that "under the current EU regulatory system, there are implementation and enforcement challenges, in particular related to the detection and differentiation of NGT products that do not contain any foreign genetic material" (EC, 2021). The Council also invited the EC to come forward with a proposal or otherwise provide information on other measures required as a follow-up to the study. The EC therefore initiated a policy action on plants produced by targeted mutagenesis and cisgenesis, involving an impact assessment. On this basis, the EC aims to present a new legislative proposal in 2023.

[8] European Court of Justice, C-528/16 - Judgement of 25 July 2018. See: http://curia.europa.eu/juris/document/document.jsf?docid=204387&mode=req&pageIndex=1&dir=&occ=first&part=1&text=&doclang=EN&cid=515140.

Against this background, the ENGL Working Group "New Mutagenesis Techniques" was established to review and to update the previous report on the detection of food and feed products obtained by new mutagenesis techniques (ENGL, 2019) to inform the future EU policy direction on targeted mutagenesis and cisgenesis. It must be clarified that the term new mutagenesis techniques and targeted mutagenesis techniques are synonymous.

This revised report additionally takes into account experimental evidence and scientific literature, published since 2019, regarding analytical approaches for the screening, detection, identification and quantification of such plant products and food and feed derived thereof. The considerations discussed relate (1) to the compliance with the GM food and feed legislation, including the requirements for method validation and provision of a suitable certified reference material (CRM) as part of the GMO authorisation procedure[2], and (2) to the provisions of the Official Controls Regulation[5] on the routine testing of food and feed by the enforcement laboratories. Along with the current EU regulatory framework, the Working Group took into consideration implications of regulatory requirements in other parts of the world, i.e. some of these products may not be regulated elsewhere and enter the EU market unnoticed. Additionally, the recommendations of the revised ENGL guidance on minimum performance requirements for analytical methods of GMO testing (ENGL, 2023) have been discussed. Finally, the report reflects upon differences between GMO detection and identification in light of the occurrence of similar mutations obtained by the various techniques used in conventional breeding, including random mutagenesis.

This document was endorsed and released for publication by the Steering Committee of the ENGL in spring 2023. It is published on the full understanding and acknowledgement that scientific knowledge and technological developments in this field are rapidly evolving. As with any scientific/technical state-of-the-art review, the content of this report is correct at the point of publication.

3 Development and validation of analytical methods for EU authorisation

Up to now event-specific methods for 89 GM events have been validated as part of the authorisation process for food and feed uses[2] (https://gmo-crl.jrc.ec.europa.eu/gmomethods/). In most of these cases, the GMOs contained one or more inserted foreign DNA sequences of up to several thousand nucleotides in length. The genetic transformation procedures employed for their generation have resulted in an 'event' of insertion of recombinant DNA sequences. Each insertion is characterised by two unique, novel DNA junction sequences, one at each end of the construct integration site linked with the plant genome. Each of the unique junctions created during a transformation event can be exploited as a unique identification marker for developing a detection method that is specific for each conventional GMO (often referred to as 'event-specific' detection method).

For feed and food marketing authorisation under the GMO regulations, the analytical method must be able to identify and quantify the presence of the GMO event at the legal thresholds (0.9 % in Regulation (EC) No 1829/2003 and 0.1 % in Regulation (EU) No 619/2011). Methods for the detection of unauthorised GMOs, however, do not need, in principle, to be quantitative or event-specific as in some cases detection of DNA sequences not present in authorised GMOs is sufficient to confirm the non-compliance of the product.

In contrast, targeted mutagenesis techniques allow different types of DNA sequence alterations such as: SNVs, substitutions, insertions and deletions each of various sequence sizes. Site-specific insertion of cisgenic or foreign genes can also be achieved. The development of identification methods for longer and unique DNA alterations is not fundamentally different from that for conventional GMOs, e.g. the 4 or 6 kb deletion in waxy corn, obtained by CRISPR-Cas, was targeted with PCR primers binding outside the deleted sequence (Gao et al., 2020) and the TALEN-induced indels of 20 bp or more nucleotides in Calyxt soybean were similarly identified by PCR (Haun et al., 2014).

Analytical challenges arise when developing detection and identification methods for SNVs or short InDels, and when applying such methods to compound food or feed products. These challenges are situated at different levels:

i.) the development;

ii.) the validation;

iii.) the implementation of the methods (see also Chapter 4).

A method targeting a sequence alteration requires prior knowledge on the modified sequence or at least the genomic region containing the modification(s). For this purpose, databases containing information on NGT products have been developed (see Text box 2). It would be beneficial to cross-reference the information coming from different initiatives and to define tools that help to develop detection strategies.

Another challenge concerns data confidentiality. Currently, PCR-based GMO detection methods are assessed by the EURL GMFF in a secure internal infrastructure, where several countermeasures are in place to ensure data security. These confidential sequences are then *in silico* compared to relevant DNA sequences from public databases (e.g. from NCBI), which are locally stored on servers due to confidentiality issues. *In silico* similarity evaluations would be much facilitated by development of publicly accessible sequence resources related to products from targeted mutagenesis and cisgenesis authorised under other jurisdictions. This may also support the GMO testing community in developing analytical tools for traceability of unauthorised GMOs entering the EU.

Box 2. Databases with information on organisms modified by NGTs

Several initiatives are being taken to gather information on the organisms modified by NGTs. The collected information may concern plants, fungi, animals or microorganisms. Some scientific papers have published partial tables on such applications (e.g. Manchanda and Suneja, 2018; Zheng et al., 2019). Having a regularly updated list of the edited organisms and of the genes modified in these organisms would provide an overview of the kind of organisms and their derived products that may enter the food, feed and seed markets. Such a database would also provide a knowledge base for developing specific detection strategies.

An interesting collection of data was composed by Parisi and Rodriguez-Cerezo (2021). Their report is very informative concerning the trends of the current and future market applications of new genomic techniques, but it does not provide details on the applications. This database has collected information on 427 applications of NGTs in plants, 1 in fungi and 59 in animals, classified in 4 categories of development: commercial stage, pre-commercial stage, advanced R&D stage and early R&D stage. Data reported in this document can be consulted using a web dashboard at: https://datam.jrc.ec.europa.eu/_datam/mashup/NEW_GENOMIC_TECHNIQUES/index.html. The web-interface allows data sorting using different filters (organism, species, country, technique used for modification, development stage, trait category, type of company, etc.). The big disadvantage of this database is that the collected information is considered confidential, hence no further details regarding the modified genes or sequences are accessible and the data is therefore not useful when it comes to developing detection strategies.

Other databases have been developed by different initiatives that are presently financed by Member States and research projects. The EU-SAGE (European Sustainable Agriculture through Genome Editing) database (www.eu-sage.eu/) presently contains more than 600 entries. EU-SAGE is a network representing plant scientists of 134 European plant science institutes and societies from 28 countries that have joined forces to provide information about genome editing. This database allows the filtering of information (traits, techniques, countries and plants) and provides a link to research papers for more information. Another database is being developed in the GenEdit project (research project gathering the partners of the Belgian NRL). This database focusses on patents and research papers and currently counts a similar number of entries as in the EU-SAGE database. Finally, the EUginius initiative, known for its extensive database for conventional GMOs, is also including organisms produced by NGTs. The EUginius database (www.euginius.eu) also indicates if detection methods are available and offers links to scientific papers or DNA/protein sequences.

3.1 Analytical technologies for the detection of DNA targets

Although genetic modifications may affect other classes of molecules such as RNA, proteins and gradually down to metabolites, which can all be targets of analytical methods, the benchmark technology for the analytical detection, identification and quantification of GMOs is typically based on **real-time PCR** (also called quantitative PCR or qPCR), a technology widely used in genetic testing to target DNA molecules. This technology provides a million-fold amplification of a selected target DNA sequence of typically 70- 150 bp, located across one of the insert-to-plant junctions. Real-time PCR can provide high sensitivity and robustness for the precise relative quantification of GMO material, even at low levels, in food and feed products. When real-time PCR is targeting the unique sequences of transformation events (which are very unlikely to be re-formed independently in another plant), it ensures the required level of specificity to comply with the legal requirements.

For the detection of SNVs or short InDels, the real-time PCR method must efficiently favour the amplification of the modified sequence over the original sequence. For such a purpose, several strategies have previously been reported, including the use of probes coupled to minor groove binders (MGBs), probes and primers modified with locked nucleic acids (LNAs) or peptide nucleic acids (PNAs) (Fouz et al., 2020; Zhang et al., 2021) and DNA polymerases that efficiently exclude an extension of primer mismatches. The nucleotide modifications significantly increase the thermodynamic stability of oligonucleotide and complementary DNA duplexes, leading to higher melting temperatures, which is more effective for perfectly matched duplexes than for mismatched duplexes (You et al., 2006). The differences in melting temperatures can be used for mismatch discrimination and thus for specific SNV or InDel detection. During method development, combinations of oligonucleotides, primer modifications and experimental conditions (i.e. annealing temperature, oligonucleotide concentration) should be identified that efficiently exploit the difference in melting temperatures between matched and mismatched annealing needed for SNV or InDel genotyping. Non-specific amplification should not occur or has to be discriminated by additional specificity effects contributed by, e.g. a hydrolysis probe containing modifications (Domingues and Kolodney, 2005; Fouz et al., 2020). RNase H-dependent real-time PCR (rhPCR) could also be an alternative approach for the development of qualitative detection methods (Dobosy et al., 2011; Ribarits et al., 2021b). It has been shown for SNV allelic discrimination assays developed in other domains than GMO testing (de Andrade et al., 2013; Feligini et al., 2014) that quantitative parameters such as PCR efficiency, slope and linearity were in line with those established by the ENGL (ENGL, 2015). Competitive allele-specific and RNase H2-dependent PCR-assays used for genotyping in plant breeding programs showed higher sensitivity and specificity in comparison to TaqMan assays (Broccanello et al., 2018). However, the materials tested in these studies were of a lower complexity and consisted of individual genotypes and plants, so their application to compound food or feed materials may be more challenging.

Although real-time PCR is widely mastered by enforcement laboratories, digital PCR (dPCR) methods are increasingly used for GMO detection and quantification (Cottenet et al., 2019; Dobnik et al., 2015; Pecoraro et

al., 2019; Košir et al., 2019). The major advantage of this technology is the absolute quantification without dependence on calibration curves derived from certified reference materials as well as its lower susceptibility to PCR inhibitors. However, CRMs are needed to determine a conversion factor that has to be used when translating GM copy number ratios measured by dPCR into a corresponding GM mass fraction (Corbisier and Emons, 2019; Corbisier et al., 2022). Previously, dPCR has been used for the screening and confirmation of particular mutations in clinical samples, namely induced pluripotent stem cells or primary cells at very low copy number (Miyaoka et al., 2016; Mock et al., 2016). Some dPCR assays (Mock et al., 2016) are particularly well designed to target SNVs as they have two probes, binding either to the mutated or the wild-type sequence, for the simultaneous identification and quantification of both wild-type and mutated sequence copies from the same PCR amplicon. This substitutes the use of taxon-specific genes for relative quantification of the GM events, as currently proposed in the new ENGL document on MPR (ENGL, 2023). However, it should be noted that the samples analysed in these studies were of limited complexity, not comparable to samples of food and feed products from plants (Fraiture et al., 2022). Digital PCR, partly in combination with an LNA-modified probe, has been used to detect and quantify genome-edited rice containing a deletion or insertion of a few nucleotides (Zhang et al., 2021). Although method robustness and specificity need further assessment, the assay format has the potential to overcome limitations associated with the detection of short InDels and SNVs. Other authors have compared the relative specificity and sensitivity of qPCR versus dPCR assays in detecting and quantifying SNVs or small InDels in transgenic mice generated by CRISPR/Cas9 mutagenesis: a lower rate of false-positive results was observed when using a dPCR assay, and locked nucleic acid probes could improve the specificity of the assay (Falabella et al., 2017). Recently, a general workflow for the development and validation of a PCR-based method was successfully used to develop a 2-plex droplet dPCR method specifically targeting a genome-edited rice carrying a single nucleotide insertion (Fraiture et al., 2022). The performance of this dPCR method was positively assessed for its specificity and sensitivity, in agreement with the ENGL minimum performance requirements for GMO testing. In addition, the method was able to deal with low admixtures of the genome-edited rice in the parental rice line as well as in mixtures with other plant species (maize or soybean). Furthermore, no impact of food processing was observed (Fraiture et al., 2022).

Theoretically, **sequencing-based strategies**, such as short- and long-read high-throughput sequencing, could potentially be applied for the detection and characterisation of (multiple) genome-edited events. Previously, targeted sequencing (Liang et al., 2014), based on PCR enrichment or probe capturing, whole-genome sequencing and, more recently, shotgun metagenomic sequencing (Buytaers et al., 2021) have been successfully investigated for detecting conventional GMOs (Kovalic et al., 2012; Barbau-Piednoir et al., 2015; Liang et al., 2017; Fraiture et al., 2017, 2018; Arulandhu et al., 2018; Debode et al., 2019; Berbers et al., 2020). However, for the detection of organisms carrying short DNA modifications introduced by targeted mutagenesis techniques, only a targeted high-throughput sequencing approach has recently been explored for compatibility with the ENGL minimum performance requirements for analytical methods of GMO testing (Fraiture et al., 2023). This approach, based on a prior PCR enrichment, was able to detect a single nucleotide insertion harboured by a gene-edited rice, even at low percentages. Moreover, no impact related to food processing nor to the presence of other crop species was observed (Fraiture et al., 2023).

The quality criteria to assess sequencing data are currently under discussion, for instance at ISO level (ISO 20397-2, 2021). This should also contribute to establishing a framework for the validation of high-throughput sequencing-based methods in the future. It should be noted that such promising high-throughput approaches are not yet validated for the quantification of DNA targets in complex mixtures.

The CRISPR/Cas technique can also be used to detect SNVs. Different CRISPR/Cas diagnostic approaches for the detection of specific nucleic acid sequences have been described as DETECTR (Broughton et al., 2020), SHERLOCK (Gootenberg et al., 2017) or HOLMES (Kellner et al., 2019; Li et al., 2018). These methods exploit the nucleic acid recognition function of Cas enzymes and their nucleolytic activity that is activated upon binding, which for some Cas enzymes includes the collateral cleavage of bystander nucleic acid probes, resulting in a detectable signal. Also multiplex methods have been developed, e.g. for SARS-CoV-2 variant detection (Patchsung et al., 2022). The methods appear to be promising for the specific detection of mutations, but none of them has yet been tested for their suitability based on the minimum performance requirements for analytical methods of GMO testing (see Section 3.3).

Although it is technically possible to detect specific DNA alterations, none of the techniques described are able to distinguish whether a SNV or short InDel is the result of genome editing, conventional breeding technologies or natural mutation (see Section 3.4 on event specificity).

3.2 Challenges for development of analytical methods for short mutations

In case of SNVs and short InDels introduced by targeted mutagenesis techniques, the difference between the altered DNA sequence and the non-modified original sequence is small. Besides establishing the required specificity of such methods, further discussed in Sections 3.3 and 3.4, several challenges exist for the development of analytical methods that allow detection, identification and/or quantification of genome-edited plants containing short mutations. Despite individual successes, as described in the previous chapter, the following challenges limit the general applicability of analytical methods targeting short mutations for enforcement purposes.

Firstly, for PCR-based measurement systems, there would be a marginal thermodynamic differentiation between oligonucleotide binding to the modified and the non-modified DNA sequences and hence the melting temperature would be similar. E.g. a single mismatch in a 25 bp long probe would create a difference in melting temperatures in the range of 0.5–3 °C (You et al., 2006). Since the melting temperature defines the temperature at which only half of the complementary bases are bound to each other, a small fraction of the non-matching primer or probe population may still anneal at the higher temperature selected for the matching oligonucleotides. This may lead to the co-amplification of highly similar but not identical sequences. The exact relationship between oligonucleotide binding and temperature is dependent, among other factors, on buffer composition, the exact oligonucleotide sequence, primer secondary structures and complementary binding effects. Moreover, the thermodynamic balance between perfect match and mismatch hybridization has to be sufficiently robust, also in a background of a large excess of unmutated DNA as well as in a complex matrix, in mixtures and in processed samples.

An example of this thermodynamic challenge was observed for the real-time PCR method developed for the detection and identification of a SNV (G to T mutation) in the AHA1SC gene which confers herbicide tolerance in Cibus rapeseed event 5715 (Chhalliyil et al., 2020). The method uses LNA-modified primers to increase its specificity for the SNV. However, experimental evidence demonstrated that the method lacked robustness and specificity for the detection of the SNV (Weidner et al., 2022). The method was therefore considered not fit-for-purpose for official control of oilseed rape products in the EU.

Secondly, the mutation size highly restricts the flexibility in designing a suitable PCR method for the mutant plant. E.g. the mutation may occur in repetitive DNA or in high GC- or AT-rich sequences, which are known as difficult targets for PCR-based methods or the surrounding DNA may have such characteristics (Hommelsheim et al., 2014). The design of a PCR-based method whose target sequence differs from the reference sequence by a few or one nucleotide may therefore not in all cases be sufficient to deliver the necessary sequence specificity (Shillito et al., 2021). Because further crossing of the mutation event to local varieties with suitable growth characteristics for a given climatic area is common practice, the detection method needs to target the mutation site and no other regions in the genome that could segregate. For some mutated sequences, this lack of flexibility in the choice of target region may make it difficult or even impossible to develop a robust and event-specific PCR method.

Thirdly, alternative methods based on next generation sequencing (NGS) may detect short mutations, particularly when using enrichment strategies, but their discriminative power when applied to mixtures or compound products is currently unknown and expected to be challenging. Also accurate quantification using NGS has not been convincingly shown yet. Implementation of NGS and bioinformatics analysis platforms require heavy investments in expensive machines and building up highly skilled expertise in data evaluation. Also because of their more time-intensive workflows, such technologies could be useful in certain specific cases, but not for routine control purposes in all enforcement laboratories.

3.3 Validation requirements for analytical methods

In the EU, the GMO producer applying for market authorisation (the 'applicant') of a GMO has to submit a complete dossier for risk assessment. This application shall also include a detection, identification and quantification method, with supporting method performance data, and control material should be provided to test the method. Applicants also need to ensure that CRMs are available to testing laboratories during the authorisation period. Applicants should follow the guidelines publicly available to prepare the 'method validation dossier' (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). In the EU authorisation and control context, it is required that analytical methods are specific to unambiguously identify the GMO event, that they provide a dynamic range around the labelling threshold (i.e. 0.9 %), and that they reach the desired level of sensitivity, robustness, ease of use and accuracy of quantification.

The EURL GMFF validates the analytical methods for GMO testing provided by applicants for market authorisation of GMO events in an interlaboratory validation exercise involving NRLs[9]. The ENGL guidance on minimum performance requirements for analytical methods of GMO testing (ENGL, 2015, 2023) provides the reference basis for the assessment of the validation study. The validated quantitative method and CRMs for calibration and quality control of the method constitute a complete 'toolkit' for the unequivocal identification and quantification of a GMO (Trapmann et al., 2010; Corbisier and Emons, 2019; Corbisier et al., 2022).

The PCR assays and technologies mentioned above for the detection of plant products obtained by targeted mutagenesis would require a significant level of method optimisation and testing under different conditions. Moreover, such approaches need to be validated in interlaboratory studies to ensure transferability of the methods across laboratories, which has not been shown up to now.

In the frame of updating this report, the scientific literature from different fields has been reviewed to evaluate if the current ENGL minimum performance requirements for analytical methods of GMO testing could be applied to methods for the detection and quantification of genome-edited products.

In 2015, ENGL elaborated the third version of the guidance document on **minimum performance requirements for analytical methods of GMO testing** (ENGL, 2015). The document is addressed to applicants submitting GMO detection methods according to Regulation (EC) No 1829/2003 and it provides criteria upon which methods for GMO detection are assessed and validated by the EURL GMFF. The document should also be considered by NRLs and official control laboratories during development of detection methods (e.g. screening or multiplex methods). The ENGL document takes into account the requirements of relevant international standards (ISO 24276, ISO 21569, ISO 21570, ISO 21571) and recommendations of the Codex Alimentarius (Codex, 2009).

The MPR document refers to real-time PCR-based methods for the detection, identification and/or quantification of GMOs, and methods for DNA extraction. It details the acceptance criteria and performance requirements for 1) DNA extraction and purification methods, 2) PCR methods for the purpose of quantification and, 3) PCR methods for the purpose of qualitative detection (Table 1). However, it also recognises that, if other technologies are developed that fulfil legal requirements, the document will need to be amended accordingly.

Table 1. Method acceptance criteria and performance requirements that need to be evaluated for official analytical methods of GMO testing (ENGL, 2015).

Criteria	DNA extraction	Quantitative PCR	Qualitative PCR
Method acceptance criteria	Applicability Practicability DNA concentration DNA yield DNA structural integrity Purity of DNA extracts	Applicability Practicability Specificity Limit of Detection (LOD) Robustness Dynamic Range Trueness Amplification Efficiency R^2 Coefficient Precision Limit of Quantification (LOQ)	Applicability Practicability Specificity Limit of Detection (LOD) Robustness
Method performance requirements		Trueness Precision	False positive rate False negative rate Probability of detection

As a direct implementation of that consideration and in consequence of the rapidly evolving targeted mutagenesis techniques and the increased knowledge on DNA-based analysis, the ENGL has recently elaborated a further part of the guidance document on minimum performance requirements for analytical methods of GMO testing (ENGL, 2023). This latter document specifies the requirements for digital PCR methods and provides additional clarifications for PCR-based methods for food and feed products obtained by NGTs. The recommendations formulated for methods for analysis of NGT products focus on the challenging short-size nucleotide changes (SNVs, InDels) that mirror those found in organisms developed by conventional breeding

[9] Commission Implementing Regulation (EU) No 120/2014 of 7 February 2014 amending Regulation (EC) No 1981/2006 on detailed rules for the implementation of Article 32 of Regulation (EC) No 1829/2003 of the European Parliament and the Council as regards the Community reference laboratory for genetically modified organisms. *Off. J. Eur. Union* L39:46-52.

techniques. It recognises that detection of the DNA alteration(s) *per se* does neither necessarily imply the unambiguous identification of the genome-edited event nor does it necessarily permit differentiation from organisms of the same species exhibiting the same DNA alteration which has been achieved through conventional breeding. The document does not address sequencing, e.g. massive parallel sequencing, where the approaches, the quality assurance parameters and uncertainty estimation still have to prove their applicability for products of targeted mutagenesis and cisgenesis, particularly when admixed in marketed food/feed samples. Up to now, no application for authorisation of plant products obtained by targeted mutagenesis or cisgenesis has been submitted in the EU. In the scientific literature, only a few methods have been proposed for the specific identification and quantification of SNVs obtained by targeted mutagenesis techniques (Fraiture et al., 2022, 2023; Zhang et al., 2021). A similar method proposed by Challiyil et al. (2020) did not pass the experimental assessment based on ENGL criteria (Weidner et al., 2022). It must be taken into account that by targeted mutagenesis or cisgenesis various types of DNA alterations can be generated (see Figure 1). The experimental evidence showing that analytical methods developed for different types of genome-edited plants comply with the legal requirements for methods of GMO testing is currently limited. Inherently, further elaboration becomes necessary following the evolving knowledge in the analytical field. Further details related to the most critical acceptance criteria are discussed below.

3.3.1 Applicability and practicability of the method

Applicability refers to the description of analytes, sample materials (matrices) and concentrations to which the method can be applied. Therefore, the evidence to demonstrate the validity and limitations of the detection method across different food and feed matrices that are expected to be placed on the market must be provided.

Specific genome editing techniques based on SDNs, such as CRISPR/Cas and TALEN, provide the possibility that all alleles of a gene or different genes can be modified simultaneously (Wang et al., 2014, 2015; Miao et al., 2018; Yu et al., 2018; Liang et al., 2017; Peterson et al., 2016). This may lead to plants bearing multiple alterations in their genome at one or more loci, which may be present in a homozygous or heterozygous state (i.e. all copies of the gene may have the same alteration or different alterations). Event-specific detection methods would be required to target each of these different alterations in the genome in case they may segregate in subsequent generations. This will considerably increase the workload for validation of all these methods and their implementation in control laboratories. Moreover it will impact the practicability of a method which is associated with the ease of operations, the feasibility and efficiency of implementation as well as with the costs. *"Most control and analytical laboratories are equipped with real-time PCR instrumentation, but may not have access to dPCR and NGS. To fulfil the requirements for practicability, it is important that methods can easily be implemented for testing purposes"* (Pecoraro et al., 2019).

Considering that targeted mutagenesis techniques are continuously evolving and already now allow multiplex editing of several genomic sites at once, the extent of mutation stacking in an increasing number of crops would put greatly increased pressure on the GMO enforcement system.

3.3.2 *In silico* and experimental specificity

As a basic requirement, the detection method must be specific to the GM event, regardless of the technique used to create the DNA alteration, and must not detect the non-modified DNA sequences nor DNA sequences from plants that have been genetically modified by techniques that are exempted from the GMO legislation such as conventional mutagenesis techniques (see also Section 3.4). The Annex III to Regulation (EU) No 503/2013 stipulates that the applicant shall provide information about the method development and the method optimisation. Importantly, the specificity "*shall be established through the submission of the full sequence of the insert(s) in a standardised electronic format, together with the base pairs of the host flanking sequences so as to enable the EURL to assess the specificity of the proposed method by running homology searches in a molecular database*". The formulation of these requirements clearly reflects the assumption that transformation events are constituted by large insertions (e.g. ranging from a few thousand to several tens of thousands of base pairs). The information relative to the genome sequences surrounding both sides of the insertion into the genome of the modified plant serves the scope of determining the specificity of the assay using *in silico approaches*. An event-specific PCR method for a conventional GMO is designed to let one primer anneal to the host genome, the other primer to the inserted sequence, and the probe ideally to the junction between the endogenous and the inserted sequence. This notion is challenged in the case of targeted mutagenesis and cisgenesis where no foreign DNA is introduced in the modified organism and no insert-to-plant junction can be targeted by an event-specific PCR method. However, the DNA region carrying the altered nucleotide(s) can be targeted by PCR approaches, some of which are described in peer-reviewed publications

(see Section 3.1). Bearing this in mind, the information on the altered sequence plus its position in the reference genome substitute for the information about the DNA insert, whilst the knowledge of the flanking DNA regions is equally decisive for evaluating primer complementarity. As a consequence, the *in silico* specificity assessment based on similarity searches becomes key to demonstrate the theoretical capacity of the method to target the intended sequence and not similar ones. Hence, the adequate choice of the databases to interrogate becomes an essential component for postulating the sequence-specificity of the method. Comprehensive and updated bioinformatics resources covering all genomic variation of all crop species would be required, in order to assess whether the same mutation is also a naturally occurring variation. Moreover, the availability of genome sequences in FASTA format for all relevant plant varieties will be needed to assess the specificity of PCR-based detection methods using *in silico* PCR predictions, whereas raw sequencing data will be useful to verify the sequencing coverage in the genomic position of interest. However, such an approach cannot solve the problem that databases will never be able to capture the complete collection of all sequence variation existing at a given moment nor the variation in the gene pool that may occur in the future.

In recent years, a number of sequencing and resequencing projects have been launched on a wide range of crops, such as rice (The 3,000 rice genomes project, 2014), maize (Chia et al., 2012; Haberer et al., 2020), and tomato (Zhou et al., 2022). The number of DNA sequences produced and stored in public databases is constantly increasing: the whole GenBank division of NCBI has reached 19.1 trillion bases in 2.2 billion sequences from whole genome sequencing projects[10], with a 37% annual increase for plant species (Sayers et al., 2019). New species-specific pan-genomic sequence databases are constantly being developed (Hu et al., 2015; Gui et al., 2020; Peng et al., 2021). For instance, the Rice Variation Map database provides curated information of more than 17 million genomic variations from sequencing data of 4,726 rice accessions, while the SolOmics Tomato database provides more than 19 million sequence variations from 838 tomato genomes. Users can explore and download the genotype information in VCF format, while the raw sequencing data are often available from the NCBI Sequencing Read Archive (SRA). Currently, NCBI hosts more than 2,000 genome assemblies for plants, covering more than 1,000 plant species, but not all genotyped varieties have a whole genome sequence submitted to NCBI. A comprehensive and updated database covering the whole genetic diversity for all the main crop species is unavailable; therefore, *in silico* specificity analyses will have to be conducted with a customized approach, depending on the species of interest. It should be stressed that plant genomes are flexible and changing all the time (natural and induced mutations), therefore, none of the current and future sequence databases are able to fully cover the plant genetic diversity (see Text box 3).

Box 3. Variability of plant genomes

Advances in whole genome sequencing in recent years have revealed that the genome sequences of plant species are diverse and dynamic. Dispensable genes may constitute a significant proportion of the pan-genome, e.g. around 20 % in soybean (Li et al., 2014). A comparison between two maize inbred lines showed that their genomes contained respectively 3,408 and 3,298 unique insertions and deletions (InDels), with an average size of approximately 20 kbp (20,000 base pairs) and a range covering 1 kbp to over 1 Mbp (Jiao et al., 2017). Currently, comprehensive knowledge on the genomic variability among commercial plant varieties of agricultural crops is not available. Moreover, it remains unclear to what extent such information would provide a substantial contribution to the detection of induced mutations, especially against the background of the high dynamics of plant genomes.

Spontaneous natural mutations are expected to change the genome at each reproduction cycle. For instance, there is a seven in 1 billion chance in the model plant Arabidopsis (*Arabidopsis thaliana*) that any given base pair will mutate in a generation (Ossowski et al., 2010), meaning that 175 new variants (SNVs) would arise per 100 individual plants per generation. In rice, more than 54,000 novel DNA sequence variants were identified in a line that went through *in vitro* culture (and 8 cycles of self-fertilisation), compared to the wild-type line, without showing any different phenotype under normal growing conditions (Zhang et al., 2014). The relatively slow rate of natural mutation has also been increased by several orders of magnitude by conventional mutagenesis, such as irradiation or chemical treatment of seeds or pollen, which have been applied in plant breeding for several decades (Jankowicz-Cieslak et al., 2016; Anderson et al., 2016). Such mutant plants, which are exempted from the GMO regulations, have been incorporated in traditional breeding programmes and have contributed to the current crop diversity.

3.3.3 Sensitivity (Limit of Detection/Limit of Quantification)

For the acceptance criterion related to the sensitivity of the method, proof of evidence is required to demonstrate that a method targeting a SNV or a short InDel has an acceptable limit of detection/quantification in different sample types. The sensitivity of the method should be determined by testing the analyte target in presence of the non-modified DNA of the corresponding species at the highest DNA amount according to the

[10] GenBank and WGS Statistics (nih.gov), https://www.ncbi.nlm.nih.gov/genbank/statistics, last access in December 2022

method protocol (ENGL, 2023; Weidner et al., 2022). This should ensure that a low target concentration in a product is not obscured by the presence of large quantities of very similar DNA sequences.

3.3.4 Robustness of the method

For methods targeting a SNV or short InDel, a critical parameter is to assess whether the method is sufficiently robust against small modifications of the testing conditions. Such modifications may occur in different laboratory settings, often without noticing them. Scientific evidence indicates that robustness tests should be conducted in a background of highly similar genomic DNA from the same species (e.g. non-modified DNA) at the highest amount per reaction according to the submitted method protocol (ENGL, 2023; Weidner et al., 2022).

3.4 Additional considerations on the event-specificity of detection methods

Specificity is the property of a detection method to respond exclusively to the target of interest. This is also clarified in Annex III to Regulation (EU) No 503/2013[11], which states that *"the method shall be specific to the transformation event (hereafter referred to as 'event-specific') and thus shall only be functional with the genetically modified organism or genetically modified based product considered and shall not be functional if applied to other transformation events already authorised; otherwise the method cannot be applied for unequivocal detection/identification/quantification*."

Several foundations in this legal paragraph need clarification and elaboration in view of the new technological developments that allow the creation of targeted mutations in the genome.

- In line with the meaning of the legislation, the notion 'transformation event' needs to be interpreted in a broader sense to refer to the genomic modification site, characterised either by a transgene (or cis/intragene) insertion or to a mutation of one or several base pairs. The detection method needs to be specific to such an event and shall only be functional with the genetically modified organism or any product derived thereof.
- The addition '*and shall not be functional if applied to other transformation events already authorised*' is a further stand-alone requirement that makes sense particularly for conventional GMOs with a unique junction sequence created upon the insertion of recombinant DNA. In such case, the detection methods employed target a sufficiently long new sequence around the insertion site with a small risk that they would also detect other GMOs. In organisms obtained by targeted mutagenesis (and their products), the major problem is that they may detect also non-mutated plants of the species, particularly when the mutation comprises only a few nucleotides.
- The legal text stating that the event-specific method '*shall only be functional with the genetically modified organism or genetically modified based product considered'* implies therefore that the method shall also not be functional with any organisms from conventional breeding or wild type organisms otherwise the link between the analytical finding on a sample and the legal status of the product would remain uncertain and '*the method cannot be applied for unequivocal detection/ identification/ quantification.*'

3.4.1 Uniqueness of GM events

For conventional GM events, the method specificity is ensured by targeting the left or right junction between the inserted transgene sequence(s) and the plant DNA, which is a unique identification marker, created *de novo* by the randomly inserted transgene sequence. Moreover, as it is highly unlikely that exactly the same junction sequence will be created *de novo* a second time, this unique marker is also ensuring traceability to the process that generated the GM event, independent of further breeding activity to cross the GM event into different genetic backgrounds (e.g. there are over 250 maize varieties containing the MON810 event in the EUPVP - COMMON CATALOGUE - Varieties of agricultural plant and vegetable species (europa.eu)).

The situation is complex for genome-edited plants. First, in the absence of foreign DNA in the genome-edited plant the altered sequence, whether short or long, may not necessarily be unique, i.e. the same DNA alteration

[11] Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. *Off. J. Eur. Union* L157: 1-47.

can be identical to those in other varieties or in wild plants of the same or other species. For instance, in rice targeted base editing technology was shown to create the same nucleotide alterations in the acetolactate synthase (ALS) herbicide resistance gene as known from natural varieties of rice and other plant species (Shimatani et al., 2017). In other plants, targeted mutagenesis techniques have reproduced traits in elite varieties that exist already in natural mutants (d'Ambrosio et al., 2018), and the corresponding DNA alterations may not be distinguishable (Chilcoat et al., 2017). This would be particularly the case for traits that confer a selective advantage to the mutated organisms, e.g. herbicide resistance, and which may result from spontaneous mutations in the active site(s) of the protein (Nandula et al., 2020). Second, as a result of the ease of use and site-specificity of the targeted mutagenesis techniques, exactly the same DNA alteration may be created independently by different operators (companies, researchers), in order to create plants with a desired phenotype such as disease resistance. Furthermore, if the DNA alterations are identical, it would be difficult to trace back the genome-edited event by current state-of-the-art technologies to a unique identification marker, developed by a certain company. In fact, an unintentionally released product with a target alteration identical to that carried by an authorised event would be indistinguishable in a mixture and the ownership of and liability for a plant resulting from targeted mutagenesis or cisgenesis therefore would be unclear.

3.4.2 Multiple sites modifications

Organisms obtained by targeted mutagenesis may harbour more than one mutation, potentially up to several dozen, either in the same gene or in genetically unlinked genes, and they may have been produced in a single targeted mutagenesis experiment. As a consequence, there is no direct association between the genome-edited organism and a single detection method targeting a (single) mutation event, but there are rather several detection methods, one for each mutation site, that together characterise the modified organism. In sexually propagated organisms, the unlinked mutations may segregate in the progeny of the genome-edited organism, similar to single GM events that segregate from a stacked GMO. However, in contrast to most conventional GMOs, multiple edited organisms may not be authorised as single events. Indeed, the technology itself may create the multiple edits in one experiment while single edits may, in some cases, not create a functional trait. For instance, Biswas et al. (2022) used CRISPR/Cas to create knockout mutations in the 4 starch-branching enzyme (SBE) genes in rice to increase the content of health-beneficial resistant starch. In this case, multiple genes were knocked out for a pathway to be (partially or completely) reverted to a different bioactive compound. As each of the mutated genes would need to be targeted by a detection method, such multiple edited organisms and their mutation sites should be considered as a stack of several (mutation) events that may segregate independently in the sexual progeny. While currently known stacked GMOs comprise up to 6 single events, plants produced by targeted mutagenesis may have a much higher complexity, comprising 20 or more site-specific modifications, and even more when stacked through breeding with other plants developed by targeted mutagenesis.

3.4.3 Genotyping technology

For pure samples, derived from a single plant variety (e.g. individual plants, whole potatoes or apples, seed for cultivation, etc.), the use of genotyping technology based on variety-specific genetic markers could contribute towards supporting the identification of genome-edited plants (although also such plants may undergo natural mutations (Adamek et al., 2021). Such molecular markers are commonly used in crop breeding programmes to characterise known germplasm. Similar technologies and markers could, in principle, be used to characterise a well-defined genotype (variety) initially used for genetic modification. The analytical combination of the genome-edited event sequence and the variety-specific markers could provide evidence that the product was obtained by targeted mutagenesis. In cases where it is known that the genome-edited event was cross-bred into another well-characterised variety, specific molecular markers could also be used for identification of such products. However, such approaches require further research specific for each crop and variety, prior knowledge of the variety (genotype) used for targeted mutagenesis (and any subsequent varieties into which the mutation was cross-bred) and dedicated technologies that are currently not available in enforcement laboratories. Furthermore, such genotyping technologies, whether based on PCR or NGS, provide qualitative (presence/absence) data only, and cannot be used for quantification. Additionally, during sexual propagation of plants, the genotyping markers would segregate in the progeny, complicating the unambiguous identification of genome-edited events by genotyping. In food and feed commodities, genotypes are largely admixed, making it difficult to assign a specific mutation to a genome-edited event, even when using technologies such as massive parallel DNA sequencing approaches (Grohmann et al., 2019). The assignment of a specific mutation to an event is even more difficult in processed food or feed where the genetic material is frequently degraded. All these elements generate additional uncertainties for the interpretation of analytical test results.

3.4.4 Genetic variation and sequence databases

For market authorisation, applicants have to submit an event-specific detection method, the sequences of the insert(s) and the flanking regions and demonstrate that the method is specific for the GM event. Demonstrating the specificity of a method for a mutation event would require full knowledge of all existing sequence variations for the edited locus for all varieties and wild plants of all species used for food or feed production, which would serve as reference basis. At present, sequence databases compiling the sequence variation of all individuals of a species, i.e. the pan-genome (Hirsch et al., 2014; Li et al., 2014; Alaux et al., 2018; Zhao et al., 2018), are being developed for several plant species (see Text box 3). In case of SNVs, it will be difficult or even impossible to guarantee that the same alteration is unique and does not exist in other varieties/populations, some of which may have been much less explored through whole genome sequencing. It can also not be concluded that the same mutation will not be created spontaneously or by random mutagenesis techniques in future plants. The same problem may exist in case of more than one SNV and even for larger InDels or cisgenic substitutions that may already exist in conventional varieties (Custers et al., 2019). Since continuously updated pan-genome databases are never complete nor available for all species, it may not be possible for applicants to demonstrate the uniqueness of the DNA alteration. The EURL GMFF after verifying the sequence-specificity of a method will only be able to conclude that the method submitted is event-specific and fulfils all minimum performance requirements for analytical methods of GMO testing on the basis of the information available at that time. However it cannot be guaranteed that the same mutation will not be obtained by conventional techniques in the future.

In conclusion, whereas the detection of genome-edited events may be technically feasible, appropriate specificity for identification may not be achieved in all possible cases. This is a matter of evaluating the probability that an induced mutation may also occur naturally or could be obtained through conventional breeding or could be developed by different applicants using targeted mutagenesis. For methods targeting genome-edited plants, it cannot be excluded that the identical DNA alterations occurred already spontaneously, were introduced by conventional (random) mutagenesis or were/will be created in an independent editing experiment. Even when all validation requirements (including current event-specificity) have been met, such methods may still not be fit for use. This uncertainty will have consequences for enforcement of the GMO legislation through analytical methods.

4 Detection of food and feed products in the context of market control

Every day, shipments of thousands of tons plant-based products are arriving at EU entry points where they await clearance for unloading the commodity. Verification of compliance with the EU food and feed legislation is achieved through a mixed system of document traceability and laboratory testing. According to EU legislation, accompanying documentation is provided with the indication on whether the lot contains GMOs or not.

Products that arrive in a harbour, such as bulk grain, food or feed, can have different origins that ultimately can lead to a non-homogeneous composition. Sampling procedures are applied by the official inspector and the samples are analysed by the official control laboratories of the EU Member States for the presence of GMOs. Real-time qualitative and quantitative PCR assays for GMOs analyses and derived products are widely used by enforcement laboratories in the EU. In recent years, digital PCR-based methods are increasingly being introduced because they allow an accurate quantification for GMO control (Noma et al., 2022; Košir et al., 2019). Methods for detection need to be robust and applicable to the typical heterogeneous nature of food and feed samples tested by enforcement laboratories. Moreover, the techniques used should meet the need of urgency for analytical results in the official control activities and especially for Customs operations.

The current first-line approach employed by enforcement laboratories to analyse samples for the presence of GMOs is mainly based on an analytical screening strategy for common DNA sequences, such as gene promoters (e.g. CaMV P-35S), gene terminators (e.g. Agrobacterium T-*nos*), or protein coding sequences (e.g. *cp4-epsps*, *pat* or *cry1Ab*). Such elements are commonly found in authorised as well as in unauthorised conventional GMOs. These methods will react positively for all GMOs that contain the genetic element-specific sequences.

When the outcome of the initial screening is positive for certain elements, the second step will be to test for the presence of authorised GMOs using event-specific methods, or for known unauthorised GMOs for which construct- or event-specific methods are available (see https://gmo-crl.jrc.ec.europa.eu/gmomethods/). In case of known unauthorised GMOs that may have been detected earlier, this strategy may lead to the direct detection of an unauthorised GMO. Alternatively it may also lead to the conclusion that the detected combination of the GMO screening targets has not been observed before. These unexplained genetic elements may point indirectly at the presence of (additional) unauthorised GMOs in the sample. Subsequent research, for example using targeted or untargeted sequencing strategies (Košir et al., 2017; Noma et al., 2022), is then required to elucidate the background of the identified GMO elements. In this way, GMOs without an EU authorisation application may be detected insofar they contain a common screening marker (ENGL, 2011). However, it must be stated that the screening approach will not work for GMOs transformed with genetic elements not used in known GMOs. (ISK-class 4 in Holst-Jensen et al., 2012) as these will not be found in a regular screening.

Alternatives to PCR approaches for the detection of unauthorised GMOs have been developed in recent years. Screening of market samples using NGS has been proposed by a few EU control laboratories for the detection of unauthorised GMOs (Fraiture et al., 2018, 2023; Chen et al., 2021). It uses the known sequences of conventional GMOs (common genetic elements or coding sequences of transgenes) as a marker to detect both authorised and unauthorised GMOs in a market sample. This NGS screening approach is dependent on the presence of combinations of foreign DNA sequences. It cannot detect NGT products, as the plants considered in this report neither contain any transgenic sequence nor any other common genetic element that can be screened for. In the absence of targets that are common and therefore specific for a large group of genome-edited plants no general screening approach is applicable or can be developed. Without robust laboratory screening methods, analytical tests cannot exclude that unknown NGT products have entered the EU market undetected. The implementation of NGS in routine GMO analysis by the enforcement laboratories is still difficult due to its relatively high costs, as well as the requirement of adequate IT infrastructures, bioinformatics experts and statisticians for dealing with the generated data (Fraiture et al, 2015). In all probability, there will be an increase in the analysis time, which is not in favour of border controls. For now, these methods are used in research projects but not implemented as routine methods in enforcement laboratories. To ensure fast, smooth, and cost-efficient implementation in enforcement control, methods for detection should be based on existing analytical systems wherever possible.

The type of samples to be analysed during enforcement will also change with the development of genome-edited crops. Conventional GMOs are often developed in high-value crops such as maize, soybean, rapeseed, rice, sugar beet, potato and cotton. It is expected that targeted mutagenesis and cisgenesis will not be restricted to those crops and will likely enlarge the array of products to be tested.

In genome-edited plants, unwanted transgenic sequences (e.g. vector backbone sequences or scaffold RNA sequences) may potentially have remained in the genome in case the targeted mutagenesis technique employed involved integration of the construct into the plant genome and this was not carefully segregated out

in subsequent crosses (Braatz et al., 2017; Li et al., 2016; Schouten et al., 2017). In the frame of market control for unknown NGT products, it may be useful to develop additional screening methods for the detection and identification of commonly used recombinant DNA sequences that unintentionally remain in plants, e.g. those resulting from integrated CRISPR-Cas vectors used during development of some products (Guertler et al., 2023). Such sequences usually segregate out and are removed prior to being placed on the market, but they may also unintentionally occur on the market as escapes from field trials.

One detection method for a commercial product containing a SNV (Cibus rapeseed event 5715) has recently been published (Chhalliyil et al., 2022). Subsequent experimental testing revealed, however, that the claimed event-specific method is not fit-for-purpose for use in official control due to unspecific reactivity with closely resembling DNA sequences. The method cannot unambiguously detect the SNV and may amplify genetically non-modified rapeseed and other variants under routine working conditions (Weidner et al., 2022). Such a plant product may be identified in a pure, homogeneous (e.g. seed batch) sample. However, in heterogeneous samples (commodities) unambiguous detection of hidden admixtures and identification of individual genotypes will not be possible in most cases (Grohmann et al., 2019).

The prior knowledge of the sequence alteration in genome-edited plants is essential for the verification of methods for the detection of these plants in the process of an application for EU authorisation. CRMs would also be necessary for quantification-based methods when the quantity is expressed as mass fraction. For sequence alterations detected by NGS-based methods, corresponding CRMs would not be necessary and knowledge about the sequence through the use of databases would be sufficient. To the best of our current knowledge, NGS-based methods are currently not applicable or fit for quantification purposes.

Worldwide regulatory frameworks defining the status of food and feed plant products obtained by targeted mutagenesis and cisgenesis are different, and international harmonisation will probably not be achieved in the near future. In the absence of a market authorisation request in the EU, validated detection methods and certified reference materials for genome-edited plants are not available to the EURL GMFF and to National Reference laboratories for GMO control. Some genome-edited plants may have been authorised in other countries and the products may have been marketed elsewhere outside the EU. In this case, several different scenarios are possible.

- At best, appropriate certified reference material and sequence information are provided by the developer, and an adequate detection method is accessible to the EURL GMFF to carry out the validation of the method.
- In other cases, reference material may not be available, but adequate sequence information on the genome-edited plants has been published in patents and/or scientific journals. If the DNA alteration in such plants is known, and would be sufficiently informative to be targeted by a detection method, the application of such a method (already published or to be developed) may allow detection of the genome-edited product.
- The worst case scenario is the complete lack of information and of reference material for the genome-edited plant. In this case, detection of such genome-edited plants and their products is not possible.

The detection of very small sequence 'signatures' by bioinformatics and of genetic or methylation 'scars', as hypothesised recently (Bertheau, 2019), does not provide realistic evidence and proof that targeted mutagenesis was applied and has created a DNA alteration. 'Scars' are mutations that may be created in plants that have been treated by any breeding technique, including conventional mutagenesis, or passed through tissue culture and are not exclusively induced by targeted mutagenesis. On the other hand, if a secondary mutation is detected in a genome-edited plant in close proximity to a targeted mutation, the combination of both mutations may provide further evidence that the product analysed was obtained by targeted mutagenesis. Moreover, it is not clear to what extent individual epigenetic changes are stable across breeding generations. Furthermore, the Central Committee on Biology Safety (ZKBS) in Germany describes that this theory, which involves “highly variable biological parameters (like epigenetic changes) that are no reliable base for identification”, is not an appropriate method to prove the use of genome editing. The example of LBFLFK oilseed rape emphasises this argument, showing that off-target changes, like “scars”, will never segregate together indefinitely (COGEM, 2020). There are efforts to investigate if there are certain features in the DNA that specifically co-localise with a genome-edited mutation site, however, such features have not been, identified so far.

Signatures like the PAM sequence (Protospacer adjacent motif - a 2-6 bp DNA sequence flanking the DNA sequence targeted by the Cas nuclease) may function as additional identification element referring to targeted mutagenesis, but only when the CRISPR technique has been used. These PAM sequences vary depending on the type of Cas protein used. The Cas enzymes are classified into two classes (defined by an organisation of effector

proteins), six types (represented by the presence of signature proteins) and thirty-three subtypes. These sequences are essential for the Cas proteins as anchor sites to find and recognise their target sequence in the genome (Nidhi et al., 2021; Wada et al., 2022). PAM sequences are short and diverse and some Cas enzymes do not need a PAM site, so their application for identification of targeted mutations is limited.

The identification of DNA alterations resulting from targeted mutagenesis and cisgenesis that are not unique remains, therefore, extremely difficult, as the altered sequences may mimic naturally occurring sequence variants, or they may not be distinguishable from those alterations obtained through conventional breeding.

An alternative approach for the detection of unauthorised GMOs has been proposed in 2010, using documentation-based screening for products that potentially contain unauthorised GMOs. This is based on web crawling and text mining technologies using descriptive keywords, to be followed by analytical confirmation (Ruttink et al., 2010; JRC, 2017). Such a laborious approach, if implemented by all actors in the field, could be considered as a way to collect world-wide information on the development and marketing of genome-edited plants, but it remains to be evaluated to what extent such an approach would be practical as it relies on open international collaboration, communication and voluntary exchange of information (Ribarits et al., 2021a; Dima et al., 2022). Moreover, analytical confirmation for enforcement of the regulations would still be required and may remain challenging.

5 Conclusions

This report has been prepared by an ENGL working group representing the official GMO analytical sector in the EU, with support from the EURL GMFF. It highlights the analytical challenges and limitations associated with the detection, identification and quantification of plant products derived from targeted mutagenesis and cisgenesis. This report builds upon a previous report from 2019 and updates the issues on the basis of current scientific and technological knowledge and experimental experience. It aims to inform the European Commission on analytical aspects of traceability and labelling, cornerstones of the current GMO legislation, and how analytical methods can be applied to products obtained by these more recent breeding techniques.

The major challenges for the detection of various plant products developed by targeted mutagenesis or cisgenesis that were identified by the working group are summarised in Table 2. This table is built upon the assumption that the modified sequence to be targeted is known and appropriate reference material is available. It mainly focusses on PCR-based methods for detection, which is the preferred technology for GMO analysis. The major issues are elaborated below.

Table 2. Analytical challenges for the development and validation of methods for detection, identification and quantification of plant products developed by targeted mutagenesis or cisgenesis.

Genomic technique	Type of modification	Method development and validation	Method implementation for enforcement
Targeted mutagenesis	SNV	Technical feasibility depends on sequence context (case-by-case), but event-specificity, robustness and quantitative use of the method generally difficult to demonstrate	Difficult/impossible to implement such methods for analytical enforcement, when the results are not reliable
	Short mutation	Technical feasibility depends on sequence context (case-by-case), but event-specificity, robustness and quantitative use of the method may in some cases be difficult to demonstrate	Difficult/impossible to implement such methods for analytical enforcement, when the results are not reliable
	Long insertion or deletion	Technically feasible, but event-specificity depends on whether the modification created a new, unique sequence junction or not	Feasible, when event-specificity is demonstrated
Cisgenesis	Targeted sequence insertion or substitution	Technically feasible, but event-specificity of the method depends on whether the altered new sequence is different (and by how many nucleotides) from similar sequences already existing in the species	Feasible when event-specificity is demonstrated. Feasible, but implementation issues may occur in case the new sequence only differs from existing sequences by a SNV or short mutation
	Random whole gene insertion	Feasible	Feasible
Intragenesis	Random whole gene insertion or targeted sequence insertion or substitution	Feasible	Feasible
Various techniques	Multiple modifications	Requires development and validation of one method per modified site, significantly increasing the workload; technical feasibility depends on the types of modifications (see above)	Analysis of each plant product with all individual methods known for the species present in the product will enormously increase the analytical workload and will rapidly make analytical enforcement impossible to continue in the same way. Also difficult to evaluate product quantity in case of multiple modifications derived from a single plant, some of which may also segregate out.

Technical restrictions

Targeted mutagenesis can result in different types of DNA alterations (deletions, insertions or substitutions) of varying sizes. SNVs - the smallest DNA sequence alteration in the plant genome - are the most challenging when developing a detection method that is specific for the mutation. Depending on the complexity of the genome and the surrounding sequences, similar technical constraints may be experienced when targeting mutations comprising a few nucleotides. Such methods may not be sufficiently sequence-specific, robust and

reliable when used for the purpose of official market control. **Methods developed for some SNVs or short mutations may fail to meet the current Minimum Performance Requirements for analytical methods of GMO testing**. In contrast, long insertions or deletions, as well as random cisgenic sequence insertions, will generally create new and unique sequence junctions that can reliably be detected and identified with current analytical capabilities.

Similarity to natural mutations

In general, alterations in the plant genome that do not involve the insertion of foreign DNA (DNA not present in the breeders' gene pool) or the insertion of cisgenic DNA in the genome in its natural location, are extremely difficult to identify by any analytical technique, especially without prior knowledge. This is particularly due to the inherent variability of plant genomes. Spontaneous mutations arise continuously in the plant genome. Most mutations are caused by errors when the DNA is replicated prior to cell division, which is why single nucleotide variants (SNVs) are the most common ones. Other small and long sequence alterations are typical natural features of plant genomes within a species. Targeted mutagenesis techniques allow the modification of the genome in a way that may mimic what can happen naturally or can be induced by conventional breeding. Additionally, the incorporation of cisgenic DNA sequences can technically be achieved with the precision of homologous recombination. In general, the major problem with analytical technologies is that they cannot discriminate products derived from plants with identical sequence modifications obtained by different techniques, e.g. targeted mutagenesis, cisgenesis, conventional breeding, natural mutation, or developed by different companies. Targeted mutagenesis may create a sequence variation already present in the breeders' gene pool. **It may be impossible to analytically evaluate whether a particular mutation is produced by targeted mutagenesis rather than the result of conventional breeding or natural mutagenesis.**

Expanding workloads for method development, validation and official control

While most conventional GMOs can be detected by enforcement laboratories due to the presence of common foreign genetic elements targeted by screening methods, such **screening methods cannot be applied to plants containing only endogenous DNA alterations or sequences from the breeders' gene pool**. Instead, only individual event-specific methods can be applied for market control for the presence of such plant products. It would mean that any food/feed product containing an ingredient from a crop to which genome-editing techniques have been applied must be analysed by all methods developed for detecting mutations in this species.

Another important feature of targeted mutagenesis techniques is that they allow the simultaneous modification of many different DNA sequences in a plant genome, possibly in the order of several dozen. As these multiple DNA alterations may segregate independently through next generations, **each genetic modification needs a separate event-specific detection method.** The validation of multiple methods for such multi-edited ("stacked") events increases the corresponding validation workload and costs for the EURL GMFF and the supporting National Reference Laboratories. It will also result in a dramatic rise of the costs and time needed for analysis by the enforcement laboratories. This increased workload is additionally fuelled by the rising diversity of crops to which these new genomic techniques are being applied. Finally this would rapidly accumulate to **hundreds of methods per plant species, which is impossible to handle** with the current human resources, available analytical technologies and laboratory equipment, and would affect the practicability of the approach.

Analytical methods have limited value for market control of unknown plant products

Detection of unknown products of targeted mutagenesis or cisgenesis without prior knowledge of the modified DNA sequence will not be possible with the current technology used by most of the enforcement laboratories. Application of NGS for this purpose is also not feasible as it still requires standardisation, development of validation concepts and ongoing massive investment in human and technical infrastructure. Furthermore, NGS may fail when analysing compound samples and usually does not allow quantification of the mutated plant ingredient. Therefore, **if unauthorised plant products obtained by targeted mutagenesis or cisgenesis enter the EU food and feed supply chains it may be difficult to identify them with analytical tools**.

This report further reinforces and augments the conclusions and outlook described in the previous report (ENGL, 2019). It newly adds concerns on the practicability of enforcement and the economic impact on the system when many products developed by targeted mutagenesis or cisgenesis will need to be analysed. It is concluded that **analytical testing to support traceability is not considered feasible for all products obtained by targeted mutagenesis and cisgenesis.**

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