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Detection of food and feed obtained by new plant mutagenesis techniques

European Network of GMO Laboratories (ENGL)

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Contents

Executive summary

The European Network of GMO Laboratories (ENGL) has reviewed the possibilities and challenges for the detection of food and feed obtained by new plant mutagenesis techniques based on genome editing.

The procedures for the validation of detection methods as part of the *market authorisation* of genome-edited plant products will in principle be similar as for the current conventional GMOs. It is, however, questionable if an event-specific quantitative detection method can be developed for such GMOs, particularly when they are characterised by a short DNA alteration consisting of one or a few base pairs. Such detection methods will probably lack the specificity required to target the unique DNA alteration in the genome-edited plant and quantification of the presence of the specific product in a complex food or feed material may not be possible. Detection methods may therefore fail to fulfil the method performance requirements and, as a consequence, may result in the rejection of the application. For genome-edited plant products with a large DNA alteration an event-specific quantitative detection method may be developed and could pass the validation process in case the DNA alteration does not also occur naturally; this will need to be demonstrated.

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 EU Reference Laboratory for GM Food and Feed (EURL GMFF) for validation of the event-specific methods. *E.g.* it is currently unclear how to demonstrate or assess the specificity of the method against all existing marketed varieties. Furthermore, it needs to be evaluated under which conditions an event-specific detection method would be required for all DNA alterations in a multi-edited plant and how to quantify such products based on measurement results for the individual genome edits.

For *market control*, considering the current knowledge and state of the art of DNA analysis, it is not possible for enforcement laboratories to detect the presence of unauthorised genome-edited GMOs in food or feed entering the EU market without prior information on the altered DNA sequences. The same kind of screening methods that are commonly used to detect conventional GMOs cannot be applied nor could be developed for genome-edited GMOs as there are often no common sequences that could be targets for such screening methods. Targeted or non-targeted DNA sequencing may be able to detect specific DNA alterations in a product, however, this does not necessarily confirm the presence of a particular genome-edited GMO since the same DNA alteration could occur naturally in other plants obtained by conventional breeding or mutagenesis techniques which are exempted from the GMO regulations.

Therefore, it is concluded that validation of an event-specific detection method and its implementation for market control will be feasible for genome-edited GMOs carrying a known DNA alteration that has been shown to be unique and can only be obtained by application of a genome editing technique. With the current technological capabilities, market control will, however, not be possible in the absence of an event-specific detection method or for unknown genome-edited food or feed products.

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 inclusive as it addresses the development of GMOs, the stepwise release into the environment, the general cultivation and seed production, marketing, labelling 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), who has issued a guidance document explaining the minimum performance requirements (MPR) for analytical methods of GMO testing[3]. Since the labelling legislation[2] 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 or, in specific cases[4], should not exceed the Minimum Required Performance Limit (MRPL) that has been set at 0.1 % in mass fraction (see Text box 1).

The EURL GMFF also has a legal mandate under the "Official Controls Regulation"[5], 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 controls 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[6] products in their national markets; this is performed by applying – when available – first-line screening methods and the detection methods validated for the authorised and known unauthorised 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] 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.

[6] In contrast to food and feed (and seed used as or in food or feed), there is a zero tolerance for GMOs present in seed lots used for cultivation.

Text box 1

Different authorisation statuses of GMOs from an EU perspective

Authorised

Authorised GM material is allowed on the EU market. Authorisation mostly concerns the import of GMOs and their use in food and feed. Few authorisations have been submitted for cultivation of GM crops and currently one GM maize 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 below 0.9% and provided that these traces are adventitious or technically unavoidable.

Non-authorised

- Pending authorisation: a GMO has been authorised for commercial use in one or more third countries, while a submitted application for authorisation in the EU is pending.
- Authorisation expired: a GMO of which the authorisation has expired and no renewal application has been submitted.

Only for feed materials and feed additives, GMOs in these two categories above may be present at a level below 0.1% related to mass fraction, under the conditions of Commission Regulation (EU) No 619/2011. A validated quantification method and certified reference material are available for these GMOs.

- GMOs that have been authorised for any other purpose than for placing on the market, under Part B of the Directive 2001/18. 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 or Regulation 1829/2003.

GMOs in these two categories are not allowed on the EU market. Zero-tolerance applies.

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 [7]). 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, resulting in single nucleotide variants (SNV) or short or large 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 part of the chromosome copies (called the alleles of a gene) may carry the alteration (*e.g.* in a tetraploid (4n) crop between one and 4 DNA copies may contain the DNA alteration).

Upon request of DG SANTE, the JRC reviewed in 2011 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 crops based on them[8]. Additionally, challenges for the detection of organisms developed through these techniques were evaluated[9]. The topic has since been discussed during meetings of the ENGL. In the past few years, a novel innovative technique for genome editing, CRISPR-Cas, with wider

[7] 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).

[8] 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).

[9] 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).

potential and easier applicability, has rapidly advanced plant biology research and the development of applications for plant breeding[7,10].

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 a long history of safety, are not exempted from the GMO legislation[11]. 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[2], including the requirements for method validation as part of the GMO authorisation procedures, and (2) to the provisions of the official controls regulation[5] 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 consulted have an in-depth expertise with respect to GMO analysis but the viewpoints mentioned here are not based on extensive experimental work on genome-edited food or feed.

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

[11] 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 GMOs obtained by recombinant DNA technology and characterised by the presence of introduced DNA sequences from the same or other species in the final plant. GMOs require an authorisation before entering the EU market, otherwise they are called unauthorised and their presence is restricted; this is further clarified in Text box 1.

Genome editing, also called gene editing, is a group of site-directed mutagenesis techniques that allows adding, removing, or altering DNA sequences at a specific location in the genome. It 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 [7]). The techniques require the presence of the SDN in the recipient host cell (in this document we will refer only to plant cells, but other organisms can be targets of genome editing), 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. When recombinant DNA has been used, it can be selected against in subsequent generations resulting in genome-edited plants that no longer contain any recombinant DNA[12,13]. In the frame of this report, plants obtained with genome editing techniques that contain inserted foreign DNA are excluded, as these will be similar to the current conventional GMOs.

Early but limited success 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[9]. 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[14].

The DNA sequence alterations introduced through any of the genome editing techniques may be single nucleotide variants (SNV), short or large insertions or deletions (called InDels), or, less frequently, gene duplications, inversions and translocations[15]. "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 a detection method, the criterion to be assessed is not the sequence length itself, but whether or not a given DNA alteration occurs already in any plant species or potentially could occur and whether or not it can be unequivocally attributed to the application of a genome editing technique. 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 plant product that contains the altered DNA sequence, as indicated above, at a specific site in the genome as a result of the genome editing technique applied. A prerequisite is that no foreign 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 result in several intended DNA alterations in the genome, each of these multi-edits, when segregating independently,

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

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

[14] 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.

[15] 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.

should be regarded as a separate genome-edited event and, for the enforcement, 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, expressed in mass fraction per total mass of the ingredient or plant species (m/m %). For 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 authorised events (0.9 m/m %). The same applies to GMOs with pending or expired authorisation status that are detected in feed[4], where it needs to be assessed if their mass fraction is below the minimum required performance limit (0.1 m/m %). Methods for the detection of unauthorised GMOs, however, do not, in principle, need to be quantitative as detection *sensu stricto* is sufficient for assessing non-compliance of the product (cf. zero tolerance policy).

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 wanting to apply for market authorisation 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. GMO producers (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 GMO legislation came into force[2].

In most of these cases, the GMOs contained one or more 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 new DNA sequence(s). For each insertion, two unique insert-to-plant junctions are generated at both ends of the integration site. The unique junctions created during a transformation event can be exploited as a unique identification marker for developing methods of detection specific for each conventional GMO (often referred to as "event-specific" detection).

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 analytical detection, identification and quantification of GMOs is typically based on real-time PCR (also called quantitative PCR or qPCR), targeting the DNA molecule. 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 trial involving qualified ENGL laboratories. 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[16,17].

In order to evaluate whether PCR-based methods may fulfil the ENGL performance criteria when applied to detection and quantification of genome-edited products, the experience from other fields of diagnosis has been reviewed. The analysis was focused on the detection and quantification of SNVs and small InDels since large insertions/deletions may constitute a likely unique sequence (to be demonstrated) with suitable unique junction regions that can be targeted by PCR.

The specificity of qPCR methods may be enhanced by use of particular probes (Minor Groove Binding (MGB) probes) as shown in other fields, *e.g.* to genotype SNVs in peripheral

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

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

blood or in bulk raw milk[18,19]. Assessment of the performance of a SNV allelic discrimination assay described in those reports indicated that quantitative parameters such as PCR efficiency, slope and linearity could be in line with those established by the ENGL. However, in those studies the target relative concentration tested (50%) was much higher than the levels required for the quantification of GMOs (i.e. 0.1%-5%). Not only the sensitivity of the method but also its specificity clearly is an issue, particularly when applied to market products with a complex composition.

Systematic studies on effects of mismatches for primers and probes[20,21] with qPCR methods have shown that non-specific amplification from non-target sequences may occur and induce quantification errors ranging between 33% and 63% or even a 658-fold underestimation of the initial copy number showing the difficulties to properly quantify SNVs. A probe-free quantitative PCR method has been shown to detect minor mutant alleles with a frequency as low as 0.1% and high specificity was obtained by adding to the reaction mix a 'T-blocker' oligonucleotide designed to prevent amplification of the wild type sequence[22].

This and other strategies would require however significant level of method optimisation to ensure successful transferability for reliable use and reproducible response across laboratories.

Droplet digital PCR (ddPCR) methodology used for the detection of off-target DNA alterations (generally InDel mutations) generated by sequence-specific nucleases (SSNs) could be similarly applied to the detection and quantification of genome-edited events. ddPCR methods have been used for the screening and confirmation of particular mutations in induced pluripotent stem cells or primary cells at very low concentrations (0.01% and 0.2% for SNV or InDel mutations[23,24]); these are levels comparable to those required by the labelling provisions of the EU legislation framework on GMOs. An internal reference probe specific for a sequence not affected by the mutation but included in the target PCR product has been used in those assays to assess the total amount of wild-type alleles present in the sample. In those ddPCR assays the simultaneous quantification of both wild-type and mutated sequences from the same PCR amplicon facilitated the precise relative quantification independently from potentially interfering parameters such as DNA quality and amplification efficiency. The correct design of two probes each binding to the mutated and wild-type sequence is therefore crucial for ensuring the required specificity of the method; 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.

[18] de Andrade, C.P., de Almeida, L.L., de Castro, L.A., Driemeier, D., da Silva, S.C. (2103) 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).

[19] 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).

[20] Süss, B., Flekna, G., Wagner, M., Hein, I. (2009) Studying the effect of single mismatches in primer and probe binding regions on amplification curves and quantification in real-time PCR. *J Microbiol Meth*. 76:316-319 (doi:10.1016/j.mimet.2008.12.003).

[21] Stadhouders, R., Pas, S.D., Anber, J., Voermans, J., Mes, T.H., Schutten, M. (2010) The effect of primer-template mismatches on the detection and quantification of nucleic acids using the 5' nuclease assay. *J. Mol. Diagn.* 12:109-117 (doi:10.2353/jmoldx.2010.090035).

[22] Kim, H., Ruby, A.E., Shandilya, H.G., Virmani, A.K., Rahman, N., Strange, C.M., Huuskonen, J. (2018) T-blocker: a simple and robust probe-free quantitative PCR assay to detect somatic mutations down to 0.1% frequency. *BioTechniques* 65:205–210.

[23] 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).

[24] 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).

Other authors[25] have compared the relative specificity and sensitivity of real-time qPCR versus ddPCR assays in detecting and quantifying SNVs or small InDels in individual founder transgenic mice generated by CRISPR/Cas9 mutagenesis; they observed a lower rate of false-positive unedited events when using a ddPCR platform. Significant higher sensitivity to all single and small InDel mutations and better accuracy were obtained also by using a locked nucleic acid probe for the real-time or ddPCR reactions that increases the discrimination of mismatch sequences and destabilises non-specific binding. In summary, the ddPCR methods seem to be preferred above qPCR methods, however the accuracy, trueness and specificity of the methods have not been systematically evaluated for genome-edited products.

Moreover, the development of event-specific PCR methods as described above may not lead to sufficiently specific quantification methods for short altered sequences (one or a few nucleotides long).

Alternatively, in biomedicine, special modelling methods using next-generation sequencing (NGS) data have been developed and validated to detect low abundant variants in cancer samples[26]. A similar framework could potentially be applied to food and feed samples. Briefly, in such approaches, high-throughput sequencing (NGS) methods are applied either to sequence entire genomes (WGS, whole genome sequencing) or multiplexed barcoded amplicons. The variant's detection specificity and selectivity are then statistically estimated in relationship to the crop natural variation. The main issue with this methodology is to avoid or limit false positives. As far as we know, such methodologies have never been formally applied on food and feed products and would require proper validation and benchmarking. As the first step in NGS is done by PCR, the same possible limitation is the inappropriate quality of the extracted DNA; however, other factors such as the variation in crop genome size and ploidy (*e.g.* hexaploid wheat or tetraploid potato) and the level of knowledge of expected variability for the entire genepool for each crop also complicate the interpretation of the results. Another issue is the sensitivity of these NGS approaches for detecting variants present at low frequency in the analytical sample. The sensitivity of NGS to detect somatic mutations as a function of sequencing depth and allelic fraction has been investigated in tumour samples[27]. Simulations show that a theoretical sensitivity of 0.58 is expected for a mutation allele fraction of 0.05 (5%) with tumor sequencing depth of 60x. These sensitivity values are not in line with the acceptance performance requirements established by the ENGL for GMO analysis.

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[27] 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 sequences 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

[25]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).

[26]Cibulskis, K., Lawrence, M.S., Carter, S.L., Sivachenko, A., Jaffe, D., Sougnez, C., Gabriel, S., Meyerson, M., Lander, E.S., Getz, G. (2013) Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat. Biotechnol.* 31:213-219.

[27] 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.

the process that generated the GMO, independent of further breeding activity to cross the GM event into different genetic backgrounds.

The situation is more 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 (see examples in Text box 2). 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. This would make it impossible to trace the genome-edited event to a unique identification marker, developed by a specific company in a specific genome-editing experiment by current state-of-the-art technologies. The ownership of and liability for a genome-edited plant may therefore be unclear.

Text box 2

Examples of genome editing applications in plants with similarity to existing commercial varieties

The Canadian company Cibus Inc. used Oligonucleotide Directed Mutagenesis (ODM, a form of genome editing) on single cells (protoplasts) followed by regeneration in tissue culture to create a mutated *Brassica napus* plant (canola) with a single nucleotide change in the *BnAHAS1* gene leading to an amino acid substitution of the corresponding protein; the mutated protein confers resistance to certain 'group 2' herbicides (*e.g.* imidazolinones and sulfonylureas). Although the mutated plant was isolated after ODM treatment, the company reported that the single nucleotide mutation was the result of a spontaneous somaclonal variation that occurred during the tissue culture process, and not due to the specific oligonucleotide used as template in the ODM protocol. The mutated plant line was subsequently crossed with a conventionally-bred imidazolinone-tolerant canola line (CLEARFIELD canola variety SP Cougar CL), which contains the same mutation in its *BnAHAS3* gene, to produce canola line 5715, homozygous for the mutation in both *BnAHAS1* and *BnAHAS3* genes[28]; these genes, residing on the C and A subgenome of *B. napus*, respectively, encode protein subunits of the acetohydroxyacid synthase (AHAS) enzyme. Another company, Pioneer Hi-Bred, developed a similar imidazolinone-tolerant canola by chemically inducing variation in the *BnAHAS2* gene using microspores. Besides canola, there are many other agriculturally-important crops, including maize, wheat and rice, as well as weeds, showing the same trait as a result of similar sequence alterations in their AHAS genes[29,30].

In tomato, genome editing has been conducted in a key gene (*psy1*) responsible for the red colour of the tomato fruit. A range of sequence alterations (from SNVs to short InDels) were produced through CRISPR-Cas9, all resulting in the yellow fruit phenotype[31]. Yellow tomato varieties also occur already on the market, either produced by conventional breeding or as a result of chemical mutagenesis. All these varieties contain mutations in the same gene but at different positions.

In maize, a high amylopectin variety was produced by CRISPR-Cas targeted deletion of the waxy (*Wx1*) gene directly in elite inbred lines[32]. *Wx*1 is one of the most studied "classical" maize genes, with over 200 spontaneous or induced mutations (deletions, insertions, translocations of various length) known to lead to the waxy phenotype[33]. A commercial natural waxy maize variety cultivated since the mid-1980s contains a sequence deletion in the middle of the gene[34].

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 all varieties and wild plants of all species used for

[28] Hu, M., Pu, H., Kong, L., Gao, J. Long, W., Chen, S., Zhang, J., Qi, C. (2015) Molecular characterization and detection of a spontaneous mutation conferring imidazolinone resistance in rapeseed and its application in hybrid rapeseed production. *Mol Breed.* 35:46, https://doi.org/10.1007/s11032-015-0227-3.

[29] Tan, S., Evans, R.R., Dahmer, M.L., Singh, B.K., Shaner, D.L. (2005) Imidazolinone-tolerant crops: history, current status and future**.** *Pest Manag. Sci.* 61:246–257.

[30] Svitashev, S., Schwartz, C., Lenderts, B., Young, J.K., Cigan, A.M. (2016) Genome editing in maize directed by CRISPR–Cas9 ribonucleoprotein complexes. *Nature Comm.* 7:13274 (DOI: 10.1038/ncomms13274).

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

[32] 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).

[33] MaizeGDB (2017) Gene Record: GRMZM2G024993/ZEAMMB73_617956 (wx1 - waxy1) [Classical Gene List] Maize Genetics and Genomics Database. Available online at: http://www.maizegdb.org/gene_center/gene/12768.

[34] Fan, L., Bao, J., Wang, Y., Yao, J., Gui, Y., Hu, W., *et al.* (2009) Post domestication selection in the maize starch pathway. *PLoS ONE* 4:e7612 (doi: 10.1371/journal.pone.0007612).

food or feed production, which would serve as reference basis[35,36,37,38] (see Text box 3). In case of single nucleotide alterations it will be difficult to guarantee that the same SNV does not exist in other varieties/populations, or will be created spontaneously 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 which may exist already in conventional varieties[39]. Without access to continuously updated pan-genome databases for all plant species, it is probably not possible for applicants to provide this information or for the EURL GMFF to verify this information and to conclude that the method submitted is event-specific.

Text box 3

Variability of plant genomes

Advances in whole genome sequencing in recent years have revealed that the genome sequences of crop species are diverse and dynamic. Dispensable genes may constitute a significant proportion of the pan-genome, *e.g.* around 20% in soybean[40]. 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[41]. 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 genome sequences.

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[42], meaning that 175 new variants (SNVs) would arise per 100 individual plants per generation. This natural mutation rate may be increased as much as 250-fold as a result of the stresses induced by *in vitro* culture conditions that are commonly used for the breeding of many commercial crops, *e.g.* 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[43]. 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[44]. 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.

Consequently, it is expected that applicants will not be able to develop an event-specific detection method for a genome-edited plant carrying a DNA alteration that may not be unique. It will need to be assessed on a case by case basis if a given DNA alteration corresponds to a specific genome-editing event that can be targeted by a detection method

[35] 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 Online* 26:121–135; Lu *et al.* (2015) High-resolution genetic mapping of maize pan-genome sequence anchors. *Nat. Comm.* 6:1-8.

[36] 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.

[37] 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.

[38] The 3,000 rice genomes project (2014) *Gigascience* 3:7; 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.

[39] 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. Bioen & Biotech.* 6:213.

[40] 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.

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

[42] 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.

[43] 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).

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

fulfilling all minimum performance requirements, including regarding specificity. It is currently unclear how this specificity can be assessed, both *in silico* and experimentally.

In conclusion, whereas the detection *sensu stricto* of genome-edited events may in a limited number of cases be technically feasible, the same level of specificity for identification as currently applicable to conventional GM event-specific methods most likely will not be achieved for methods targeting most genome-edited plants. This will have important consequences for enforcement of the GMO legislation.

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[45]. The document is addressed to applicants submitting detection methods according to Regulation (EC) No 1829/2003 and it provides criteria upon which methods for GMO analysis are assessed and validated by the EURL GMFF. The ENGL document takes into account all the requirements of the relevant international standards (ISO 24276, ISO 21570, ISO 21571) and recommendations of the Codex Alimentarius[46].

Method validation is an essential component of the measures that a laboratory, operating its methods under accreditation to ISO/IEC 17025, shall implement before releasing analytical data. The standard requires that the analysis of a sample is performed by using "fully validated" methods. 'Full' validation of an analytical method usually includes an examination of the characteristics of the method in an inter-laboratory method performance study.

It is important to underline that the ENGL document refers to polymerase chain reaction (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).

Table 1. Acceptance criteria and method performance parameters considered in the ENGL document on minimum performance requirements for methods of GMO testing (version 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

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

[45] European Network of GMO Laboratories (2015) Definition of minimum performance requirements for analytical methods of GMO testing (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).
[46] Codex Alimentarius Commission (2009) Foods derived from modern biotechnology. FAO/WHO, Rome, Italy.

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, *e.g.* 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: small nucleotide changes may not be significant enough to generate a unique sequence that can be exploited to develop a detection method that is specific for identification of the genome-edited event; which databases and plant samples need to be used for demonstration of the event-specificity of the method?
- Robustness of the method: it needs to be assessed if methods targeting a SNV would be sufficiently robust against small modifications to the testing conditions.
- Sensitivity (Limit of Detection/Limit of Quantification): what is the proof of evidence required to demonstrate that a method has an acceptable specificity and limit of detection also in complex food or feed samples?

Further consideration is 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[47,48,49,50,51,52]. This may lead to plants having 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 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 as that experienced for stacked transformation events (which also cannot be specifically detected 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. Furthermore, the quantification of multi-edited plants cannot be performed using the current state of the art, and only the single events can be measured, similarly as for conventional GMO stacks. Therefore, when two or more single genome-edited events belonging to the same

[47] 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.

[48] Wang, Z.P., Xing, H.L., Dong, L., Zhang, H.Y., Han, C.Y., Wang, X.C., Chen, QJ. (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.

[49] 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.

[50] 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, https://doi.org/10.1111/jipb.12622.

[51] Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., … Gao, C. (2017). Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nature Publishing Group, 8, 1–5. http://doi.org/10.1038/ncomms14261*

[52] 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*(9), 1–11. http://doi.org/10.1371/journal.pone.0162169

ingredient are found in a food or feed sample, it cannot be concluded if these originate from a multi-edited plant or from separate single-event plants.

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 crop 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 based on an analytical screening strategy for DNA sequences, such as gene promoters (*e.g.* CaMV *p35S*), 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 and further testing will need to identify the specific GMO(s) present in the sample.

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. This strategy may lead to the direct identification 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; additional research, for example using targeted sequencing[53], is required to elucidate the background of the identified GMO elements. In this way GMOs without an EU authorisation request, with or without prior information on the modification, may be detected[54].

For genome-edited crops, such screening methods generally are not possible, as such crops 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.

Alternative 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

[53] 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. (2014) 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).

[54] ENGL (2012) 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).

control laboratories for the detection of unauthorised GMOs[55,56,57]. 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 edits (of any length of insertion or deletion). As a consequence there are no robust laboratory methods to assure that unauthorised genome-edited products could be prevented from entering the market.

While unlikely to occur in marketed crops, unwanted transgenic 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. 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 crops 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 had been found fit for the intended purpose by the EURL GMFF, then the validated method may be generally applied for control purposes.

In the absence of a market authorisation request in the EU, some genome-edited plants may have been authorised in other markets, or information could have been published in scientific journals. When 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 EURL.

The detection of very small sequence 'signatures' by bioinformatics and of genetic or methylation 'scars', as hypothesised recently[58], 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 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, therefore, remains extremely difficult as the altered sequences may mimic naturally occurring sequence variants, or they cannot be distinguished 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 using web crawling and text mining technologies using descriptive keywords, to be followed by analytical confirmation[59]. Such laborious approach, if

[55] 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.*

[56] 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.*

[57] Arulandhua, 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.

[58] 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)

[59] 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.

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 crops, 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 would still be very challenging to enforce the regulations.

5 Conclusions

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

The ENGL has issued guidelines specifying the minimum performance requirements of methods for GMO testing. The 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. While this document will need to be reviewed to clarify the implications for methods for genome-edited GMOs, it is on the basis of the current knowledge and technical capabilities unlikely that a method for a genome-edited GMO would fulfil the performance requirements. It would need to be demonstrated that such methods provide the level of applicability, selectivity and specificity needed for the enforcement of legislation. The largest 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 single developer. This may be possible for large DNA alterations, *e.g.* a large sequence deletion, not mimicked by identical alterations that have been detected already in the (natural) plant pan-genome. According to the current state of the art, for small DNA alterations affecting one or a few DNA base pairs, the specificity of a detection method cannot be ensured. In all cases, it will be challenging to demonstrate the specificity of a detection method for a genome-edited GMO, as this would require access to a substantial proportion of the genetic variation in the germplasm of all plant species that are used for food and feed production at any time.

In the absence of prior knowledge on the potential genome-edited alterations in a crop, their detection and identification by the enforcement laboratories does not seem to be feasible. A general analytical screening strategy, as employed for conventional GMOs, cannot be developed for most genome-edited GMOs. When a DNA alteration has been detected, there are also no procedures that allow to unambiguously conclude that genome editing has created the alteration. At the same time, it may be possible to set up screening strategies for particular sequences of interest, for instance when the sequences are unique and known to result from genome-editing.

Therefore, many products obtained by genome editing may enter the market undetected. Moreover, if a suspicious product would be detected at the EU market, it is unclear how to provide legal proof on whether or not a modified sequence originated from a genome editing technique.

In conclusion, implementation of the GMO legislation will be possible for authorised genome-edited plants for which an event-specific detection method could be validated. With the current technical capabilities, market control will, however, not be possible in the absence of an event-specific detection method or for unknown genome-edited food or feed products.

602

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