# Detection of food and feed plant products obtained by new mutagenesis techniques

*European Network of GMO Laboratories (ENGL)*

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## Executive summary

The European Network of GMO Laboratories (ENGL) has reviewed the possibilities and challenges for the detection of food and feed plant products obtained by new directed mutagenesis techniques leading to genome editing. The focus of this report is on products of genome editing that do not contain any inserted recombinant DNA in the final plant.

The procedures for the validation of detection methods as part of the **market authorisation** application process for genome-edited plant products will in principle be the same as for the current conventional GMOs. It is, however, questionable if event-specific identification and quantitative detection methods can be developed readily for all genome-edited plants. For instance, detection methods for those plant products that are characterised by a non-unique DNA alteration will probably lack the specificity required to identify the genome-edited plant. Moreover, accurate quantification may be challenging if only changes of just one or a few basepairs are introduced.

The EU Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) assisted by the ENGL will need to review the minimum performance requirements that are applied for GMO method validations in view of the specific characteristics of genome-edited plants. This should provide further guidance to applicants for market authorisation and to the EURL GMFF for validation of the event-specific methods. For example, it is currently unclear how to demonstrate or assess the specificity of the method if the mutation could also occur spontaneously or could be introduced by random mutagenesis techniques. Furthermore, it needs to be emphasised that specific detection methods would be required to cover all DNA alterations in a multi-edited plant.

For **market control**, considering the current knowledge and state of the art of GMO testing, it is highly improbable for enforcement laboratories to be able to detect the presence of unauthorised genome-edited plant products in food or feed entering the EU market without prior information on the altered DNA sequences. The PCR (polymerase chain reaction)-based screening methods that are commonly used to detect conventional GMOs cannot be applied nor could be developed for genome-edited plant products. The reason is that the currently used screening methods are targeting common sequences which are not occurring in genome-edited plants.

DNA sequencing may be able to detect specific DNA alterations in a product. However, this does not necessarily confirm the presence of a genome-edited plant product. The same DNA alteration could have been obtained by conventional breeding or random mutagenesis techniques, which are exempted from the GMO regulations.

**In conclusion**, validation of an event-specific detection method and its implementation for market control will only be feasible for genome-edited plant products carrying a known DNA alteration that has been shown to be unique. Under the current circumstances, market control will fail to detect unknown genome-edited plant products.

Several issues with regard to the detection, identification and quantification of genome-edited products are currently based on theoretical considerations only and lack any experimental evidence. Therefore, they will require further consideration.

# 1 Introduction

In the European Union the authorisation system for the introduction of 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, marketing, labelling, enforcement and the whole agro-food chain, up to the consumption by humans and animals.

The EU Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF), hosted by the Joint Research Centre (JRC) of the European Commission, is legally mandated to assess and validate the detection methods submitted by the applicants (GMO producers) for authorisation of GMOs[2]. For this task, the EURL GMFF is assisted by a consortium of national reference and enforcement laboratories, known as the European Network of GMO Laboratories (ENGL), which has issued a guidance document explaining the minimum performance requirements (MPR) for analytical methods of GMO testing[3]. Since the labelling and traceability legislation[2,4,5] is based on the GMO content present in the food or feed product, one of the requirements refers to the accurate quantification of the 'GM fraction' in such products. GMOs or GM food and feed products that do not meet the requirements of the legislation should not be present on the market (see Text box 1).

The EURL GMFF also has a legal mandate under the 'Official Controls Regulation'[6], which defines harmonised rules on official controls and, among others, activities performed to ensure compliance to the food and feed laws related to the presence of GMOs. In that context, official enforcement should control the implementation of the labelling requirements and prevent infringement of the legislation due to the presence of unauthorised GMOs on the market. To implement this Regulation, Member States have appointed National Reference Laboratories (NRLs) and official laboratories to perform analyses on food, feed and seed products in their national markets; this is performed by applying – when 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 the authorised GMOs.

[1] In line with Directive (EU) 2015/412 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] European Network of GMO Laboratories (2015) Definition of minimum performance requirements for methods of GMO testing (http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf).

[4] 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.

[5] 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.

[6] 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.

Text box 1

**Different authorisation statuses of GMOs under Directive 2001/18/EC[7] and Regulation (EC) No 1829/2003[2]**

Authorised for placing on the market

Authorised GM material is allowed on the EU market. Authorisation mostly concerns the import of GMOs and products thereof and their use in food and feed. Few authorisations have been submitted for cultivation of GM plants and currently only one GM maize event is authorised for cultivation.

GMOs in this category 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 is at or below 0.9% and provided that these traces are adventitious or technically unavoidable.

Non-authorised 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 Directive Part C of 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 applies.

For feed use only, and under the conditions of Commission Regulation (EU) No 619/2011[5], GMOs in the latter two categories shall be considered non-compliant at or above the Minimum Required Performance Limit (MRPL) of 0.1% related to mass fraction, and findings below the MRPL shall be notified to the Commission and other Member States. For pending authorisations, the requirements are that the GM material must be authorised for commercialisation in a third country, a valid application had been submitted to the EU and has been pending for more than three months, no adverse effects have been identified by EFSA when present under the MRPL, and a validated quantification method and certified reference materials are available. For expired authorisations, certified reference materials have still to be available.

During the past years, several new plant breeding techniques, including targeted mutagenesis techniques generically called 'genome editing', have been employed to create diversity for exploitation in plant breeding (reviewed in [8]). Instead of the random mutation of many genes at the same time (as in conventional mutation breeding techniques) or the random insertion of new genes (as in conventional GMOs), genome editing allows the site-specific alteration of the DNA sequence of one or a few selected genes; this can result in single nucleotide variants (SNV) or sequence insertions or deletions (InDels). These DNA alterations may be present either in a homozygous or heterozygous state in the genome, *i.e.* all or only a fraction of the copies of a given gene (called the alleles of a gene) may carry the alteration (*e.g.* in a tetraploid (4n) plant the same DNA alteration can be present as DNA copy between one and 4 times)[9,10,11].

[7] Directive 2001/18/EC of the EuropeanParliament 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.

[8] Scientific Advice Mechanism (2017) New techniques in Agricultural Biotechnology. European Commission (https://ec.europa.eu/research/sam/pdf/topics/explanatory_note_new_techniques_agricultural_biotechnology.pdf#view=fit&pagemode=none).

[9] Clasen, B.M., Stoddard, T.J., Luo, S., *et al.* (2016) Improving cold storage and processing traits in potato through targeted gene knockout. *Plant Biotechnol. J.* 14:169-176.

[10] Haun, W., Coffman, A., Clasen, B.M., *et al.* (2014) Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. *Plant Biotechnol. J.* 12:934-940.

[11] Demorest, Z.L., Coffman, A., Baltes, N.J., *et al.* (2016) Direct stacking of sequence-specific nuclease-induced mutations to produce high oleic and low linolenic soybean oil. *BMC Plant Biol*. 16:225.

In 2011, upon request of DG SANTE, the JRC reviewed the state-of-the-art of some of the emerging new plant breeding technologies, their level of development and adoption by the breeding sector and the prospects for a future commercialisation of plants created by these techniques[12]. Additionally, with support of several ENGL experts, the challenges for the detection of organisms developed through these techniques were evaluated[13]. The topic has since been discussed also during meetings of the ENGL. In the past few years, a novel innovative technique for genome editing, CRISPR-Cas, with wider potential and easier applicability, has rapidly advanced plant biology research and the development of applications for plant breeding[8,14].

In 2018, the European Court of Justice ruled that organisms obtained by new mutagenesis techniques, *i.e.* genome editing, in contrast to conventional mutagenesis techniques *"that have conventionally been used in a number of applications and have a long safety record"*[15], are not exempted from the GMO legislation[15]. 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.

This document addresses questions related to the new analytical challenges for the detection, identification and quantification of genome-edited food and feed products of plant origin. Those may relate (1) to the compliance with the GM food and feed legislation, including the requirements for method validation as part of the GMO authorisation procedures[2], and (2) to the provisions of the Official Controls Regulation[6] on the routine testing of food and feed by the enforcement laboratories.

This document has been endorsed and released for publication by the Steering Committee of the ENGL.

The ENGL experts who mentioned their viewpoints here have an in-depth expertise with respect to GMO analysis for many years. It is noted, that, at the current state, own experimental work on detectability of genome-edited food or feed products of plant origin has not been conducted.

[12] Lusser, M., Parisi, C., Plan, D., Rodríguez-Cerezo, E. (2011) New plant breeding techniques. State-of-the-art and prospects for commercial development. Luxembourg, *Publications Off. Eur. Union*, 184 p. (https://publications.europa.eu/en/publication-detail/-/publication/12988d6d-c6a4-41b2-8dbd-760eeac044a7/language-en).

[13] Lusser, M., Parisi, C., Plan, D., Rodríguez-Cerezo, E. (2012) Deployment of new biotechnologies in plant breeding. *Nature Biotechnology* 30:231–239 (doi:10.1038/nbt.2142).

[14] Khatodia, S., Bhatotia, K., Passricha, N., Khurana, S.M.P., Tuteja, N. (2016) The CRISPR/Cas genome-editing tool: Application in improvement of plants. *Front. Plant Sci.* 7:506 (doi: 10.3389/fpls.2016.00506).

[15] 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.

# 2 Terminology used in this document

The term **conventional GMOs** will be used throughout this report to refer to plant GMOs obtained by recombinant DNA technology and characterised by the presence of introduced DNA sequences from the same or other species in the final organism.

**Genome editing**, also called gene editing, is a group of new directed mutagenesis techniques that facilitate addition, removal, or alteration of DNA sequences at a specific location in the genome. This is mostly achieved with the aid of the cell's natural DNA recombination/repair system activated with the use of a site-directed nuclease (SDN), creating a double-strand DNA break at a defined location, a repair template sequence consisting of an added nucleic acid molecule (*e.g.* an oligonucleotide or longer nucleic acid sequence with partial sequence similarity to the target site), or the combination of both (modified from [8]). The techniques require the presence of the SDN in the recipient host cell, either following stable integration of recombinant DNA into the plant genome, or by transient expression or delivery of a protein/nucleic acid complex into the cell. In this document we will refer only to plant cells, but also other organisms could be targets of genome editing. When recombinant DNA has been used, it can be segregated away in subsequent generations, resulting in genome-edited plants that no longer contain any recombinant DNA[16,17]. In the frame of this report, plants obtained with genome editing techniques that contain inserted recombinant DNA or unintentionally remaining insertions of the transformation vectors are excluded, as these will be similar to the current conventional GMOs.

Early but limited success of genome editing 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[8]. Editing of single nucleotides can also be achieved using a specific set of enzymes referred to as 'base editors', which aim at modifying DNA at specific sites without involving double-strand breaks[18].

The DNA sequence alterations introduced through any of the genome editing techniques may be single nucleotide variants (SNV), insertions or deletions (called InDels), or, less frequently, gene duplications, inversions and translocations[19]. 'Short' DNA alterations, as mentioned in this report, are referring to changes in one or a few base pairs, while 'large' alterations refer to alterations of several dozen base pairs. However, there is a grey zone between 'short' and 'large' 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 alteration is unique or occurs already in any plant species, or potentially could occur, and whether or not it can be unequivocally attributed to the application of genome editing. This may need to be assessed on a case-by-case basis using approaches which should be defined by the ENGL.

By analogy to the term 'transformation event' used in GMO legislation[2], we propose here to use '**genome-edited event**' to refer to the altered DNA sequence, as indicated above, at a specific site in the genome as a result of the genome editing technique. A prerequisite is that no recombinant DNA remained in the genome of the final plant (from vector backbone or other 'unwanted' integrations), which was not removed by segregation. Furthermore, as genome editing may create several intended DNA

[16] Zhang, Y., Liang, Z., Zong, Y., *et al.* (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. *Nat. Commun.* 7:12617.

[17] Liang, Z., Chen, K., Li, T., *et al.* (2017) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat. Commun.* 8:14261.

[18] Zong, Y., Wang, Y., Li, C., Zhang, R., Chen, K., Ran, Y., Qiu, J.-L., Wang, D., Gao, C. (2017) Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nat. Biotechnol.* 35:438-440.

[19] Zhu, C., Bortesi, L., Baysal, C., Twyman, R.M., Fischer, R., Capell, T., Schillberg, S., Christou, P. (2016) Characteristics of genome editing mutations in cereal crops. *Trends Plant Sci.* 22:38-52.

alterations in the genome simultaneously, each of these multi-edits, when segregating independently, would require a specific detection method.

The term '**detection**' as referred to in this report encompasses different aspects:

(1) the 'finding' of a target sequence, *i.e.* detection *sensu stricto*, without necessarily being specific for the genome-edited event;

(2) the identification of the detected sequence as a specific genome-edited event;

(3) and the quantification of the genome-edited event.

For feed and food marketing authorisation under the GMO regulations, all three aspects of the broader interpretation of 'detection', *i.e.* including quantification, need to be fulfilled as the detection method needs to be able to quantify the presence of the genome-edited event at the GMO labelling threshold for adventitious or technically unavoidable presence of authorised events (0.9 m/m % expressed in mass fraction per total mass of the ingredient or plant species). When GMOs with pending or expired authorisation status are detected in feed[5], it needs to be assessed if their mass fraction is below the minimum required performance limit (MRPL) of the analysis method (0.1 m/m %). Methods for the detection of unauthorised GMOs, however, do not, in principle, need to be quantitative or event-specific as detection *sensu stricto* is sufficient for assessing non-compliance of the product.

# 3 Validation of detection methods for genome-edited events under an EU authorisation request

## 3.1 Possibilities and challenges for analytical methods

In an authorisation context, the GMO producer applying for market authorisation (the 'applicant') of a GMO has to submit a complete dossier for risk assessment. This dossier shall include a detection, identification and quantification method, with supporting method performance data, and the reference material should be made available. 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, that they provide a dynamic range around the labelling threshold (*i.e.* 0.9 m/m %), and that they reach the desired level of sensitivity, robustness, ease of use and accuracy of quantification.

At the time of writing, more than 150 applications for authorisation of mostly plant GMOs for food or feed uses have been submitted in the EU since the GM food and feed legislation came into force[2].

In most of these cases, the GMOs contained one or more inserted foreign DNA sequences of up to several thousand nucleotides long. The genetic transformation procedures employed for their generation have resulted in an 'event' of insertion of recombinant DNA sequences. For each insertion, two unique insert-to-plant junctions are generated, one at each end of the integration site. Each of the unique junctions created during a transformation event can be exploited as a unique identification marker for developing a method of detection specific for each conventional GMO (often referred to as 'event-specific' detection method).

Although genetic modifications may affect other classes of molecules such as RNA and 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 method widely used in molecular biology to target DNA molecules. This technology provides a million-fold amplification of a selected target DNA sequence of typically 70-150 base pairs, located across one of the insert-to-plant junctions. qPCR can provide high sensitivity and robustness for the precise relative quantification of GM material, even at low levels, in food and feed products. When qPCR is targeting the unique sequences of transformation events, it ensures the required level of specificity to be in compliance with the legal requirements.

The EURL GMFF validates the detection methods provided by applicants for market authorisation in an interlaboratory validation exercise involving National Reference Laboratories[20]. The ENGL guidance on minimum performance requirements[3] provides the reference basis for the assessment of the validation study. The validated quantitative method and certified reference materials (CRMs) for calibration and quality control of the method constitute a complete 'toolkit' for the unequivocal identification and quantification of a GMO[21,22].

In the frame of establishing this report, the scientific literature from different fields has been reviewed to evaluate if the current ENGL method performance criteria could be applied to methods for the detection and quantification of genome-edited products.

[20] Commission Implementing Reguation (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.

[21] Trapman, S., Corbisier, P., Schimmel, H., Emons, H. (2009) Towards future reference systems for GM analysis. *Anal. Bioanal. Chem.* 396:1969-1975.

[22] Corbisier, P., Emons, H. (2019) Towards metrologically traceable and comparable results in GM quantification. *Anal Bioanal. Chem.* 411:7-11.

It has been shown for SNV allelic discrimination assays developed in other domains[23,24] that quantitative parameters such as PCR efficiency, slope and linearity are in line with those established by the ENGL. Other assay types such as 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[25]. However, in those studies the materials tested were of a lower complexity and consisted of individual genotypes and plants. Both the sensitivity of the method for a genome-edited product and its specificity are challenging issues for food and feed products with a complex composition.

The assays mentioned above and other strategies would require a significant level of method optimisation and experience which is currently not available. 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.

Digital PCR (dPCR) methods have been used for the screening and confirmation of particular mutations in clinical samples, namely induced pluripotent stem cells or primary cells at very low concentrations[26,27]. In some dPCR assays[27] two probes, binding to the mutated or wild-type sequence, were used for the simultaneous 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 ENGL document on Minimum Performance Requirements[3]. 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.

Other authors have compared the relative specificity and sensitivity of qPCR versus dPCR assays in detecting and quantifying SNVs or small InDels in individual founder transgenic mice generated by CRISPR/Cas9 mutagenesis: a lower rate of false-positive unedited events was observed when using a dPCR assay, and locked nucleic acid probes could be used to enhance the specificity of the assay[28]. Overall, the dPCR methods seem to be preferred in comparison to qPCR methods, however the precision, trueness and specificity of the methods have not been systematically evaluated for genome-edited plant products.

Theoretically, sequencing-based strategies, such as Next Generation Sequencing (NGS), could potentially be applied for the simultaneous detection of (multiple) genome edited events. On a case by case basis, target enrichment or probe capturing NGS approaches may be considered, for which a proof of concept has been reported for the detection of conventional GMOs[29,30]. The quality criteria to assess sequencing data are currently under

[23] de Andrade, C.P., de Almeida, L.L., de Castro, L.A., Driemeier, D., da Silva, S.C. (2013) Development of a real-time polymerase chain reaction assay for single nucleotide polymorphism genotyping codons 136, 154, and 171 of the *prnp* gene and application to Brazilian sheep herds. *J Vet. Diagn. Invest.* 25:120-124 (doi: 10.1177/1040638712471343).

[24] Feligini, M., Bongioni, G., Brambati, E., Amadesi, A., Cambuli, C., Panelli, S., Bonacina, C., Galli, A. (2014) Real-time qPCR is a powerful assay to estimate the 171 R/Q alleles at the *PrP* locus directly in a flock's raw milk: a comparison with the targeted next-generation sequencing. *J. Virol. Meth.* 207:210-4 (doi: 10.1016/j.jviromet.2014.07.017).

[25] Broccanello, C., Chiodi, C., Funk, A., McGrath, J.M., Panella, L., Stevanato, P. (2018) Comparison of three PCR based assays for SNP genotyping in plants. *Plant Meth.* 14:28 (doi: 10.1186/s13007-018-0295-6).

[26] Miyaoka, Y., Berman, J.R., Cooper, S.B., Mayerl, S.J., Chan, A.H., Zhang, B., Karlin-Neumann, G.A., Conklin, B.R. (2016) Systematic quantification of HDR and NHEJ reveals effects of locus, nuclease, and cell type on genome-editing. *Sci. Rep.* 6:23549 (doi:10.1038/srep23549).

[27] Mock, U., Hauber, I., Fehse, B. (2016) Digital PCR to assess gene-editing frequencies (GEF-dPCR) mediated by designer nucleases. *Nat. Protoc.* 11:598-615 (doi: 10.1038/nprot.2016.027).

[28] Falabella, M., Sun, L., Barr, J., Pena, A.Z., Kershaw, E.E., Gingras, S., Goncharova, E.A., Kaufman, B.A. (2017) Single-step qPCR and dPCR detection of diverse CRISPR-Cas9 gene editing events in vivo. *G3: Genes/Genomes/Genetics* 7:3533-3542 (doi: https://doi.org/10.1534/g3.117.300123).

[29] Fraiture, M.A., Herman, P., Papazova, N., De Loose, M., Deforce, D., Ruttink, T., Roosens, N.H. (2017) An integrated strategy combining DNA walking and NGS to detect GMOs. *Food Chem*. 232:351-358.

[30] Arulandhu, A.J., van Dijk, J., Staats, M., Hagelaar, R., Voorhuijzen, M., Molenaar, B., van Hoof, R., Li, R., Yang, L., Shi, J., Scholtens, I., Kok, E. (2018) NGS-based amplicon sequencing approach; towards a new era in GMO screening and detection. *Food Control* 93:201-210.

discussion, for instance at ISO level[31]. This should also contribute to establishing a framework for the validation of NGS-based methods in the future. It should be noted that NGS approaches are currently not sufficiently validated for the quantification of targets in complex mixtures.

Although it is technically possible to detect specific DNA alterations, without prior knowledge, none of the techniques described are able to distinguish whether the SNV or InDel is caused by genome editing, by classical breeding technologies or by natural mutation (see Chapter 3.2).

## 3.2 The event-specificity requirement of detection methods

Specificity is the property of a detection method to respond exclusively to the target of interest. Annex III to Regulation (EU) No 503/2013[32] 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*."

For current transformation events, the method specificity is ensured by targeting the junction between the inserted transgene sequence(s) and the plant DNA, which is a unique identification marker created *de novo* upon the randomly inserted transgene sequence. Moreover, as it will be highly unlikely that exactly the same transgenic genome sequence will be created *de novo* a second time, this unique marker is also ensuring traceability to the process that generated the GMO, independent of further breeding activity to cross the GM event into different genetic backgrounds.

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 may already exist 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[33]. In other plants, genome editing has reproduced traits in elite varieties that exist already in wild plant species, and the corresponding DNA alterations may not be distinguishable[34,35].

Secondly, as a result of the ease of use and site-specificity of the genome-editing techniques, exactly the same DNA alteration may be created by different operators (companies, researchers) independently, in order to create plants with a desired phenotype such as disease resistance. If the DNA alterations are identical, it would be impossible to trace back by current state-of-the-art technologies the genome-edited event to a unique identification marker, developed by a specific company in a specific genome-editing experiment. The ownership of and liability for a genome-edited plant may therefore be unclear.

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[31] ISO/WD 20397-2 Biotechnology - General requirements for massive parallel sequencing - Part 2: Methods to evaluate the quality of sequencing data (https://www.iso.org/standard/67895.html).

[32] 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.

[33] Shimatani, Z., Kashojiya, S., Takayama, M., Terada, R., Arazoe, T., Ishii, H., Teramura, H., Yamamoto, T., Komatsu, H., Miura, K., Ezura, H., Nishida, K., Ariizumi, T., Kondo, A. (2017) Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. *Nat. Biotechnol.* 35:441-445 (doi:10.1038/nbt.3833).

[34] D'Ambrosio, C., Stigliani, A.L., Giorio, G. (2018) CRISPR/Cas9 editing of carotenoid genes in tomato. *Transg. Res.* 27:367–378.

[35] Chilcoat, D., Liu, Z.B., Sander, J. (2017) Use of CRISPR/Cas9 for crop improvement in maize and soybean. *Prog. Mol. Biol. Transl. Sci.* 149:27–46 (doi: 10.1016/bs.pmbts.2017.04.005).

For market authorisation, applicants have to submit an event-specific detection method and demonstrate that the method is specific for the GMO. This would require full knowledge of all existing sequence variations for the genome-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[36,37,38,39], are being developed for several plant species (see Text box 2). In case of single nucleotide alterations it will be difficult or even impossible to guarantee that the same alteration is unique and does not exist in other varieties/populations, or will be created spontaneously or by random mutagenesis techniques in future plants. The same problem may exist in case of more than a single nucleotide alteration, and even for larger gene deletions or duplications that may exist already in conventional varieties[40]. If continuously updated pan-genome databases are not available, it may not be possible for applicants to demonstrate the uniqueness of the DNA alteration or for the EURL GMFF to verify this information and to conclude that the method submitted is event-specific.

Consequently, it could be difficult for applicants to develop an event-specific detection method for a genome-edited plant not carrying a unique DNA alteration. It will need to be assessed on a case-by-case basis if a given DNA alteration corresponds to a specific genome-edited event that can be targeted by a detection method fulfilling all minimum performance requirements, including specificity. It is currently unclear how this specificity could be assessed, both *in silico* and experimentally.

In conclusion, whereas the detection *sensu stricto* of genome-edited events may be technically feasible, the same specificity for identification as currently applicable to conventional GM event-specific methods may not be achieved in all possible cases. For methods targeting genome-edited plants, it cannot be excluded that the identical DNA alterations occurred already spontaneously, were introduced by random mutagenesis or were/will be created in an independent editing experiment. This uncertainty will have consequences for enforcement of the GMO legislation.

[36] Hirsch, C.N., Foerster, J.M., Johnson, J.M., Sekhon, R.S., Muttoni, G., Vaillancourt, B., Penagaricano, F. (2014) Insights into the maize pangenome and pan-transcriptome. *Plant Cell* 26:121–135.

[37] Li, Y.-H., Zhou, G., Ma, J., *et al.* (2014) *De novo* assembly of soybean wild relatives for pan-genome analysis of diversity and agronomic traits. *Nat. Biotechnol.* 52:1045-1054.

[38] Alaux, M., Rogers, J., Letellier, T., *et al.* (2018) Linking the International Wheat Genome Sequencing Consortium bread wheat reference genome sequence to wheat genetic and phenomic data. *Genome Biol.* 19:1-10.

[39] Zhao, Q., Feng, Q., Lu, H., *et al.* (2018) Pan-genome analysis highlights the extent of genomic variation in cultivated and wild rice. *Nat. Genet.* 50:278–284.

[40] Custers, R., Casacuberta, J.M., Eriksson, D., Sagi, L., Schiemann, J. (2019) Genetic alterations that do or do not occur naturally; consequences for genome edited organisms in the context of regulatory oversight. *Front. Bioeng. Biotech.* 6:213.

Text box 2

***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[41]. 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[42]. 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 genome-edited events, 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[43], 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[44]. 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[45,46]. 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 The minimum performance requirements for analytical methods of GMO testing

The European Network of GMO Laboratories (ENGL) elaborated in 2015 the third version of the guidance document on minimum performance requirements for analytical methods of GMO testing[3]. The document, inter alia, is addressed to applicants submitting 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 ENGL document takes into account the requirements of the relevant international standards (ISO 24276, ISO 21569, ISO 21570, ISO 21571) and recommendations of the Codex Alimentarius[47].

Method validation is an essential component of the measures that a laboratory, operating its methods under accreditation to ISO/IEC 17025[48], shall implement before releasing test results. The standard requires that the analysis of a sample is performed by using 'validated' methods.

It is important to underline that the ENGL document refers to PCR-based methods since those are generally applied across applicants and control laboratories for GMO analysis. 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).

[41] Li, Y. H., Zhou, G., Ma, *et al.* (2014) De novo assembly of soybean wild relatives for pan-genome analysis of diversity and agronomic traits. *Nat. Biotechnol. 32:*1045-1052.

[42] Jiao, Y., Peluso, P., Shi, J., *et al.* (2017) Improved maize reference genome with single-molecule technologies. *Nature* 546:524-527.

[43] Ossowski, S., Schneeberger, K., Lucas-Lledó, J.I., Warthmann, N., Clark, R.M., Shaw, R.G., Weigel, D., Lynch, M. (2010) The rate and molecular spectrum of spontaneous mutations in *Arabidopsis thaliana*. *Science* 327:92-94.

[44] Zhang, D., Wang, Z., Wang, N., Gao, Y., Liu, Y., Ying, W., Yan, B., Zhibin, Z., Xiuyun, L., Yuzhu, D., Xiufang, O., Chunming, X., Bao, L. (2014) Tissue culture-induced heritable genomic variation in rice, and their phenotypic implications. *PLoS ONE* 9:e96879 (doi:10.1371/journal.pone.0096879).

[45] Jankowicz-Cieslak, J., Tai, T.H., Kumlehn, J., Till, B.J. (2016) Biotechnologies for Plant Mutation Breeding. SpringerLink ISBN 978-3-319-45019-3.

[46] Anderson, J.A., Michno, J.-M., Kono, T.J.Y., Stec, A.O., Campbell, B.J., Curtin, S.J., Stupar, R.M. (2016) Genomic variation and DNA repair associated with soybean transgenesis: a comparison to cultivars and mutagenized plants. *BMC Biotechnol.* 16:41.

[47] Codex Alimentarius Commission (2009) Foods derived from modern biotechnology. FAO/WHO, Rome, Italy.

[48] ISO/IEC 17025:2017, General requirements for the competence of testing and calibration laboratories. International Organization for Standardization, Geneva, Switzerland.

**Table 1.** Method acceptance criteria and performance parameters considered in the ENGL document on minimum performance requirements for methods of GMO testing (version 2015)[3].

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

It should thus be considered to which extent the analytical methods proposed for genome-edited plants would (1) comply with the current provisions of the ENGL document as it is, and (2) if additional explanatory notes or amendments need to be made in order to provide a quality and compliance framework for analytical approaches not yet covered. The most critical aspects for consideration include the following elements:

- **Applicability/Practicability of the method**. For new technologies, *e.g.* next-generation sequencing, the equipment may not be widely available, the quality assurance parameters and uncertainty estimation are still under development, and training may be required in the enforcement laboratories to make sure the methods can be applied in a reliable way.
- **Specificity to be demonstrated *in silico* and experimentally**. In order to develop a detection method that is specific for identification of the genome-edited event, a unique and sufficiently long sequence is required. SNV and short InDels may not provide such a unique sequence. It also needs to be specified which databases and which plant samples have to be used for demonstrating the event-specificity of the method.
- **Robustness of the method**. It needs to be assessed whether methods targeting a SNV or short InDel are sufficiently robust against small modifications to the testing conditions.
- **Sensitivity (Limit of Detection/Limit of Quantification)**. Proof of evidence is required to demonstrate that a method targeting a SNV or short InDel has an acceptable limit of detection in different sample types.

Further considerations are necessary in order to provide guidance on the requirements for detection methods for genome-edited products containing multiple DNA alterations. A characteristic of genome editing techniques such as CRISPR-Cas and TALEN is the possibility to simultaneously modify all alleles of a gene or different genes simultaneously[49,50,51,52,53,54]. This may lead to plants having multiple alterations in their

[49] Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., Qiu, J.-L. (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* 32:947-952.

[50] Wang, Z.P., Xing, H.L., Dong, L., Zhang, H.Y., Han, C.Y., Wang, X.C., Chen, Q.J. (2015) Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in Arabidopsis in a single generation. *Genome Biol.* 16:144.

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 all different alterations in the genome in case they may segregate in subsequent generations. Analysing the performance of multiple methods on a single genome-edited plant makes it more laborious for the EURL GMFF to perform the method validation in an interlaboratory trial and for the enforcement laboratories to carry out the verification of these methods when they are implemented in the laboratory. The case of multiple genome-editing events is to some extent similar to the detection of stacked transformation events in food and feed, with the difference that in the latter case, the regulatory approach demands the validation of a detection method for each of the single transformation events composing the stack, before the validation of the same methods on the stacked product can be started. For genome-edited plants, the 'single events' may not exist independently when multiple alterations have been created at once. Therefore, when two or more single genome-edited events belonging to the same ingredient are found in a food or feed sample, it cannot be concluded if these originate from a multi-edited plant or from segregated single-event plants.

[51] Miao, C., Xiao, L., Hua, K., Zou, C., Zhao, Y., Bressan, R.A., Zhu, J.-K. (2018) Mutations in a subfamily of abscisic acid receptor genes promote rice growth and productivity. *PNAS* 115:6058–6063.

[52] Yu, Z., Chen, Q., Chen, W., Zhang, X., Mei, F., Zhang, P., Zhao, M., Wang, X., Shi, N., Jackson, S., Hong, Y. (2018) Multigene editing via CRISPR/Cas9 guided by a single-sgRNA seed in Arabidopsis. *J. Integr. Plant Biol.* 60:376-381 (doi.org/10.1111/jipb.12622).

[53] Liang, Z., Chen, K., Li, T., *et al.* (2017) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat. Commun.* 8:14261 (doi.org/10.1038/ncomms14261).

[54] Peterson, B. A., Haak, D. C., Nishimura, M. T., Teixeira, P. J. P. L., James, S. R., Dangl, J. L., & Nimchuk, Z. L. (2016) Genome-wide assessment of efficiency and specificity in CRISPR/Cas9 mediated multiple site targeting in *Arabidopsis*. *PLoS ONE* 11:1–11 (doi.org/10.1371/journal.pone.0162169).

# 4 Detection of genome-edited events in the context of market control

Every day, shipments of thousands of tons are arriving at EU harbours 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. Moreover, custom inspectors collect and prepare a sample for laboratory analyses (controlling for GMOs, mycotoxins, heavy metals, pesticides, etc.) according to the applicable sampling schemes and recommendations.

Bulk grain that arrives in a harbour, and similarly any food or feed product produced from it, is a compound product composed of different source materials, including plant varieties with different genetic backgrounds, cultivated by various farmers in various regions of the world and present in different proportions. Samples taken from these products are analysed by the official control laboratories of the EU Member States for the presence of GMOs. Real-time PCR-based methods are well-established analytical techniques adopted by all control laboratories in the EU. Methods for detection need to be robust and applicable to the typical heterogeneous nature of food and feed samples tested by enforcement laboratories.

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.* T-*nos*), or protein coding sequences (*e.g. cp4 epsps*, *pat* or *cry1Ab*) that are commonly found in authorised as well as in unauthorised conventional GMOs. These methods will react positively for all GMOs that contain the element-specific sequences.

Based on the outcome of the initial screening, 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 (http://gmo-crl.jrc.ec.europa.eu/gmomethods/). This strategy may lead to the direct detection of an unauthorised GMO (in the case of known unauthorised GMOs that may have been detected earlier), but it may also lead to the conclusion that some of the detected GMO screening targets could not be explained in this way. These unexplained elements may point indirectly at the presence of (additional) unauthorised GMOs in the sample. Subsequent research, for example using targeted or untargeted sequencing[55,56], is then required to elucidate the background of the identified GMO elements. In this way GMOs without an EU authorisation application, with or without prior information on the modification, may be detected insofar they contain a common screening marker[57].

For genome-edited plants such screening methods generally are not possible, as the plants considered in this report do not contain any transgene sequence nor any other common 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. As a consequence, it can be asssumed that in the near future the distinction between detection by screening and subsequent identification may not be applicable as for conventional GMOs. Instead, detection and

[55] Košir, A.B., Arulandhu, A.J., Voorhuijzen, M.M., Xiao, H., Hagelaar, R., Staats, M., Costessi, A., Žel, J., Kok, E.J., van Dijk, J.P. (2017) ALF: a strategy for identification of unauthorized GMOs in complex mixtures by a GW-NGS method and dedicated bioinformatics analysis. *Sci. Rep.* 7:14155 *(*doi:10.1038/s41598-017-14469-8).

[56] Wahler, D., Schauser, L., Bendiek, J., Grohmann, L. (2013) Next-Generation Sequencing as a tool for detailed molecular characterisation of genomic insertions and flanking regions in genetically modified plants: a pilot study using a rice event unauthorised in the EU. *Food Anal. Meth.* 6:1718-1727.

[57] ENGL (2011) Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified materials. Guidance document of the ENGL. (http://gmo-crl.jrc.ec.europa.eu/doc/2011-12-12%20ENGL%20UGM%20WG%20Publication.pdf).

identification will coincide, as the detection of genome-edited events already requires targeting the unique sequence in the analysis.

Alternative approaches to PCR 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[30,59,58]. It uses the known sequences of conventional GMOs (common elements or coding sequences of transgenes) as a 'bait' to detect both authorised and unauthorised GMOs in a market sample. This screening approach is dependent on the presence of combinations of foreign DNA sequences and cannot detect genome-edited events. As a consequence there are no robust laboratory methods to assure that unknown unauthorised genome-edited products could be prevented from entering the market.

If marketed genome-edited plants are not sufficiently assessed during development, unwanted transgenic sequences (*e.g.* vector backbone sequences) may potentially have remained in the genome in case the genome editing technique employed involved integration of the construct into the plant genome and it was not carefully segregated out in subsequent crosses[59,60,61]. This will require developing additional screening methods for the detection and as well the identification of such unintentionally remaining recombinant DNA sequences.

The implementation of methods for the detection of genome-edited plants in the process of an application for EU authorisation depends strongly on the prior knowledge of the sequence alteration and on the availability of reference material. Only if the analytical procedure for detection, identification and quantification of a genome-edited product has been found fit for the intended purpose by the EURL GMFF, then the validated method may be generally applied for control purposes. The genotype of such plant product from a homogeneous sample might be identified in a homogeneous (reference) sample. However, in heterogeneous samples (commodities) unambiguous detection of hidden admixtures and identification of individual genotypes will be not possible in most cases[62].

In the absence of a market authorisation request in the EU, some genome-edited plants may have been authorised in other markets, and information could have 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 method, already published or to be developed, may allow detection of the genome-edited product. However, at the current state no assessment has been carried out for any method for the detection of any genome-edited plant product by the ENGL or the EURL.

The detection of very small sequence 'signatures' by bioinformatics and of genetic or methylation 'scars', as hypothesised recently[63], does not provide realistic evidence and proof that a new breeding technique was applied and has caused a detected DNA alteration. Signatures like the PAM sequence (PAM- Protospacer adjacent motif - a 2-6 bp

[58] Fraiture, M.A., Saltykova, A., Hoffman, S., Winand, R., Deforce, D., Vanneste, K., De Keersmaecker, S.C.J., Roosens, N.H.C. (2018) Nanopore sequencing technology: a new route for the fast detection of unauthorized GMO. *Sci. Rep. 8:7903.*

[59] Braatz, J., Harloff, H.J., Mascher, M., Stein, N., Himmelbach, A., Jung, C. (2017) CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different homoeologous gene copies in polyploid oilseed rape (*Brassica napus*). *Plant Physiol.* 174:935-942.

[60] Li, W.X., Wu, S.L., Liu, Y.H., Jin, G.L., Zhao, H.J., Fan, L.J., Shu, Q.Y. (2016) Genome-wide profiling of genetic variation in *Agrobacterium*-transformed rice plants. *J. Zhejiang Univ. Sci*. B 17:992–996.

[61] Schouten, H.J., vande Geest, H., Papadimitriou, S., Bemer, M., Schaart, J.G., Smulders, M.J.M., Sanchez Perez, G., Schijlen, E. (2017) Re-sequencing transgenic plants revealed rearrangements at T-DNA inserts, and integration of a short T-DNA fragment, but no increase of small mutations elsewhere. *Plant Cell Rep.* 36:493–504.

[62] Grohmann, L., Keilwagen, J., Duensing, N., Dagand, E., Hartung, F., Wilhelm, R., Bendiek, J., Sprink, T. (2019) Detection and identification of genome editing in plants – challenges and opportunities. *Front Plant Sci.* 10:236 (doi:10.3389/fpls.2019.00236).

[63] Bertheau, Y. (2019) New Breeding Techniques: Detection and Identification of the Techniques and Derived Products. In: *Reference Module in Food Science, Encyclopedia of Food Chemistry, pp.* 320-336 (doi.org/10.1016/B978-0-08-100596-5.21834-9).

DNA sequence immediately following the DNA sequence targeted by the Cas nuclease) are relevant only for the CRISPR technique and vary depending on the type of Cas protein used. 'Scars' are potentially created in cells that have been directly treated by any mutagenesis technique or passed through tissue culture and are not exclusively induced by genome editing. Moreover, it is not clear to what extent epigenetic changes are stable across breeding generations.

The identification of DNA alterations from genome editing 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 with conventional mutagenesis.

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[64]. 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. Moreover, analytical confirmation for enforcement of the regulations would still be very challenging.

[64] Ruttink, T., Morisset, D., Van Droogenbroeck, B., Lavrac, N., Van Den Eede, G.L.M., Zel, J., De Loose, M. (2010) Knowledge-technology-based discovery of unauthorized genetically modified organisms. *Anal. Bioanal. Chem.* 396:1951-1959.

# 5 Conclusions and outlook

This report highlights analytical challenges and limitations related to the detection, identification and quantification of genome-edited food and feed products of plant origin.

Similarly to conventional GMOs, products of genome editing can only be readily detected and quantified in commodity products by enforcement laboratories if prior knowledge on the altered genome sequence, a validated detection method and certified reference materials are available.

The ENGL has issued a guidance document specifying the minimum performance requirements (MPR) of methods for GMO testing[3]. This document is informative for applicants submitting an event-specific detection method for a GMO as part of a request for market authorisation and provides the acceptance criteria for the EURL GMFF when validating the detection method. The document will need to be reviewed to clarify the implications for methods for genome-edited plant products. On the basis of the current knowledge and technical capabilities, it is unlikely that a method for a genome-edited plant product with only single nucleotide variations or short InDels would fulfil the performance requirements for methods of GMO testing, *e.g.* regarding applicability, sensitivity, specificity and quantification aspects.

The major bottleneck relates to providing proof for the origin of a detected DNA alteration, *i.e.* to be able to demonstrate that it was created by genome editing and refers to a unique genome-edited event that can be traced back to a specific genome-editing process. This may in principle be possible for unique DNA alterations, *e.g.* a large sequence deletion not mimicked by an identical alteration that has been identified already in the (natural) plant pan-genome. However, for non-unique DNA alterations affecting one or a few DNA base pairs, an applicant may not be able to develop an event-specific method.

In the absence of prior knowledge on the potential genome-edited alterations in a plant, their detection and identification by the enforcement laboratories does not seem to be feasible by using routinely applied detection methods and established analytical instrumentation. The general analytical screening strategy, as employed for conventional GMOs, cannot be applied for genome-edited plant products, as no common sequences are present that could be targeted for screening. In case a DNA alteration has been detected, there are currently no procedures established that facilitate an unambiguous conclusion that genome editing has created the alteration.

Therefore, plant products obtained by genome editing may enter the market undetected. Moreover, if a suspicious product with an unknown or non-unique DNA alteration would be detected on the EU market, it would be difficult or even impossible to provide court-proof evidence that the modified sequence originated from genome editing.

Several issues with regard to the detection, identification and quantification of genome-edited products cannot be solved at the present time, for example due to a lack of experimental verification, and will require further consideration. Technologies different from the currently applied qPCR methods may need to be implemented in the enforcement laboratories; additional resources will need to be made available and experience has to be developed. For known genome-edited events, alternative screening strategies targeting all known genome-edited events simultaneously may have to be developed to facilitate routine enforcement. Furthermore, under the current regulatory system the event-specific detection method is linked to a specific product application for market authorisation. However, the targeted mutagenesis techniques allow to reconstruct exactly the identical genome-edited product in another plant. Thus, the detection method for the food or feed product is no longer specific for the original genome-edited product, but would also detect the reconstructed product which has not received a market authorisation. The implications of this need to be further investigated.