Challenges for the detection and identification of NGT products

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Who needs GMO detection methods?

- Official control laboratories
- Food and feed producers
- Plant breeders
- GMO developers/producers
- Non GMO certification agencies

- GMO traceability!
- GM food/feed labelling
- Post Market Monitoring
- Post Market Environmental Monitoring (to some extent)

The European Network of GMO Laboratories (ENGL)

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ENGL

The European Network of GMO Laboratories (ENGL) is a consortium of official enforcement laboratories designated by the EU Member States plus Norway, Switzerland and Turkey.
The primary purpose of the ENGL is to assist the EURL GMFF in its tasks laid down in Regulation (EC) No 1829/2003 and to help solving the challenges of detection, identification and quantification of GMOs.

This objective is achieved through various activities:

- Organisation of plenary meetings for the exchange of experience;
- Preparation of guidance documents on topics of interest to the enforcement laboratories;
- Co-operative research, exchange of scientists, training;
- Technology transfer between ENGL members;
- Exchange of scientific literature.

The network was inaugurated in Brussels on December 4th 2002 and is governed by the ENGL consortium agreement. The EURL GMFF chairs the ENGL and provides its secretariat.

The ENGL chairman with representatives of the 24 GMO laboratories from Accession Countries who officially signed the ENGL agreement

https://gmo-crl.jrc.ec.europa.eu/ENGLabs#inline-nav-engl-circabc

Working group established by the European Commission in **2010** evaluated whether certain new techniques constitute techniques of genetic modification and if the resulting organisms fall within the scope of the EU GMO legislation.

Report published in 2011

Science based report

Evidence based research

Challenge – new types of GMOs

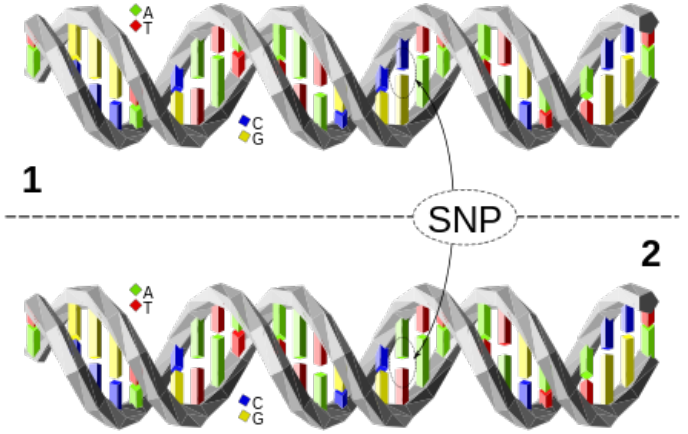
NGTs – new types of DNA alterations

Altered DNA sequence, at a specific site in the genome resulting from the use of particular technique (**no recombinant DNA remain in the genome of the final plant**).

NGT - UMBRELA TERM – various techniques – different products

- **Single nucleotide variants (SNV)**
- Insertions or deletions (InDels)
- Gene insertion and duplication
- Inversions and translocations

- Large alterations (several dozen base pairs) - unique
- Short alterations (one or few base pairs) – unique?



substitution of a single nucleotide that occurs at a specific position in the genome

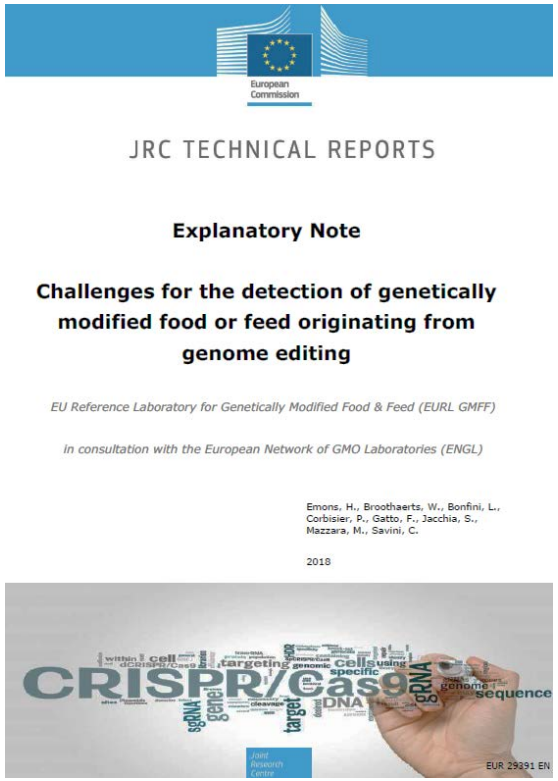
https://commons.wikimedia.org/wiki/File:Dna-SNP.svg

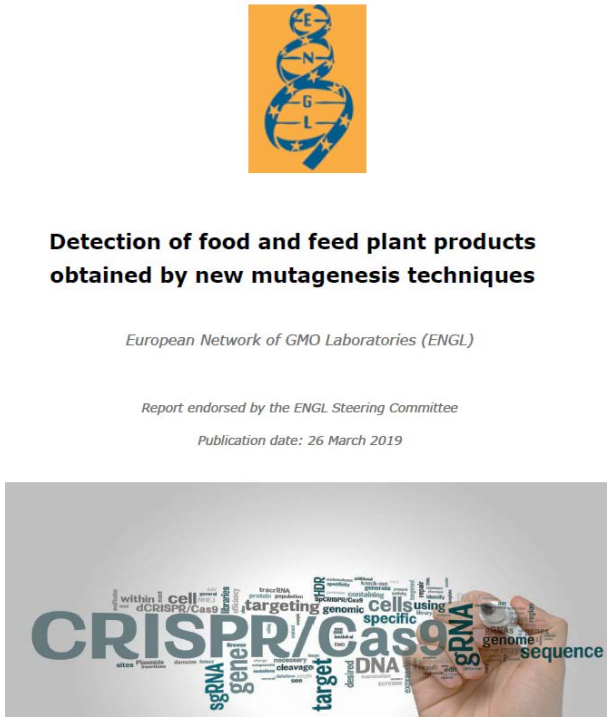
Evaluation of NPBT identification possibility (2011).

New Plant Breeding Technique Name	New Plant Breeding Technique Description	Identification Possibility With Prior Knowledge	Identification Possibility Without Prior Knowledge	Information Requirements for Identification	Identification Methods	Comments
Zinc finger nuclease (ZFN) technology						
ZFN-1	Genes encoding ZFNs are delivered without a repair template. The ZFN generates a site-specific double strand break. The natural DNA-repair process leads to (short) site-specific mutations (change, deletion or insertion of one or few bp)	NO	NO	See part 1 chapter 2 - the reference/baseline for identification of a genetic modification is the PCR-method approach used for GMO detection - a minimum of information about the target DNA sequence needs to be available (DNA sequence introduced by genetic modification and neighbouring genomic DNA sequence)	PCR	No differentiation possible with products from mutation techniques (chemical, radiation mutagenesis) or natural mutations
ZFN-2	Same as ZFN-1 but genes encoding ZFNs are delivered together with a short DNA repair template (one or few bp), which generate site-specific mutations through homologous recombination.	NO	NO	Idem	PCR	Idem
ZFN-3	Genes encoding ZFNs are delivered together with a long DNA stretch (several kbp), which is inserted in the genome in a site-specific manner	YES	NO	Idem	PCR	

JRC 63971; ISBN 978-92-79-19715-4, ISSN 1018-5593, doi:10.2791/54761
Luxembourg: Publications Office of the European Union 2011

New scientific and technical reports since 2018





Court of Justice of the European Union
PRESS RELEASE No 111/18
Luxembourg, 25 July 2018

Press and Information

Judgment in Case C-528/16
Confédération paysanne and Others v Premier ministre and Ministre de l'Agriculture, de l'Agroalimentaire et de la Forêt

New ENGL Working Group

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

2023

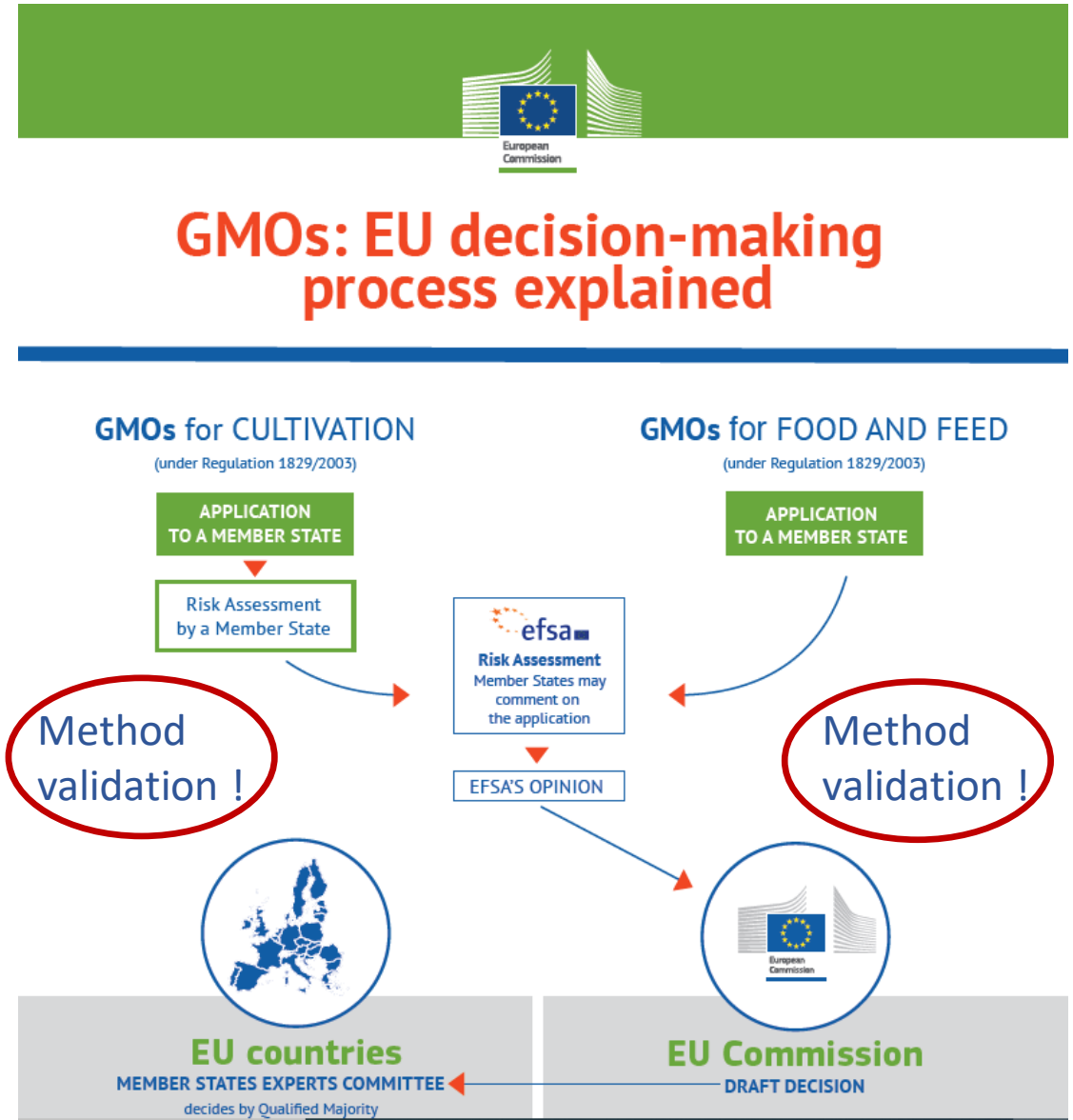
EU GMO legislation and GMO methods

1. **Protect human and animal health and the environment**
2. Ensure clear labelling of GMOs placed on the market
3. Ensure the traceability of GMOs placed on the market

- **Directive 2001/18/EC** on the deliberate release of GMOs into the environment.
- **Regulation (EC) 1829/2003** on genetically modified food and feed.
- **Directive (EU) 2015/412** amending Directive 2001/18/EC as regards the possibility for the MS to restrict or prohibit the cultivation of GMOs in their territory.
- **Regulation (EC) 1830/2003** concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced GMOs
- **Regulation (EU) No 503/2013** on applications for authorisation of genetically modified food and feed
- **Directive 2009/41/EC** on contained use of genetically modified micro-organisms.
- **Regulation (EC) 1946/2003** on transboundary movements of GMOs.

https://ec.europa.eu/food/plant/gmo/legislation_en

Validation of GMO identification and quantification method by EU Reference Laboratory is a part of GMO authorization in EU



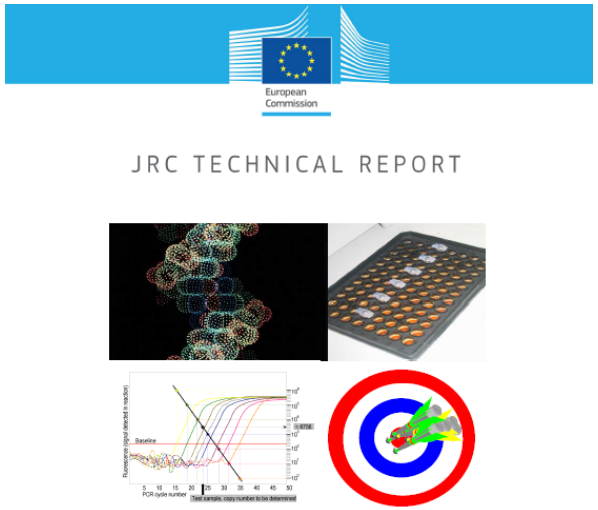
Methods:

- developed by the applicants !
- validated by EURLGMFF

- Validation means verification of minimum performance requirements

- Validation fails – no authorisation of GMO event!

Challenges for the applicant (method developer)



To develope a GMO detection method that meets the MPR requirements

Current MPR based on PCR techniques

The method provider should submit data demonstrating the positive evaluation of the detection method:

- Applicability
- Practicability
- **Specificity**
- Limit of Detection (LOD)
- Robustness

For quantitative methods (Regulation (EC) No 1829/2003):

- Dynamic Range
- **Trueness**
- Amplification Efficiency and R2 Coefficient
- Repeatability Standard Deviation (RSDr)
- Limit of Quantification (LOQ)

Challenge - provide evidence based data demonstrating specificity and trueness of the method. (MPR)

Specificity - Event-specific method should exclusively detect the targeted GM event.

To be demonstrated by a) similarity searches against databases (e.g. EMBL, GenBank, Patent, etc.)

b) experimental results from testing the method with non-target transgenic events, non-transgenic material and target material.

- a unique and sufficiently long DNA sequence is required,
- **SNV and short InDels may not provide such a unique sequence.**

Trueness (quantitative methods)

- Trueness is usually expressed in terms of bias obtained from a large series of test results and an accepted reference value.
- The trueness should be within ±25% of the accepted reference value over the whole dynamic range.

For some genome edited plants (SNVs) higher bias must be accepted!

ACS AGRICULTURAL SCIENCE & TECHNOLOGY
pubs.acs.org/acsagscitech
Review

Occurrence and Nature of Off-Target Modifications by CRISPR-Cas Genome Editing in Plants

Mark H. J. Sturme,*,⊥ Jan Pieter van der Berg,⊥ Lianne M. S. Bouwman, Adinda De Schrijver, Ruud A. de Maagd, Gijs A. Kleter, and Evy Battaglia-de Wilde

Cite This: https://doi.org/10.1021/acsagscitech.1c00270
Read Online

The size of off-target and on-target changes

Table 1. Details of the Reported off-Target and Unwanted on-Target Changes by CRISPR-Cas Genome Editing in Plant Species for the 28 Peer-Reviewed Studies Performing an Off-Target Analysis

plant species	Cas variant	off-target detection method: biased (B) unbiased (U)	description of method (s)	target gene	off-target or unwanted on-target changes[a]	number of mismatches off-target with gRNA	indel size (bp) and frequency or change at off-target/on-target location	location (coding/ noncoding)	off-target gene	reference
Apple (*Malus domestica*), pear (*Pyrus communis*)	Cas9	B	*In silico* prediction followed by PCR amplification and sequencing	TFL1	"Off-T"[b]	0[b]	Insertions: +1 (1x), +7 (1x) Deletions: −1 (8x), −2 (2x), −4 (1x), −6 (1x)	Coding	PEBPMD12	44
Arabidopsis thaliana	A dCas9-SunTag system; dCas9 with the catalytic domain of the *Nicotiana tabacum* DRM methyltransferase (NtDRMcd)	U	Whole genome bisulfite sequencing (WGBS) to screen DNA methylation	FWA	Off-T: genome-wide epigenetic off-target effects were observed such as CHH hypermethylation (where H = A, T, or C) and chloroplast DNA methylation	Not specified	DNA methylation	Not specified	Not specified	29
Arabidopsis thaliana	Cas9	B	Digenome-seq and targeted amplification deep sequencing of potential off-target sites	TRY	Off-T	2	Insertions: +1 (88.4−90.7%) Deletions: −1 (2.2−3.1%)	Coding	Not specified	45
Barley (*Hordeum vulgare*)	Cas9	B	*In silico* prediction followed by PCR/ Sanger sequencing	HvPM19-1	Off-T	1	Indels: size not specified	Coding	HvPM19−3 (target homologue)	32
Brassica oleracea				BolC.GA4.a	Off-T	2	Indels: size not specified	Coding	BolC.GA4.b (target homologue)	
Cassava (*Manihot esculenta*)	Cas9	B	PCR amplification of 504 bp of the target sequence followed by Sanger sequencing	MePDS	On-T and On-T-ns	1	Mainly 1 bp insertions (+1) and deletions (−1) on-target. Nucleotide substitutions also indicated on-target, but outside of target site. Deletions of 16 bp and 101 bp also observed	Coding	MePDS (on-target CDS)	27
	Cas9	B	*In silico* prediction followed by PCR amplification and sequencing	nCBP-2	Off-T	2 or 3	Deletions: −1, −3, and −11 bp	Coding	Not specified	46
Cotton (*Gossypium hirsutum*)	Cas9	U	Whole genome sequencing (WGS), assessment of off-target mutations at predicted potential off-target sites	MYB44 and ARC	Off-T	3 (for MY44) and 2 (for ARC)	Indels of 1−4 bp at Crd1, 1-bp at MYB77. 1-bp deletions with the ARC gRNA.	Coding and noncoding	Promoter dicarboxylate diiron gene (Crd1. First exon of MYB77 (MYB44 target homologue)	22
	nCas9 cytidine base editing system	U	Whole genome sequencing as well as targeted deep sequencing of potential off-target sites	GhCLA and GhPEBP	Off-T	1 to 5	Base edit: less than 0.1% single nucleotide substitutions	Coding	Not specified	47
Maize (*Zea mays*)	Cas9	B	A three-step approach: (1) *in silico* prediction, (2) combination of in silico predictions with CLEAVE-	MS26, MS45, Lig1	Off-T	1 or 2	Not specified	Not specified	Not specified	25

https://doi.org/10.1021/acsagscitech.1c00270
ACS Agric. Sci. Tech

NGTs In the pipeline

SDN1 >90%

Challenge – to provide evidence based data demonstrating robustnes ans sensitivity of the method. (MPR)

Robustness

- Is the method targeting a SNV or short InDel sufficiently robust against small modifications to the testing conditions?

Sensitivity (Limit of Detection/Limit of Quantification).

- If the method targeting a SNV or short InDel has an acceptable LOD and LOQ in different sample types? (stacked events, multiple events, composed samples etc)
- 0,1% Regulation 619/2011
- 0,9% Regulation 1829/2003

Challange - can new analytical techologies meet current minimum performance requirements for analytical methods of GMO testing?

e.g. next-generation sequencing (NGS),

Applicability/Practicability of the method.

- the equipment is not broadly used, (expensive to buy and to run – this may change)
- the quality assurance parameters and uncertainty estimation are still under development,
- training is required in the enforcement laboratories to make sure the methods can be applied in a reliable way.

- **Can a detection method be developed and optimized for any DNA sequence? (any NGT)**
- **Are those MPR fit for purpose for any method and any product?**

Challenges for control laboratories

To answer following questions

- Is there a GM event in the sample?
- Which GM event is present in the sample?,
- Is this event authorised in EU?

(Directive 2001/18, Regulation 1829/2003, Regulation 619/2011)

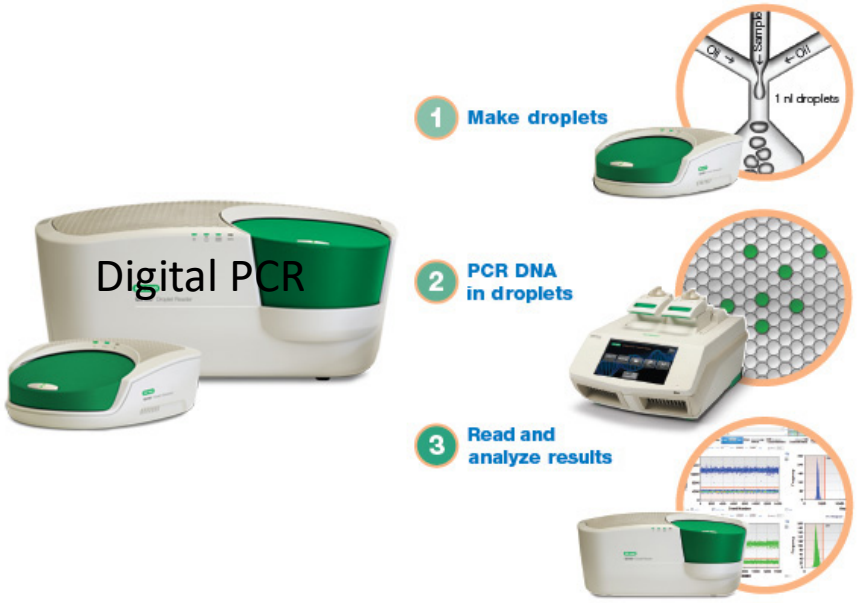
in case of authorised GM event(s):

- What is the quantity (%) of the GM event(s)?.

- Sanitary inspection, Veterinary inspection, Seed inspection
- Custom inspection

- Is there a GMO that fall under the EU GMO legislation in the sample?

- Zero tolerance policy for GMOs in seeds



Challange - conventional GMO screening can’t be applied

Can screening for known NGTs be developed?

Methods targeting the most frequently present genetic elements and constructs

1. **P-35S**- Cauliflower mosaic virus (CaMV) 35S promoter
2. **T-nos** - nos terminator derived from Agrobacterium tumefaciens
3. **cp4-epsps** - ctp2-cp4epsps junction of the chloroplast-transit peptide (CTP2) from Arabidopsis thaliana and the epsps gene from Agrobacterium tumefaciensstrain CP4
4. **bar gene** from Streptomyces hygroscopicus
5. **P-35S-pat** - junction of the CaMVP-35S promoter and the synthetic pat gene
6. **P-FMV** - promoter Figwort mosaic virus
7. **cry1Ab/Ac** - modified cry1Ab/Ac gene from Bacillus thuringiensis
8. **nos** - promoter from Agrobacterium tumefaciens (P-nos).

(Waiblinger et al., 2010).

Detection of known genome-edited events in the context of market control

Screening strategy applied for conventional GMOs is not possible !

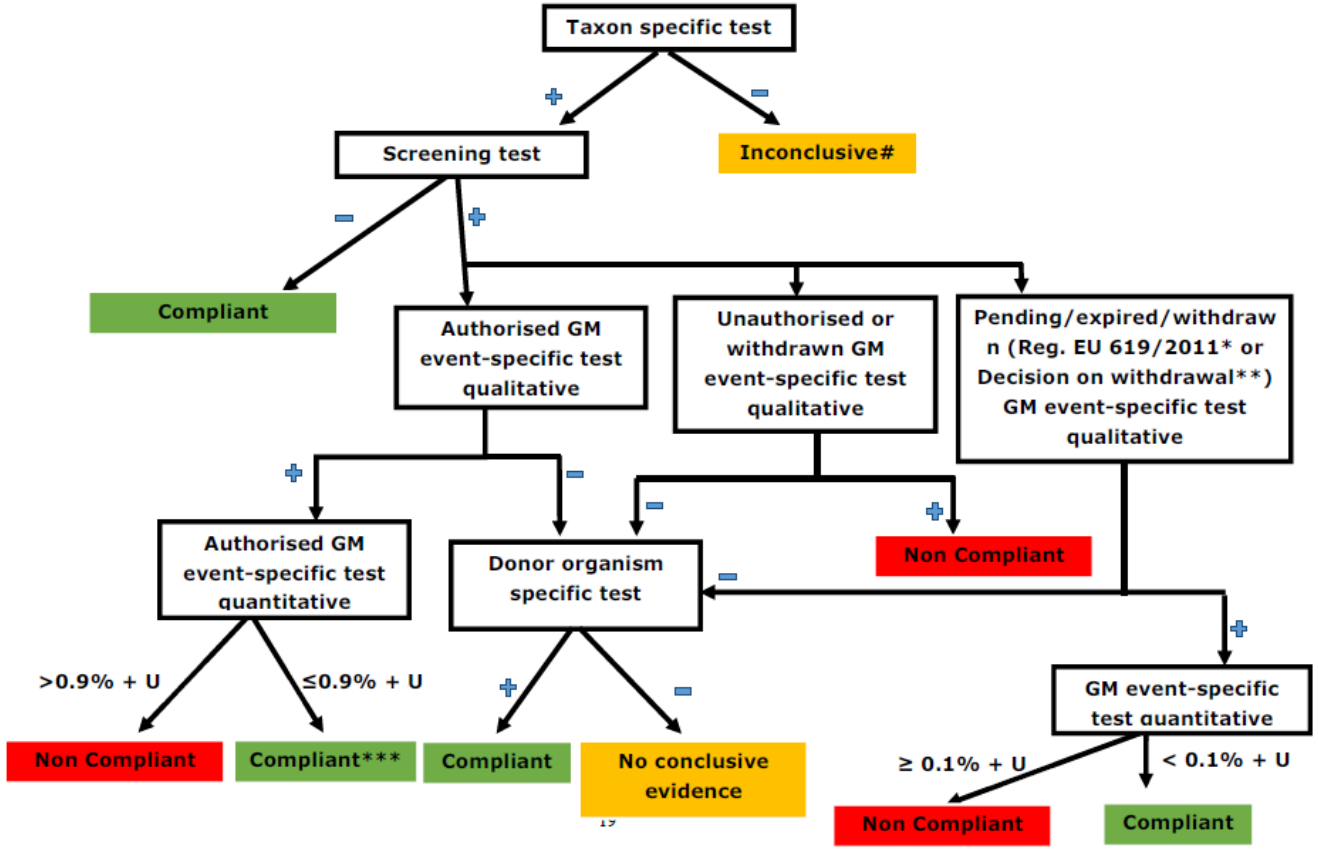
- genome edited plants do not contain any transgene DNA sequence
- common DNA element (promotors, terminators) are not present

The detection of genome-edited events already requires targeting the unique sequence in the analysis

detection = identification

Detection of unauthorised conventional GMOs is basicly based on screening strategy

Challange - can screening for unknown NGTs be developed?



For genome edited plants event specific method must be used.

Zero tolerance policy for unauthorised GMOs

Overview on the detection, interpretation and reporting on the presence of unauthorised genetically modified materials Prepared by the ENGL ad hoc working group on “unauthorised GMOs” December 2011 JRC ENGL Technical Report

Challanges for identification of genome edited event

- **In the absence of foreign DNA 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.

- 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 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.

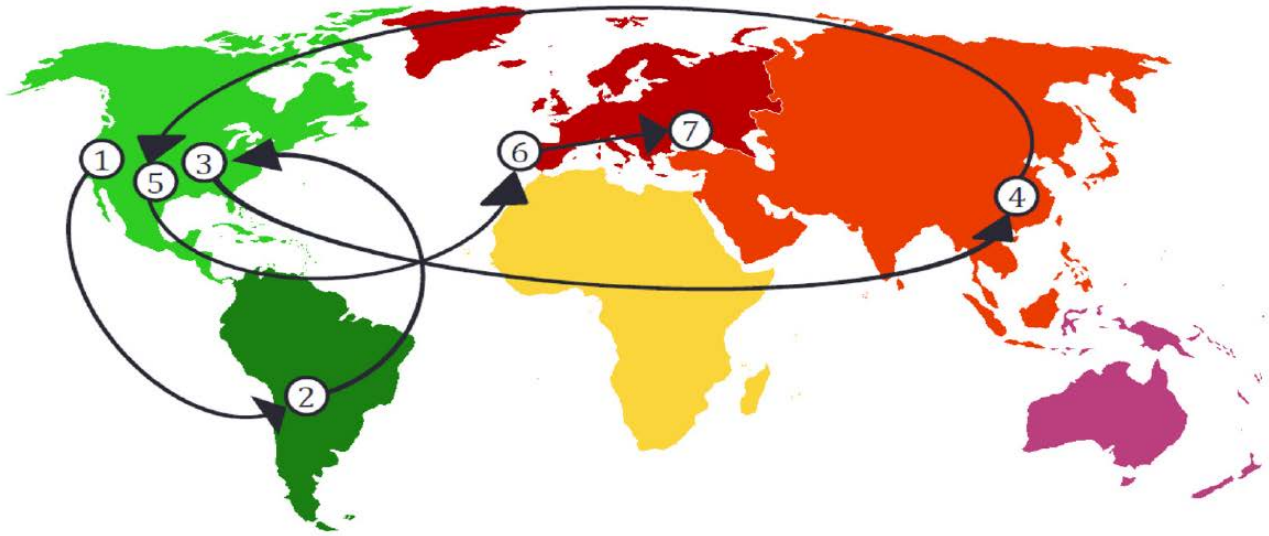
European Network of GMO Laboratories (ENGL), *Detection of food and feed plant products obtained by new mutagenesis techniques, 26 March 2019 (JRC116289)*

Challange - How to ensure breeding without GMOs?

Plant breeding is conducted globaly – the risk of NGTs admixtures

Production of tomato seeds:

1. **Production of breeding lines**
2. **Production of elite lines**
3. **Seed storage,**
4. **Certified seeds production,**
5. **Cleaning and treatment,**
6. **Delivery to the place of storage,**
7. **Delivery to the importer.**



Podlaski. S, Chomontowski Ch. 2021 Biuletyn IHAR Nr 294 / 2021 : 13–26
(za Dunkle, 2015)

Challenge – develope novel methods
Detection of genome editing crops by Next Generation Sequencing (NGS)

Practicability of new analytical methods?

- costly and time consuming (WGS),
- require experienced staff and expensive equipment,
- demand genome data management services and bioinformatics expertise.
- NGS application would require constantly updated crop database with reference pan-genomes including sequence variations.

These factors currently limit the implementation of NGS in many official control laboratories in EU.

Detection and identification of unknown genome-edited crops in complex samples is currently not achievable in routine practice !

https://upload.wikimedia.org/wikipedia/commons/7/7b/Illumina_MiSeq_sequencer.jpg

Challenges for detection and identification of NGTs – market control

- Growing number of NGT products
- Growing number of modified species (plants, animals, microorganisms…)
- Increasing problem of asymmetric authorisation (unknown GMOS)
- Availability of CRMs and the price
- New methods (epigenetics, synthetic biology etc)
- Availability of comprehensive sequence database
- Detection, identification and quantification of stacked NGT products or multiple edits
- Ensuring coexistence of NGTs
- Food, feed, seed control for organic production
- Zero tolerance policy for unathorised GMOs

Conclusions

- Authorisation in EU NGT products with small genetic alterations (SNV) might not be possible as the method will lack required specificity or fail validation according to current MPR,
- Currently NGT products could only be detected by control laboratories with prior knowledge on the altered DNA sequence,
- Many of the mutations induced by genome editing cannot be without a doubt distinguished from natural mutations or from those induced by conventional mutagenesis techniques,
- Zero tolerance for unauthorised GMOs is far more difficult to achieve compared to conventional GMOs,
- The growing number of regulated and deregulated genome edited plants worldwide put the GMO detection in a new dimension requires adaptation of current GMO legislation,

- Need of adaptation of minimal performance requirements for detection methods,
- Need for legal harmonisation - which organisms should be classified as GMOs.

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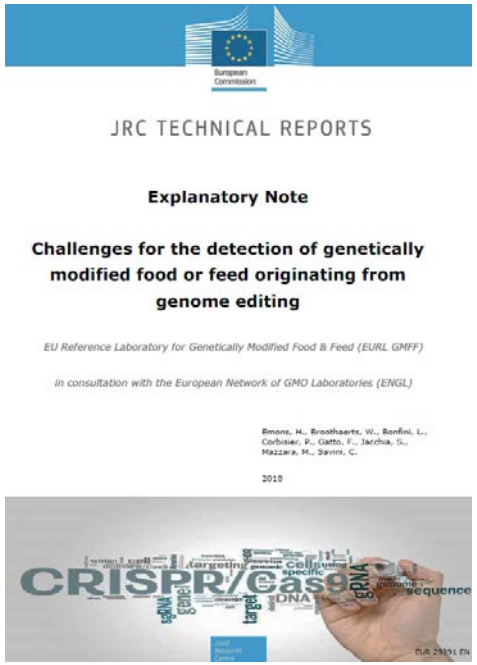

New ENGL Working Group

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

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