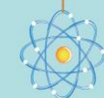
32nd ENGL Meeting

Report

19 November 2021

The European Commission's science and knowledge service

Joint Research Centre



32nd ENGL Meeting
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1. Welcome of the Chair

The Chair Hugues Crutzen welcomed the participants and informed that the meeting was recorded for the sole purpose of drafting the minutes. He notified to chair the meeting as acting Unit Head before the appointment on 1 December 2021 of Ms Ursula Vincent as Head of the F.5 Food and Feed Compliance Unit. The Chair reported that the Agenda was recently updated and kindly asked the speakers to respect the allotted times. He remarked that the comments in the chat would be shared at the end of the meeting. The Agenda (Annex 1) was finally approved.

2. GMO risk assessment and the challenges of NGTs (A. Afonso, EFSA)

The speaker remarked that the role of the European Food Safety Authority (EFSA) is to provide independent scientific advice, risk assessment and communication in various areas.

She informed that twenty-three new applications for authorisation of Genetically Modified (GM) food and feed and eight applications for renewal were submitted to EFSA. She noted a change in the traits displayed by the new genetically modified organisms (GMOs), from agricultural performance to nutritional aspects and an increase in complexity and stacking of elements.

EFSA concluded that no potential novel hazards are posed by synthetic biology and new genomic techniques (NGT) products and that current European Union (EU) regulatory requirements are adequate and sufficient although not always applicable for their risk assessment.

The current EFSA guidance documents are applicable to plants produced by cis/intra-genesis and Site Directed Nuclease 3 (SDN-3) techniques and partially applicable to SDN-1, -2 and Oligonucleotide Directed Mutagenesis (ODM) techniques.

The speaker announced the recent publication of a scientific report “Overview of EFSA and EU national authorities scientific opinions on the risk assessment of plants developed through NGTs”.

EFSA is currently verifying if the conclusions in the 2012 opinion on cis-genesis/intra-genesis are still applicable considering the current state-of-the-art and available knowledge on NGTs.

Discussion

A participant requested if bioinformatics tools are available for evaluating off-target effects. The speaker explained that off-target effects have limited value and that the current legislation does not require whole genome sequencing for products derived from genome editing. Bioinformatics analysis are currently performed for assessing only the insertion sequences. She clarified that NGT applications have not yet been submitted.

Another participant enquired on the effects of Regulation (EU) 2019/1381 (Transparency Regulation) on the timeline of GMOs approvals. The speaker claimed to expect some initial difficulties in understanding the new rules, but not major challenges.

3. Current and future market applications of new genomic techniques (NGTs) (E. Rodríguez Cerezo, JRC)

The speaker reminded that in November 2019 the EU Council requested the Commission to submit a study on the status of NGTs. As part of the study, the JRC was also tasked with providing an overview of current and future scientific and technological development in NGTs. The scope covered all agriculture, bio-based industry and medical sectors and included plants and

mushroom, animals, microorganisms and human cells. The report on current and future market applications of NGTs has been published in April 2021.

The JRC developed a database of NGT products by searching scientific literature, clinical trials databases and performing expert consultation and survey of public and private technology developers. The database is currently containing 645 items and is published on a web dashboard at https://datam.jrc.ec.europa.eu/datam/embed/NEW_GENOMIC_TECHNIQUES/.

Commercially there are two plants on the market: a soybean – high oleic (Calyno oil) cultivated in USA, generated by Transcription Activator-Like Effector Nucleases (TALEN) and developed by Calyxt (USA) and a tomato containing high level of Gamma-Aminobutyric Acid or GABA (Sicilian Rouge High GABA) – generated with CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats)/Cas systems, developed by Sanatech (Japan). Only one animal generated by NGT (red sea bream (Madai)) is commercially available. NGTs are already applied commercially in microorganisms for the bio-production of industrial molecules and are becoming standard tools to improve microbial strains. One Pivot-bio-soil bacteria-N fixing nitrogen associated to maize is at a commercial stage. Cancer, viral diseases and haematology applications are already in clinical trials, some in early or advanced Research and Development (R&D) stages.

Discussion

Participants requested if the database will be kept updated and enquired on the profile of the companies developing NGT products, on the possibility of identifying the GMOs listed in the database and on the risks associated with global seeds trade.

The speaker answered that the database will be probably updated every two years, and underlined that the products in development are expected on the market within a decade, while the ones in early development may be commercially available after 2030. Some profiling in the report is provided for the type of company; the players in this case are different from those involved in the development of conventional GMOs. The details of the project and the crop could not be released for confidentiality reasons. Finally, the NGT crops are usually premium products that will not enter the international trade market.

4. Detection Methods for NGT Products: first efforts and developments (L. Grohmann, BVL, DE)

The speaker remarked that *a priori* knowledge of the product placed on the market, detailed information on the modification and the availability of (a) reference material(s) are pre-requisites for designing detection methods for NGTs derived organisms. Molecular information on 46 NGT products has been collected in the EUginius database in collaboration with WFSR. As a first effort, a working group according to the German Genetic Engineering Act, which is hosted by the BVL, has started to develop methods for detecting:

- generic element of the CRISPR/Cas9 machinery (scaffold RNA part)
- CRISPR/Cas waxy maize event
- CRISPR/Cas GABA tomato
- TALEN Calyxt soybean

To design the generic element targeting method, the scaffold guide RNA highly conserved sequence parts derived from *Streptococcus pyogenes* and *Staphylococcus aureus,* which are the source bacteria for this part of the CRISPR/Cas editing machinery, were selected. The working group is validating the method using different types of segregants with/without inserts of the CRISPR/Cas machinery and is planning to run a collaborative study, if the results are promising.

The working group started testing the performance of a published method for the detection of the waxy maize event on reference material received by Corteva.

The working group retrieved a report from the Japanese Ministry of Agriculture and Fisheries describing the modification in the GABA tomato. This is a one base pair (bp) insertion in a GABA synthesis gene introducing a frameshift stop codon. The potential target region for designing the method was identified in the SlGAD3 gene. The working group developed synthetic plasmid DNA material which is corresponding to the wild type or the potentially modified sequence, because the developer has refused to provide reference material.

The working group also tried to develop a detection method for the "Calyxt" soybean lines presenting a knock-out of the FAD3a/b gene. It is possible to design primers and probes on the FAD3a and b genes according to the information provided in the patent and the publication, respectively. It was not possible to receive reference material from Calyxt and therefore again synthetic plasmid DNA materials are used for positive control.

The speaker requested general support by the JRC and the Commission for the provision of (a) reference material(s) for NGT products placed on the market in other regions. He also concluded by remarking that *a priori* knowledge of the edited sequence could be obtained from publications, patents and notifications. Availability of reference materials depends on the cooperation of developers. Specific detection of an edited sequence/identification of an event is feasible but the key factor is the uniqueness of the modified sequence or the presence of additional edits/event-specific markers stably inherited. He remarked that further specifications and criteria are needed for these products in the Method Performance Requirement (MPR) document drafted by the European Network of GMO Laboratories (ENGL).

The Chair appreciated the concrete actions in the ENGL for detection of NGTs.

<u>Discussion</u>

It was enquired on the efficiency of methods detecting deletions instead of insertions as for conventional GMOs. In addition, information was requested on the EUginius NGTs entries and their stage of development. The problem of uniqueness for a GMO presenting a single base pair modification was further raised for the GABA tomato line.

The speaker remarked that for discrete deletions, distinguishing the wild type from the deleted product is not challenging. The first results are promising since there is amplification from the edited but not from the wild type product. The speaker further explained that the EUginius entries are more relevant for commercial products for which information could be retrieved from dossiers, patent or publications. He finally acknowledged the difficulty in designing detection methods for commercial lines having an identical base pair modification present in conventional breeding lines.

5. Assessment of the method for detection of the Cibus herbicide-tolerant oilseed rape (Chhalliyil *et al.*, Foods 2020) (C. Weidner, BVL, DE)

The authors Chhalliyil *et al*. claimed in their article to have developed a quantitative Polymerase Chain Reaction (qPCR) method specific for the GM plant Cibus canola that contains a single point mutation conferring herbicide resistance. The article was discussed at the previous ENGL meeting and addressed from a theoretical point of view in the ENGL report "Detection of food and feed plant products obtained by new mutagenesis techniques".

Under request of the German Federal Ministry, the German NRL located at the Federal Office of Consumer Protection and Food Safety (BVL) evaluated the performance of the Chhalliyil *et al*. method according to the ENGL MPR document (qualitative method) focusing on sensitivity, specificity and robustness. The BVL was supported by the EURL GMFF and by the Swiss Federal Food Safety and Veterinary Office (FSVO).

The laboratories designed four different DNA templates each specific for each variant genotype and used genomic reference material comprising oilseed rape (OSR) GM lines (transgenic and conventional), three Cibus OSR lines, raphanus weed and 56 from other species.

The laboratory assessed the sensitivity with ten copies/reaction in sixty replicates. The Limit of Detection (LOD) resulted to be lower or equal than ten copies, but it was not tested in presence of wild-type material as recommended in the MPR (Part 2) document.

Primer-blast analyses indicated the presence of many non-redundant sequences in *Brassica napus* and *Ethiopian rape* database collection of the National Center for Biotechnology Information (NCBI). Almost identical target sequences where also found in wild type variants of *B. napus,* raphanus and other species as a result of selection in fields treated with herbicide.

The method performed adequately with the intended target but provided non-specific amplification with the wild type or Clearfield DNA sequences or with different Cibus lines not yet on the market, that were containing the same mutated sequence without the use of mutagenesis techniques. The Chhalliyil *et al*. method therefore cannot be considered as sequence-specific due to non-specific amplification with other sequences. The method also cannot be considered event-specific as the species or GM lines tested contain the same DNA sequence variation.

The laboratory studied the robustness with a multifactorial design approach and concluded that the method was not sufficiently robust on mixed samples.

The method therefore could not be considered fit for purpose for EU official control.

Discussion

It was asked if the laboratory tried to increase the annealing temperature of the PCR reaction to avoid non-specific amplification; the speaker answered that the temperature was increased but that the improvement was nevertheless insufficient.

6. GM yeast and filamentous fungi used for food industry purposes (D. Mojzita, VTT, FI)

The speaker from VTT Technical Research Centre, Finland, provided an overview of the industrial basic and commercial research on filamentous fungi. He explained the definition of GMOs and related labelling requirements in EU and US, the applications of GM fungi in the food industry and emerging trends in fungal GM-food applications.

GM fungi are used for food production as ingredients or as processing aids. The latter are substances exerting a technological function during food processing, but not present in the final food product. He provided examples of GM fungal host (*Saccharomyces cerevisiae*, *Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei*) in food industry and applications for food ingredients (enzyme) production.

The speaker presented a list of microbial-produced food processing enzymes being assessed in the EU for food applications (March 2015). More than half of the applications regard fungi and 37 % bacteria, however more than 50 % of the applications are not GM.

A big trend in the GM fungi-based food field is the development of microbial production platforms for milk and egg proteins and other ingredients currently obtained from animals (proteins as ovalbumin, collagen, gelatin, lacto globulin). Other trends are the re-programming of yeast metabolism from alcoholic fermentation to lipogenesis and the production of elements as vitamins or cellular agriculture. In the future GM-based fungal manufacture systems could help reaching sustainability, availability, consistency and safety-contained production.

Discussion

A question was raised as to whether antimicrobial resistance (AMR) genes were also used for the development of GM fungi and their detection strategies.

The speaker clarified that AMR genes are typically used for research, not for commercial production. CRISPR/Cas sequences are usually not detected in GM fungi because the latter are modified by transient introduction of CRISPR proteins. Off-target modifications are quite random and could be detected only by whole genome sequencing. The detection method should be provided in the applicant's documentation but strains are being constantly improved and may not necessarily remain detectable.

7. Characterisation of genetically modified microorganisms using short- and long-read whole-genome sequencing (K. Vanneste, Sciensano, BE)

The speaker illustrated the Specenzym project coordinated by the Belgian Federal Public Service of Health, Food Chain Safety and Environment. The aim of the project is to study the purity of food enzymes (FE) for the development of general purity criteria. Genetically Modified Microorganisms (GMM) are used to obtain higher production efficiency and/or yield of e.g. enzymes, vitamins and additives, but such products are unauthorised in the EU food and feed chain, if the GMM or derived DNA is present (Regulation (EC) No 1829/2003). It was underlined that GMM often contain antimicrobial resistance (AMR) genes as selectable markers and may spread those genes via horizontal transfer after ingestion, especially if they are inserted in plasmids. Several GMM in FE products were found during the pilot monitoring study. The laboratory designed a screening approach to detect and characterise the GMM present in the samples in four possible steps described thereafter.

- 1st Step: screening for AMR gene detection; if positive, there is GMM suspicion,
- 2nd Step: screening for GMM proteases; if positive, there is GMM confirmation,
- 3rd Step: viability test followed by
- 4th Step: whole genome sequencing (WGS) of isolates to characterize viable GMM or metagenomics sequencing of all DNA in the sample to characterize not culturable GMM.

Protease-producing GMM were detected by qPCR in FE products and viable protease GMM strains were isolated.

The laboratory demonstrated that short-read sequencing can enable GMM detection. They used a short and long sequencing strategy to characterize the GMM and were able to identify a 6.7 kb plasmid pUB110-protease (Berbers *et al.*) carrying several AMR genes which were posing serious public health concerns. To characterize the isolates origin, they performed single nucleotide polymorphisms (SNP) phylogeny (small scale) and whole genome (large scale) comparison and alignment. Isolates differed from each other by at most a few dozen of SNPs and some shared a 115 kb rearrangement. The laboratory concluded that the isolates were closely related and likely derived from the same parental strain. D'Aes *et al.*, 2021 demonstrated therefore, that this approach could be used for tracing GMM outbreaks.

The laboratory also detected alpha-amylase by qPCR in FE products from which in some cases could not be retrieved viable GMM strains. The laboratory performed metagenomics sequencing and hybrid assembly (Buytaers *et al.*, 2021) and concluded that three species were present in the samples with the protease deriving from one species and the alpha-amylase from one or more species. They showed that metagenomics with both short and long read sequencing may allow to characterise unculturable organisms in complex samples and used SNP phylogeny to establish the origin of the samples.

Discussion

A participant underlined the importance of bioinformatics analysis for GMM detection and characterisation. It was also asked whether the manufacturers were aware of contamination problems even with viable organisms.

The speaker commented that the producers may not know EU regulations and that the safety of these products could not be taken for granted.

Another participant remarked that EFSA dossiers are confidential on the parts regarding the sequences and that as a result they are not able to correlate them with the findings on the market samples. The difficulty of performing official control on these types of products was highlighted.

8. ddPCR strategy to detect a gene-edited plant carrying a single variation point (M.A. Fraiture, Sciensano, BE)

It was tested whether a PCR–based method could specifically detect and quantify a gene-edited organism carrying a single base pair variation. The speaker proposed a general workflow in three consecutive steps.

In step 1 "*in silico* nucleotide sequence analyses", verification whether sequences related to the single variation and its flanking sequences are available and if it is possible to design oligonucleotides for the region of interest. It is verified if it is possible to discriminate the gene-edited organism from its natural counterpart based on the targeted single variation.

In step 2 "method performance assessment", verification whether the method is in line with the ENGL MPR document on specificity, sensitivity and applicability.

In step 3 the laboratory uses sequencing-based approaches if the previous steps are not feasible.

To test the feasibility of a ddPCR strategy for NGTs detection, the authors used a parental wild type rice line and a mutant rice line generated by CRISPR/Cas9 with a single nucleotide difference (an additional A inserted in the gene OsMADS26). The workflow described above was followed to develop a method for the mutant line.

In step 1 the authors designed a duplex system with competing probes, specific for the mutant and the wild type sequences. It was verified that the single nucleotide insertion was detected only in the GMO and not in the natural variants. In step 2 the authors assessed the performance of the method and confirmed that it could specifically detect and quantify the NGT product.

The method development for detection of NGT products is a case-by-case process and unfortunately is not always feasible. Information on the sequences of interest is not necessarily available, it may not be technically possible to design the method, or the mutation may be present in natural varieties. In the latter case it is necessary to confirm the findings by using e.g. additional off-target sites. As an alternative a target Next Generation Sequencing (NGS) amplification approach could be used. These approaches are currently promising for food control but challenging, since several optimisations are needed.

Discussion

A participant enquired on the probe design: the speaker informed that basic probes were not able to discriminate the mutant from the wild type sequence while MGB probes were more specific.

Other participants enquired over the extent of analyses needed for excluding the presence of a natural mutation, on the strategy applied for defining the positive and negative reactions in ddPCR and requested whether the robustness of the method was tested.

The speaker informed that the mutation of interest leads to the inactivation of the gene and is therefore not largely spread in nature. She recommended having at least 10000 positive droplets and three replicates. She also clarified that the robustness was not tested since the proof-of-concept project intended only to show the assay ability to discriminate between the mutant variation and the wild type sequence.

9. Interlaboratory study for the validation of a ddPCR method (C. Savini, JRC)

The speaker presented the results of a JRC study on the accuracy of a droplet digital PCR (ddPCR) method in quantification of GMOs. A ddPCR method for MON810 was first assessed by the EURL GMFF in a single laboratory validation study and then in an international collaborative trial. The study followed the ENGL guidance "Overview and recommendations for the application of digital PCR" (Pecoraro *et al*., 2020) and used the conversion factors (CF) for dPCR available from the EURL GMFF website for converting the number of copies to mass fractions. The measurement of uncertainty (MU) was estimated according to the "Guidance document on Measurement Uncertainty for GMO Testing Laboratories" (Trapmann *et al*., 2020). The EURL GMFF assessed the LOD, dynamic range, linearity, robustness, trueness and precision. The impact of simplex and duplex setting formats, of the DNA extraction step and of decreasing ingredient content were evaluated in the collaborative study.

The outcome of the collaborative study was overall positive, with the method fulfilling all performance requirements.

Discussion

A participant enquired on the platform used for the study and on the transferability of the validated method to other dPCR platforms. It was also requested if new applications for authorisation of GM food and feed providing digital PCR methods were foreseen, and if applicants would need to provide equipment transferability data in their dossier. Potential problems were underlined also for laboratories not holding the necessary equipment.

The speaker specified that the study was performed on the instrument identified as being widely spread within the ENGL community, in order to guarantee a sufficient number of participants. More laboratories are expected to be equipped with digital PCR instruments in the near future and it is therefore feasible that detection and quantification methods will be performed on these platforms. The speaker underlined nonetheless that the ENGL MPR document requires the transferability of the method to different instruments.

10. European Joint Action TERROR: synthetic biology, DIY biology and DNA synthesis identified as novel threat of concern (F. Limonier, Sciensano. BE)

The speaker presented a European joint action for preventing and preparing for biological and chemical terror attacks. It is involving partners from eighteen EU countries and includes partners as DG SANTE, DG HOME and DG ECHO. The main objective of the action is to address gaps in health preparedness and strengthen cross-sectoral work with security, civil protection and health sectors. The project, coordinated by Norway and running up to April 2024, has been divided in eight Work Packages (WPs). The WP8 on novel threats has the task to identify 1) Novel biological agents 2) Synthetic opioids and 3) Dual use technologies. The members will design a questionnaire and review the literature to produce recommendations for future governance guidelines. ENGL members may contribute by responding to the questionnaire or by reviewing the deliverables. This contribution may be discussed at the following SC meeting.

Discussion

A participant enquired if other targets, e.g. insects could be considered. SANTE noted that the literature review is very broad and that it should have a reasonable target. The scope could be defined once the guideline is produced.

11. AOB

The Chair communicated that the information in the EURL GMFF database for distributing the plasmid control samples is not up-to-date. He asked to kindly send to the Secretariat e-mail the updated contacts for distributing the plasmid samples.

He thanked the participants, expressed the hope of organising the following event in-presence and closed the meeting.

Annex 1 – Agenda

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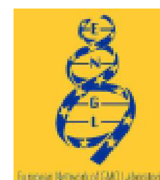

	Time	Topic	
1	8:45	▪ Welcome of the Chair	
2	9:00	▪ GMO risk assessment and the challenges of NGTs (A. Afonso, EFSA)	
3	9:45	▪ Current and future market applications of new genomic techniques (NGTs) (E. Rodríguez Cerezo, JRC)	
	10:30	*Break*	
4	11:00	▪ Detection Methods for NGT Products: first efforts and developments (L. Grohmann, BVL, DE)	
5	11:45	▪ Assessment of the method for detection of the Cibus herbicide-tolerant oilseed rape (Chhalliyil *et al.*, Foods 2020) (C. Weidner, BVL, DE)	
	12:30	*Break*	
6	13:45	▪ GM yeast and filamentous fungi used for food industry purposes (D. Mojzita, VVT, FI)	
7	14:30	▪ Characterisation of genetically modified microorganisms using short- and long-read whole-genome sequencing (K. Vanneste, Sciensano, BE)	
	15:15	*Break*	
8	15:45	▪ ddPCR strategy to detect a gene-edited plant carrying a single variation point (M.A. Fraiture, Sciensano, BE)	
9	16.15	▪ Interlaboratory study for the validation of a ddPCR method (JRC)	
10	17:00	▪ European Joint Action TERROR: synthetic biology, DIY biology and DNA synthesis identified as novel threat of concern (F. Limonier, Sciensano. BE)	
11		▪ AOB	
	17:30	End of meeting	

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