Literature review on analytical methods for the detection of precision bred products

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Here is the FSA's response to 'Literature review on analytical methods for the detection of precision bred products'.

Executive Summary

The Genetic Technology (Precision Breeding) Act (England) aims to develop a science-based process for the regulation and authorisation of precision bred organisms (PBOs). PBOs are created by genetic technologies but exhibit changes which could have occurred through traditional processes.

This current review, commissioned by the Food Standards Agency (FSA), aims to clarify existing terminologies, explore viable methods for the detection, identification, and quantification of products of precision breeding techniques, address and identify potential solutions to the analytical challenges presented, and provide recommendations for working towards an infrastructure to support detection of precision bred products in the future.

The review includes a summary of the terminology in relation to analytical approaches for detection of precision bred products. A harmonised set of terminology contributes towards promoting further understanding of the common terms used in genome editing.

A review of the current state of the art of potential methods for the detection, identification and quantification of precision bred products in the UK, has been provided. Parallels are drawn with the evolution of synergistic analytical approaches for the detection of Genetically Modified Organisms (GMOs), where molecular biology techniques are used to detect DNA sequence changes in an organism’s genome. The scope and limitations of targeted and untargeted methods are summarised.

Current scientific opinion supports that modern molecular biology techniques (i.e., quantitative real-time Polymerase Chain Reaction (qPCR), digital PCR (dPCR) and Next Generation Sequencing (NGS)) have the technical capability to detect small alterations in an organism’s genome, given specific prerequisites of a priori information on the DNA sequence of interest and of the associated flanking regions. These techniques also provide the best infra-structure for developing potential approaches for detection of PBOs. Should sufficient information be known regarding a sequence alteration and confidence can be attributed to this being specific to a PBO

line, then detection, identification and quantification can potentially be achieved.

Genome editing and new mutagenesis techniques are umbrella terms, incorporating a plethora of approaches with diverse modes of action and resultant mutational changes. Generalisations regarding techniques and methods for detection for all PBO products are not appropriate, and each genome edited product may have to be assessed on a case-by-case basis. The application of modern molecular biology techniques, in isolation and by targeting just a single alteration, are unlikely to provide unequivocal evidence to the source of that variation, be that as a result of precision breeding or as a result of traditional processes. In specific instances, detection and identification may be technically possible, if enough additional information is available in order to prove that a DNA sequence or sequences are unique to a specific genome edited line (e.g., following certain types of Site-Directed Nucelase-3 (SDN-3) based approaches).

The scope, gaps, and limitations associated with traceability of PBO products were examined, to identify current and future challenges. Alongside these, recommendations were made to provide the infrastructure for working towards a toolkit for the design, development and implementation of analytical methods for detection of PBO products. Recognition is given that fully effective methods for PBO detection have yet to be realised, so these recommendations have been made as a tool for progressing the current state-of-the-art for research into such methods.

Recommendations for the following five main challenges were identified. Firstly, PBOs submitted for authorisation should be assessed on a case-by-case basis in terms of the extent, type and number of genetic changes, to make an informed decision on the likelihood of a molecular biology method being developed for unequivocal identification of that specific PBO. The second recommendation is that a specialist review be conducted, potentially informed by UK and EU governmental departments, to monitor those PBOs destined for the authorisation process, and actively assess the extent of the genetic variability and mutations, to make an informed decision on the type and complexity of detection methods that need to be developed. This could be further informed as part of the authorisation process and augmented via a publicly available register or database.

Thirdly, further specialist research and development, allied with laboratory-based evidence, is required to evaluate the potential of using a weight of evidence approach for the design and development of detection methods for PBOs. This concept centres on using other indicators, aside from the single mutation of interest, to increase the likelihood of providing a unique signature or footprint. This includes consideration of the genetic background, flanking regions, off-target mutations, potential CRISPR/Cas activity, feasibility of heritable epigenetic and epitranscriptomic changes, as well as supplementary material from supplier, origin, pedigree and other documentation.

Fourthly, additional work is recommended, evaluating the extent/type/nature of the genetic changes, and assessing the feasibility of applying threshold limits associated with these genetic changes to make any distinction on how they may have occurred. Such a probabilistic approach, supported with bioinformatics, to determine the likelihood of particular changes occurring through genome editing or traditional processes, could facilitate rapid classification and pragmatic labelling of products and organisms containing specific mutations more readily.

Finally, several scientific publications on detection of genome edited products have been based on theoretical principles. It is recommended to further qualify these using evidenced based practical experimental work in the laboratory environment.

Additional challenges and recommendations regarding the design, development and implementation of potential detection methods were also identified. Modern molecular biology-based techniques, inclusive of qPCR, dPCR, and NGS, in combination with appropriate bioinformatics pipelines, continue to offer the best analytical potential for developing methods for detecting PBOs. dPCR and NGS may offer the best technical potential, but qPCR remains the

most practicable option as it is embedded in most analytical laboratories.

Traditional screening approaches, similar to those for conventional transgenic GMOs, cannot easily be used for PBOs due to the deficit in common control elements incorporated into the host genome. However, some limited screening may be appropriate for PBOs as part of a triage system, should a priori information be known regarding the sequences of interest.
The current deficit of suitable methods to detect and identify PBOs precludes accurate PBO quantification. Development of suitable reference materials to aid in the traceability of PBOs remains an issue, particularly for those PBOs which house on- and off-target mutations which can segregate. Off-target mutations may provide an additional tool to augment methods for detection, but unless these exhibit complete genetic linkage to the sequence of interest, these can also segregate out in resulting generations. Further research should be conducted regarding the likelihood of multiple mutations segregating out in a PBO, to help inform the development of appropriate PBO reference materials, as well as the potential of using off-target mutations as an additional tool for PBO traceability.

Whilst recognising the technical challenges of developing and maintaining pan-genomic databases, this report recommends that the UK continues to consider development of such a resource, either as a UK centric version, or ideally through engagement in parallel EU and international activities to better achieve harmonisation and shared responsibilities. Such databases would be an invaluable resource in the design of reliable detection methods, as well as for confirming that a mutation is as a result of genome editing.

PBOs and their products show great potential within the agri-food sector, necessitating a science-based analytical framework to support UK legislation, business and consumers. Differentiating between PBOs generated through genome editing compared to organisms which exhibit the same mutational change through traditional processes remains analytically challenging, but a broad set of diagnostic technologies (e.g., qPCR, NGS, dPCR) coupled with pan-genomic databases and bioinformatics approaches may help contribute to filling this analytical gap, and support the safety, transparency, proportionality, traceability and consumer confidence associated with the UK food chain.

Introduction

The Genetic Technology (Precision Breeding) Bill (England) was announced in the Queen’s Speech on 10th May 2022, highlighting the scope for a future regulatory approach for food and feed (1). This Act, which was passed in March 2023, aims to develop a science-based process for the regulation and authorisation of PBOs.

Precision breeding involves the use of technologies such as genome editing to make changes to the DNA of plant or animal species which could also come about through traditional processes. The use of genetic techniques in this way is increasing due to the range of beneficial outcomes (e.g. improved health or environmental sustainability).

With the implementation of The Genetic Technology (Precision Breeding) Act, the FSA need to ensure a framework for regulation of precision bred products considers safety, transparency, proportionality, traceability and consumer confidence. Exploring the viability of scientific methodologies to routinely identify and trace food and feed produced by the techniques of precision breeding will support traceability, particularly in relation to authenticity and enforcement of precision bred products. This will help build capabilities in Public Analyst laboratories for official controls and assist in steering future work on analytical methods.

Previous reviews considered methods for the analysis of genome edited products and discussed limitations to their detection (2, 3). It has been identified that current techniques are not able to determine whether certain small genetic events are a result of genome editing or have come

about through traditional processes. With this, the continued development of analytical technologies and the emerging concepts of using genetic tags/barcodes and genomic databases, the FSA require a more in-depth and up-to-date review of the full range of traditional and novel analytical techniques available, which addresses the challenges presented.

This current review, commissioned by the FSA, clarifies existing terminologies, and explores the scope and limitations of potential methods for the detection, identification, and quantification of products of precision breeding techniques. The review provides recommendations to address both the analytical challenges presented, as well as progressing the infrastructure for working towards a toolkit for the design, development and implementation of analytical methods for detection of PBO products.

Aims and Objectives

The aim of this report is to review analytical methods for the detection of the products of precision breeding, culminating in recommendations towards addressing the gaps for further research and development of methods for testing for these products in the UK. The individual objectives of the report are:

Review of standardised terminology: A review of national and international expert working groups, standards, regulatory texts and practices has been conducted in order to further refine and harmonise the terminology involved (Annex I).

Review of current and novel methods: A review of the potential of current and emerging methods for the detection, identification and quantification of products of precision breeding techniques was completed. This included PCR, sequencing and untargeted techniques, as well as detection of site directed nuclease mediated activity, off-target effects and cisgenic changes. As a benchmark, the evolution of analytical methods for detection of GMOs was examined.

Review of the published and updated ENGL guidance of the original ENGL 2019 report (or equivalent): A review of the updated European Network of GMO Laboratories (ENGL) report of the original ENGL 2019 (3) was not published within the timeframe of this project. As an alternative, current best measurement practice guidance and international approaches in the evolving area of methods for the detection of genome edited products and PBOs were assessed and evaluated for impact and implementation from a UK centric perspective.

Review supportive tools: A literature review was conducted regarding other tools to augment analytical detection methods. The availability, viability and (where feasible) associated costs/resources of tools such as reference databases and reference and control materials, as well as genetic varietal markers/tags/barcodes, were explored.

Knowledge gaps and challenges: An assessment of knowledge gaps, limitations, and current and future challenges in detecting products of precision breeding, was conducted. Practicalities of delivering methods for enforcement bodies and the cost implications for food businesses in the UK were hard to estimate, based on the current deficit in fully effective methods for the detection of PBO products.

Recommendations: Recommendations towards establishing a toolkit for the design, development and implementation of analytical methods for these products, have been provided. Consideration was given to a focus on current knowledge gaps, state of the art and scope/limitations of the science, as well as the need to provide objective experimental based experimental evidence to support any of the findings.

Terminology

The advent of new genomic techniques (frequently abbreviated as NGTs), which use genome editing for targeted and precise genomic changes within an organism, is now a reality and can be effective in a relatively short space of time. Genome editing is an umbrella term, encompassing a range of different techniques, all of which are characterised by production of specific targeted alterations in a genome without the introduction of foreign DNA (4). The concept and practice of genome editing a plant or animal to possess a beneficial and heritable trait, has gained much scientific, socio-economic and political interest recently. As a result of this, a number of scientific consortia, working groups, standards, etc., have been formed, often using diverse but overlapping terminology in relation to the genome editing process.

Annex I provides a summary of the key relevant terms used by national and international expert working groups, standards, regulatory texts and practices, and takes into account key published texts from the EU and UK with the aim of harmonising and providing further clarity on common terms. Annex I has been generated in order to further define and harmonise the terminology involved, and to ensure consistency with such terms in the rest of the review.

Current and emerging methods

Evolution of GMO analytical methods

Overview

For identification and quantification of GMOs, the largest common denominator in the global framework of GMO analysis are DNA based techniques (5). Quantitative real-time PCR (qPCR)-based analysis is the current preferred DNA-based technique for routine GMO analysis (5). For quantitative determination, the validated protocols for event specific detection of GM varieties (produced by the EU Reference Laboratory for GMOs in food and feed (EURL-GMFF)) provide unequivocal target identification (6). Central to these is the specificity afforded by the unique integration region between the transgene insert and the host genome. Such GM detection methods are subject to intensive intra-laboratory (single lab) validation and inter-laboratory collaborative trials, to assess the method’s fitness for purpose (7).

Specificity

GMO testing can range from low specificity tests (for example, identification of the cauliflower mosaic virus 35S promoter (P35S) to infer a GMO is present), to medium specificity construct specific tests, which may provide information about the lines being used (for example, regulatory element CTP4 combined with glyphosate tolerance gene will infer the likelihood of a RoundUp Ready® resistant line). The highest level of specificity for GMOs is afforded through event specific tests, providing identification based on the unique integration region between the transgene insert and the host genome

Screening

A current challenge for GMO analysis is the increasing number of EU/UK authorised and unauthorised lines. In 2021 the total number of approved GM lines was over 90 (8), making effective detection resource intensive.

Low specificity tests (e.g., looking for common control elements indicative of a range of GMO transformation events, such as p35S, tNOS, ctp2/cp4 epsps, bar, p35S-pat, Cry1Ab/Ac, pFMV and pNOS) are often used as an effective screening strategy for GM lines. This approach, coupled with a bioinformatics type “matrix” work flow (9) can reduce the analytical burden by removing those GM lines which do not have a strong likelihood of occurring based on the

presence of control elements, from further testing. Common GMO screening approaches include the wet-lab based techniques coupled with a “matrix” approach and reference to a database (10), to more ready-to-use “kit” based approaches using lyophilized primers and probes for authorised GM events in a 96-well micro-titre plate (11). These screening assays are qualitative in nature, indicating presence or absence of the target.

Quantification

Real-time PCR underpins the majority of all EU validated methods for GMO detection (6). These event specific tests also afford accurate quantification of the GMOs, with reference to a labelling threshold underpinned by the relevant EU and UK legislation. The quantitative amount of a GMO in a food or feed sample is usually expressed as a relative amount, taking into account the amount of GM and non-GM material arising from a particular taxon (e.g. soya).

Established methods for GMO analysis

PCR based strategies

The polymerase chain reaction (PCR) is a technique used to amplify small amounts of DNA, to larger, more measurable amounts (12). Such traditional “end-point” PCR is facilitated through careful selection of forward and reverse primers specific to the target. Extracted DNA, subject to PCR, will provide a qualitative result of either “presence” or “absence” of the target DNA sequence.
Real-time PCR adds further functionality in that it can also be used for accurate quantitative estimation of the target sequence relative to a calibration curve (12). Real-time PCR monitors the accumulation of a product in real time, typically by measuring the response of a fluorescent signal from a labelled probe/DNA-binding dye, which increases in direct proportion to the amount of PCR product being generated.

New and emerging methods for GMO analysis

Digital PCR (dPCR)

Digital PCR (dPCR) is gaining increasing traction as a reliable technique for the accurate quantification and value assignment of GMOs (13). dPCR is a technique used to quantify the amount of target DNA in a sample by counting amplifications from single molecules and permits absolute single molecule quantitation. It is based on the theory of limiting dilutions, where the PCR reaction is partitioned into thousands of smaller individual reactions. The concentration of any sequence in a DNA sample can be calculated based on the number of positive reactions.

Next Generation Sequencing (NGS)

NGS can encompass a range of different approaches, from single molecule sequencing through to massive parallel sequencing and whole genome sequencing. NGS has been used for GMO analysis inclusive of screening and detection of authorised and unauthorised GMOs, using both targeted and untargeted approaches. However, the application of NGS is far from routine due to a number of factors inclusive of costs, skill-base needed, requirement for bioinformatics infrastructure and storage, and the need for further harmonisation.

Other untargeted methods

Aside from NGS, the vast majority of other untargeted techniques are broadly characterized as a suite of imaging/spectroscopic tools, based on the reflectance or absorbance of a range of

applied wavelengths (14) for example near-infrared, Fourier transform infrared and Raman spectroscopy. Their utility in the area of GMO analysis has only met with limited success, as the profiles generated can be impacted upon by age, tissue type, variety/cultivar, seasonal, environmental and processing influences. Such approaches are also not ideally suited for quantitative purposes, may have limited sensitivity, sample size is an issue, and there may be only limited differences in reflectance/absorption profiles between GM and wild-type plants/grains.

Protein based methods are only useful should a genetic modification produce an observable response in terms of creating, changing the protein structure/levels or otherwise removing a protein product. Should the genetic modification not be expressed at the protein level, protein-based methods would not be applicable. Their utility is also based on a priori information on the new/changed protein, and proteins can often be adversely affected when present in processed food samples (15). For these reasons, protein-based methods are not commonly used for GMO analysis.

Methylation status (a change in the physical make-up of the DNA whilst it still retains the same DNA sequence) has been postulated as another way to detect GMOs (16). However, a lack of information regarding the methylation mechanism precludes this, and more research in this area is needed prior to any conclusions being drawn.

Analysis of PBOs: detection and quantification

Detection, identification and quantification of PBOs will be dependent upon methods which provide full specificity for the genetic variation occurring as a result of modern biotechnology. Analytical challenges associated with developing detection methodologies for products as a result of NGTs have previously been reported, as described by the ENGL in 2019 (3), which this review also supports.

In the previous section, the main molecular biology technologies for GMO analysis were briefly described. Each of these is further elaborated upon below, with a focus on their potential for applicability for PBO analysis.

PCR potential

For PCR to be effective, the altered genome sequence present in the PBO needs to be known a priori, and the sequence needs to be specific to that particular PBO (15). PCR instrumentation is commonplace within a number of analytical laboratories and is inexpensive to run, making PCR very practicable. End-point PCR is qualitative only and lacks additional specificity compared to real-time PCR.

Real-time PCR potential

Real-time PCR boasts better specificity and is quantitative in nature. Should the PBO possess an altered genome sequence which has been characterized and shown to be unique to that PBO, then real-time PCR has the potential to be used for routine PBO analysis. Real-time PCR instrumentation is relatively commonplace in an analytical laboratory, particularly so in GMO enforcement laboratories, demonstrating good practicability as a detection method. The skill-set required to use real-time PCR is higher than traditional end-point PCR.

Digital PCR potential

Digital PCR is sensitive, precise and quantitative in nature, providing good potential for PBO analysis, should the PBO possess a unique sequence alteration. Digital PCR instrumentation is less common in analytical laboratories, but recent years have seen an increased uptake (13). The

cost of the instrumentation is decreasing, but still generally remains higher than that of real-time PCR instrumentation. Assays designed for real-time PCR can be relatively easily adapted for dPCR, providing flexibility to move from a real-time PCR to a dPCR based format in the future if required. Specialist training is required to operate digital PCR instrumentation.

Next Generation Sequencing potential

NGS approaches have excellent potential for identifying both authorised and unauthorised PBOs in complex food and feed samples, should the altered PBO sequence information be known and also be specific to that PBO. The utility of NGS approaches for these purposes is dependent upon the type of NGS methodology being used, as well as the availability of appropriate reference genomes/databases. For example, use of short read NGS sequence technologies may not enable appropriate diagnostics without access to a highly contiguous reference genome sequence. Without a highly contiguous reference genome sequence the use of NGS technologies would likely require the use of long-read NGS technologies. NGS instrumentation is less frequent in analytical laboratories, and the cost of this, as well as the associated infrastructure (storage and bioinformatics) is often prohibitive, although cloud-based computing/storage may help alleviate this issue. Most sequencing approaches should also only be regarded as qualitative in nature.

Untargeted methods potential

Imaging and spectroscopic untargeted methods have not proven particularly effective for GMO analysis, being dependent upon observable phenotypic differences between the test sample and a reference material. They are therefore unlikely to prove effective for PBO analysis. Protein based methods and methylation status, having not proven effective for GMO analysis, would exhibit the same limitations for PBO analysis.

Certified reference materials

There are currently no certified reference materials (CRMs) available for PBOs, precluding effective controls and quantification. Furthermore, the choice of an applicable PBO reference material requires further discussion. A reference material needs to be stable and representative, and should a PBO possess multiple on- and off-target mutations, these may segregate out in subsequent generations.

Potential screening approaches

There is a general deficit of common control elements introduced into PBOs as a result of genome editing, precluding development of effective general screening approaches (3). However, it is theoretically possible to develop a limited screening approach for specific PBOs whose sequence is known a priori, by targeting the sequence of interest. Such a screening approach would provide some confidence whether further analytical work is necessary. If the sample does not possess a genetic variation consistent with the profile of the PBO, then no further analysis is required. Should the sample exhibit a profile consistent with that of the PBO (but potentially also consistent with genetic variation that could have occurred as a result of traditional processes), then further analysis would be needed.

Potential for quantitative estimation

Should a quantitative approach be required, further elaboration of the expression units and measurement response would be a pre-requisite. The Genetic Technology Act (1) does not prescribe whether a quantitative measurement is required.
Based on current best practice for GMO analysis, it may not be an incorrect assumption that there

would be zero tolerance of any unauthorised PBOs found in the food and feed supply chains in Great Britain. Thus, the positive confirmation of the presence of any unauthorised PBO, no matter how small an amount, would be considered non-compliant with UK legislation. In these instances, it is likely that a full quantitative approach may not be required.
A future possibility is that PBOs, which are authorised for placement on the UK market for food and feed use, may require the product which contains them to be labelled. For GMOs, traceability is maintained by a threshold level for labelling, to distinguish between unavoidable adventitious contamination and deliberate adulteration. For authorised GMOs, this labelling threshold is set as 0.9% m/m of a particular ingredient in that food or feed product. Should such a labelling threshold be defined by UK regulations for PBO controls, then a validated and fully quantitative method for PBO analysis will likely be a requirement. Currently, there are no approved analytical methods available for the specific detection of a particular PBO, precluding the development of quantitative methods.

Monitoring off-target effects

Unintended (off-target) mutations can be introduced into the genome of an organism, through traditional breeding and genome editing. The impact of such off-target mutations is the subject of much current research and debate (17).
Methods for detection of off-target mutations are subject to the same limitations as any “on-target” mutation: prerequisites being a priori knowledge of the sequence and evidence that the mutation is unique. If evidence is presented that a number of off-target mutations afforded by a genome edit have occurred, some scientists have speculated that there is potential to use these to provide a unique genetic signature/foot-print for a particular PBO (16). However, as off-target mutations are often unlinked to the intended mutation, they may typically segregate out in subsequent generations and their utility as an additional tool for traceability would be lost.

Detection of products as a result of Site-Directed Nuclease (SDN) activity

Methods for targeted mutagenesis (including SDN-1, SDN-2 and SDN-3) as well as methods for cisgenesis, may result in changes to an organism’s genome classifying it as a PBO. Detection of SDN or cisgenic activity could help infer the potential presence of a PBO. Changes mediated by these mechanisms are still subject to the same limitations of the sequence of interest being known a priori and that sequence being unique to a PBO, making inferences very difficult. However, in some instances, SDN-3 type changes may introduce larger DNA sequence changes (up to several kilobase pairs long) into a predefined insertion region on the recipient genomic locus. The combination of the larger DNA sequence change and its unique integration region on the genome, could provide the basis for specific PCR or sequencing based detection methods, similar to event-specific methods for GMO analysis (16, 18).

International approaches

Experiences from international expert groups and the evidence in the published literature has been reviewed with respect to potential detection methodologies. Account has been taken of current European Commission studies, expert working group publications, and guidance published by the European Network of GMO Laboratories (ENGL).

HORIZON-CL6-2023-FARM2FORK: New detection methods on products derived from new genomic techniques to enable safe innovation in the food system

This is part of the Horizon Europe - Work Programme 2023-2024 Food, Bioeconomy, Natural Resources, Agriculture and Environment. Project consortium responses were invited, with a

deadline of the end of March 2023 (19). Proposals were asked to address exploring innovative ways for detecting NGT products, to develop and validate reliable methods for these purposes, to qualify these in terms of wide applicability and potential use as screening approaches, to examine off-target mutations, to consider artificial intelligence (AI) learning models, and to support international standardisation. Such calls reinforce the topical nature of the subject, as well as the requirement for further R&D in this area and the expectation that there is some likelihood of success in the development of appropriate detection methods.

Target analyte for potential detection of genome editing: DNA

There is general international scientific consensus that DNA remains as the preferred target analyte with the greatest potential to help detect genome edited organisms or products (2, 18).

DNA hybridizations assays (e.g. microarrays) require large amounts of genetic material and have known sensitivity and specificity issues. Protein based methods require further confirmation that the protein change is as a result of a change in the genetic sequence. Metabolite-based methods are largely considered inappropriate, as the profile generated can fluctuate in response to developmental and environmental conditions, and they are only generally applicable for detecting qualitative differences if the sample is homogenous and unprocessed (2).

Detection of genome edited organisms and products

An overview of the analytical challenges associated with developing detection methodologies for products as a result of New Genomic Techniques was provided by the ENGL in 2019 (3), which this review also ascribes to. Additional pivotal scientific papers since the publication of this report have been cited below, elaborating further on some of the key aspects involved.

Technical analytical strategies for the detection of mutations focus on the similarities between sequence data and not on determining how the alteration was introduced. In the event that a difference in the genome sequence between two plants was detectable, it is still challenging to prove whether this was as a result of genome editing techniques (2). Provided that several preconditions apply, bioinformatics and statistical approaches may be useful in estimating the probability of whether this occurred due to genome editing, but this is again dependent upon knowledge of the DNA sequence exhibiting the variation. Additional information such as supplier, origin, pedigree and other appropriate documentation may be necessary (2).

Several published papers support the use of molecular biology methods to provide identification of a specific genome edited line. A qPCR method has been described to detect a targeted Single Nucleotide Variation (SNV) with respect to the first commercialized genome-edited oilseed rape line (20). However, other scientists have suggested that this method does not provide unequivocal evidence that the targeted SNV was as a result of genome editing (21).

Other publications have cited the use of “minimum qualifying information” and the concept of “scars”, “signatures” or “footprints”, to build up a unique profile associated with a genome edited line, and therefore provide unequivocal identification and traceability of that PBO (16). This is based on the concept of using a weight of evidence approach and assessing such aspects as the sequence of the target site of interest, the flanking regions, the genetic background, off-target mutations, and potential epigenetic and epitranscriptomic changes which are heritable between generations. There is a requirement to further investigate the potential utilisation of such approaches as an aid to traceability, by experimental led evidence.

One paper describes the concept of “scars” – the unintended effect caused by application of any technique, including genome editing (16). This paper suggests that these scars can manifest themselves as genetic, epigenetic and epitranscriptomic changes, and can occur as stable and heritable off-target mutations and effects. It was postulated that collectively these scars, along

with the modification of interest, may provide a unique profile or “signature”, allowing the potential identification of a specific genome edited line.

A published paper has reported on the use of a CRISPR/Cas analytical approach as a tool for the detection of specific nucleic acid sequences (22) principally for the detection of SARS-CoV-2. The approach is based on the specific nucleic acid recognition function of Cas enzymes and their related activities which can be activated upon binding. This activity can sometimes result in unintended cleavage of nucleic acid probes, providing a measurable signal. Such approaches may provide viable methods for the detection of SNVs, but additional research is required to assess their applicability for GMO and PBO detection.

Others have proposed approaches to identify genome edited lines based on a multiplex ligation-dependent probe amplification method (23). This approach is designed to target both on-target and off-target CRISPR/Cas induced mutations as well as screening for naturally occurring mutations. Such approaches are attractive due to the potential use of the same primers and uniform assay conditions, as well as the ability to run assays in tandem. The method gives promising results, but is also subject to the same caveats associated when testing for genome edited products, inclusive of a priori information on the sequence of interest and access to a relevant reference to establish the sequence is unique.

A recent paper by Fraiture and co-workers, 2022 describes the development of a general PCR workflow to specifically detect and quantify a genome-edited rice line carrying a SNP (24). The authors use a two-step droplet digital PCR approach, based on an in silico nucleotide sequence analyses, followed by evaluating the performance of the PCR assay against international guidelines. The work was demonstrated using a genome-edited rice line with a single nucleotide insertion. The authors were careful to highlight the scope and limitations of this approach, inclusive of a requirement for a priori knowledge of the genetic variation and associated sequence information, and the potential inability to confirm the source/origin of the variation to be a genome edit as opposed to natural variation/traditional breeding. Although not a limitation specific to this technique alone, the assay is also specific to one particular mutation only, and should additional on and off-target mutations need to be monitored, additional assays would need to be developed and validated.

To further augment this, the same expert research group proposed a pioneering sequencing-based strategy aligned with database searching, to enrich regions of interest, to provide further additional evidence of a genome-edited organism (25). The authors proposed and successfully implemented a proof-of-concept targeted high-throughput sequencing approach, making use of a prior PCR-based enrichment step to amplify regions of interest, a sequencing step, and application of a data analysis methodology to identify SNVs of interest. Whilst successful, this approach was currently only demonstrated as viable in a closed system consisting of limited and well characterised breeding lines (rice) to help identify a genome edited line. The authors were also careful to highlight that a pre-requisite of such approaches is access to detailed sequence information and appropriate databases to compare reference genomes.

Another publication described the development of a screening method to detect and identify genome-edited rice lines, based on a combination of qPCR and high resolution melt curve analysis (26). The authors stressed the usual caveats of a requirement for a priori information regarding the DNA sequence at the site of interest. Whilst successful, this approach was qualitative only, and the central issue remained in that such a method cannot necessarily distinguish between a genome edited plant/animal and those that had the same mutation arising from traditional processes.

A further paper demonstrated the utility of a duplex dPCR method for the detection and evaluation of genome editing frequencies in plants, using rapeseed as an example (27). Based on a balanced appraisal of the results the authors highlighted that a priori information of the target and

flanking sequences were needed to provide confidence in the results, and the dPCR method alone does not provide information regarding the source of the mutation.

Supportive tools

Supportive tools can augment the successful application of detection methods for GMOs and could provide additional options to consider as a supportive structure for potential PBO detection. A range of supportive tools are available to augment the analytical and decision-making process which include areas such as biobanks and bioresources, specific and pan-genomic databases, reference and control materials, as well as other genetic markers.

Genetic varietal markers/tags/barcodes

Researchers have investigated the application of genetic varietal markers, tags or barcodes to allow traceability (28, 29). This involves the insertion or creation of a unique molecular tag (sequence) into the genome of an organism, as a way to further augment traceability (and to guard against possible infringement on a manufacturer's intellectual property). Roy and co-workers, 2018 (29) developed a CRISPR-Cas9 based system for multiplexed accurate genome editing with short, trackable, integrated cellular barcodes (MAGESTIC) in Saccharomyces cerevisiae. The method allows for a plasmid barcode to be stably integrated into the genome which allows for rapid screening and supports traceability. Later researchers such as Garrigues et al, 2021 (28) demonstrated that CRISPR/Cas9 genetically edited Apergillus niger strains incorporating genetic barcodes did not impact the performance of resulting strains which is crucial to the successful application of this technology.

From a technical angle, the literature supports the potential application of genetic markers to PBO development to provide an alternative approach to confirm the presence of a specific PBO product. However, the main issue with this for PBO traceability is that insertion of any foreign DNA into the organism's genome will be considered as transgenic recombinant DNA, and by definition the organism would be classified as a conventional GMO and therefore subject to usual GMO controls and legislation. Further technical complications could occur as well, including that unless the molecular tag exhibited complete genetic linkage to the mutation of interest, then there is a strong likelihood of it segregating out in subsequent generations. The natural genetic drift (mutations) may also occur over time, causing the sequence of the molecular tag to potentially change. In plants, which can have various levels of zygosity and ploidy, the genetic tag would have to be stable and potentially inserted on all genetic material to ensure traceability.

For these reasons, it is unlikely that artificially introduced genetic varietal markers, tags or barcodes would be plausible for PBO detection.

Blockchain

Blockchain technology (30) has been proposed as a general tool to help compliment traceability in the food and feed supply chain (31-33). Blockchain was developed as the technology underpinning cryptocurrency and essentially comprises a public tamper-proof ledger detailing all transactions taken place across a peer-to-peer network. Linked blocks of data identifiable by its hash ensures the integrity of transactions.

The application of blockchain technology within the agri-food sector has been discussed in recent literature (31-33) and highlights the use of blockchain to increase transparency and accountability in supply chain networks, help in the detection of counterfeit products and facilitate product traceability. The integration of blockchain technologies into the analytical approach to detect precision bred organisms could help maintain up to date and traceable methodologies whilst ensuring broad availability.

The implementation of blockchain for GMO/PBO traceability may be operationally challenging and only fully effective in a closed and well-established system. However, as an additional tool to help augment traceability as part of a weight-of-evidence approach, blockchain could be used to provide supplementary electronic and documentary evidence to support traceability provided through molecular biology methods.

(Pan-genomic) databases

The efficacy of any analytical approach is completely dependent upon the availability of (curated) databases or reference materials, and fit for purpose methods for the detection of PBOs are not excluded from this. A comparator is required in order to provide positive confirmation that a sequence variation exists.

The scientific community has discussed the generation of pan-genomic data bases (2, 34), incorporating both genome edited and wildtype lines, in order to affirm that an observed mutation is due to genome editing only, thus facilitating development of molecular biology methods specific to that mutation.

Woodhouse and co-workers, 2021 (34) demonstrated the development of a pan-genomic database called the Maize Genetics and Genomics Database (MaizeGDB) which hosts genomic data covering a large number of maize genomes, including reference genomes and Nested Associated Mapping (NAM) population founder lines. These PacBio genome assemblies of the NAM population founder lines represent the underpinning genomic data (high quality reference datasets are crucial) which enables analysts to explore the complexity and diversity of the maize genome. Representative pan-genomic databases are important supportive tools for PBO analysis as they provide reference genomic sequences to aid in the detection of genome edited events by highlighting natural sequence variation and facilitating biostatistical/bioinformatics approaches.

However, production of pan-genomic databases will be challenging for a number of reasons. One reference genome per crop species will not be feasible due to naturally occurring genetic variability. A pan-genomic database would need to capture all appropriate sequence variability (e.g. commercial crop cultivars as well as wild type examples) and would require significant input in terms of financial resources, infrastructure and bioinformatics (4). Such databases will require continuous and active curation, as new lines are developed and existing ones are sequenced. Complications to reference sequences can occur through sexual reproduction, introgressions, induced mutagenesis, naturally occurring mutations and other evolutionary processes. The database should incorporate all naturally occurring mutations, not exhibit any sequencing bias or misrepresentation of sequences, and sequencing errors and false positive rates should be kept to a minimum. The infrastructure required to store huge volumes of data, as well as its security, backup and disposal, in adherence to national and international laws, must be considered. It would be important to ensure that such databases were also freely accessible on an international basis.

The production of such databases would provide strong technical challenges, being resource intensive and requiring constant curation to have any effectiveness. Nevertheless, the generation of such a central repository, if created, could be used to share data and would create an unparalleled scientific resource and opportunity.

Reference Materials

Reference materials (35) are crucial to underpinning the accuracy and comparability of analytical results between analysts, laboratories and over time. Existing GMO labelling regulations are supported by a wide variety of CRMs that range from mass fraction assigned powder CRMs to genomic DNA with metrological traceability at their core. Validated analytical methods for the detection of PBO products would benefit greatly from the availability of appropriate reference

materials, including sequence data, cell lines and genomic DNA.

Because PBOs are produced by genome editing, there may be multiple on-target and off-target mutations present in just one PBO. Unless all of the mutations exhibit strong genetic linkage, they are not likely to be inherited in the progeny as one coherent unit, and are likely to segregate out. In these instances, it may be challenging to produce an appropriate and representative reference material for a PBO. This may ultimately put additional pressure on the control system, should individual analyses need to be conducted for each mutation and type of PBO mutation.

Biobanks and related resources

Biobanks

The term biobank is typically applied to biological collections of human, animal, plant or microbial samples; and associated information (e.g., sample data) that are professionally managed and represent important resources that are currently being standardised/harmonised internationally through ISO working groups. ISO activities have led to the development of standards focussed on the clinical sector such as ISO 20387:2018 (36) which describes the “general requirements for biobanking” and ISO 21899:2020 (37) covering the “general requirements for the validation and verification of processing methods for biological material in biobanks”. The general concepts and guidance are directly applicable to agri-food related applications. Biobanking resources have been developed to support genome editing applications such as CRISPR (38).

Bioresources

The term bioresources typically describes the laboratory animals, plants, cells, genes, and microorganisms used for research and is historically focussed on the clinical field through large bioresources such as the National Institute for Health and Care Research (NIHR) BioResource. However, plant-based bioresources are increasingly important within the sector such as the European Cooperative Programme for Plant Genetic Resources (ECPGR) which is a collaborative programme among many European countries aimed at ensuring the long-term conservation and facilitating the increased utilization of plant genetic resources in Europe.

Biorepositories

The term biorepository typically refers to a facility that collects, catalogues, and stores samples of biological material (e.g., cells, tissues, DNA/RNA and proteins) from humans, animals, or plants for research. Biobank and biorepository terms are frequently interchangeable within the scientific community.

Role as a support tool

Biorepositories, biobanks and bioresources could represent important resources to support the detection of PBOs by providing access to high quality collections of curated and annotated biological materials (e.g., cell lines, gDNAs) to support the development and validation of methodologies. In addition, biobanks/bioresources provide developers with potential routes for external quality assessment (EQA) schemes. Unless part of the authorisation process, agreement would have to be sought with the producers of such PBOs for permission to house such materials, which may provide operational and legal challenges.

Cost implications of the methods

One of the objectives of the current report was to provide an indication of the costs associated with the development and deployment of detection methods for PBOs, for example through

implementation of such methods in official laboratories. However, since no effective methods for the detection of PBOs have yet to be developed or agreed upon, it is challenging to provide any cost estimates with any certainty, and more research is required.

The cost structure associated with a particular methodology is dependent on a complex set of factors which include the analytical technology employed (e.g., qPCR has a lower cost base than NGS-based approaches), the availability of reference materials (externally sourced or in-house) and access to suitable biobanks/bioresources to support method development and validation. Commonality exists across methodologies in areas such as access to biobanks/bioresources which are frequently available on an international basis for research and commercial purposes, e.g., the Riken BioResource Research Center provides a wide variety of bioresources, including plant seed and clones, with an example fee for a set of individual plant DNA (per 100 clones) costing JPY 264,200 (~£1600 for academic research and JPY 528,400 (~£3300) for-profit research.

Modern molecular biology techniques remain the most viable option for detection of PBOs, with qPCR being imbedded into the infra-structure of a number of analytical laboratories, thus offering good practicability. Both digital PCR and NGS have also been proposed as techniques for potential detection of PBOs. Whilst these technologies are increasingly becoming more accessible, the cost and skill base requirement make these less practicable, but perhaps the only, options at the current stage.

Challenges and recommendations

This section provides an assessment of knowledge gaps, limitations, and challenges in detecting products of precision breeding. Current and future analytical challenges have been examined, as well as some of the potential implications of the current version of the Genetic Technology (Precision Breeding) Act. Alongside these challenges, recommendations are made to provide the infrastructure for working towards a toolkit for the design, development and implementation of analytical methods for detection of PBO products. It is important to recognise that fully effective methods for PBO detection are yet to be demonstrated, so these recommendations have been made as a tool for progressing the current state-of-the-art for research into such methods.

Current state of the art in molecular biology based methods

This review has established that modern molecular biology techniques (qPCR, dPCR, and NGS), have the technical potential to detect small DNA alterations. The review also highlights that it is currently very challenging to distinguish mutations obtained by genome editing, from those obtained through traditional processes.

Modern techniques inclusive of qPCR, dPCR, and NGS currently offer the best analytical potential for detecting products of genome editing, but the confidence in applying these techniques will be dependent upon the efficacy of databases and the availability of suitable reference materials. As per conventional GMOs, products of genome editing can only be readily detected and quantified in commodity products by enforcement laboratories if prior knowledge on the altered genome sequence is known, alongside a validated detection method and access to CRMs.

Main recommendations for providing an infrastructure towards the design, development and implementation of analytical methods for the detection of PBO products

Diversity of techniques encompassed by the term “genome editing”

PBOs may be generated as result of the multitude of techniques incorporated by the umbrella term of genome editing, all of which may differ in their mode of action and the resultant mutational changes. Because of this, generalisations regarding techniques and methods suitable for detection of all PBOs are not appropriate. For example, developing methods for the specific detection of a PBO containing just one SNV will be extremely challenging. However, some SDN-3 activity, which may result in larger DNA sequence changes at unique insertion regions at a genomic locus, may be more suitable for detection methods. It is therefore recommended that each PBO be treated on a case-by-case basis in order to evaluate the likely success of a method providing unequivocal detection of a particular PBO.

Assessment of PBOs intended for submission for authorisation

Assessment of the extent, nature and number of mutations associated with PBOs intended for submission as part of the authorisation procedure, should be conducted. If the majority of these consist of large sequence mutations present at unique points on the genome of an organism (e.g. following certain SDN-3 type activities), then there is increased confidence that molecular methods could be used to provide unequivocal identification of that specific PBO. This report recommends additional work be conducted, in parallel with UK and international authorities, to monitor those PBOs potentially destined for authorisation, and actively assess the extent of the genetic variability and mutations, in order to make an informed decision on the type and complexity of detection methods that may be effective. This could be further informed as part of the official authorisation process and results on sequence information be held in a publicly available database or register.

Weight of evidence approaches

Some scientists support a weight of evidence approach as an aid to traceability of genome edited products. The theory is to capitalise upon other heritable markers, aside from the mutation of interest, in order to increase the likelihood of providing a unique signature or footprint. This includes the sequence of interest, flanking regions, the genetic background, the concept of “scars” (the unintended effect caused by application of any technique, including genome editing), strongly linked off-target mutations, use of CRISPR/Cas to actually detect SNVs (22), and documentary evidence such as supplier, origin, pedigree and other appropriate supporting information.

The theory behind using such a weight of evidence approach is currently hypothetical only, and opponents to this approach cite that not all changes are heritable, and those that are may also segregate out in subsequent generations. It is therefore recommended that further research and development, allied with laboratory-based evidence, is required to evaluate the likelihood of success for such approaches.

Probabilistic based approaches on genetic changes and causation factors

Up to this point, the opinions and evidence for the efficacy of modern molecular biology techniques in detecting PBOs has been based on the ability of such approaches to detect small changes as well as determine the source of that change. An alternative line of thought is to use an approach to model the likelihood of the extent, type, amount and number of changes occurring as a result of traditional processes, based on historical datasets. Although limitations to this approach exist, inclusive of the requirement for a priori information on the sequence of interest and access to historical datasets, it may be possible to use a probabilistic approach (27) inclusive of bioinformatics and statistics (2) to determine the likelihood of a mutation occurring as a result of genome editing. This approach may provide a more pragmatic solution to the labelling issue,

whereby agreed thresholds are assigned to whether a defined change is more likely to be as result of genome editing as opposed to traditional processes. It was outside the mandate of the current review to assess all available information to make an informed decision regarding any correlation between number/type of genetic change and the source of this.

Additional work is recommended, to further examine any evidence of the feasibility of applying limits/thresholds associated with genetic changes as part of a probabilistic model to make any distinction on how they may have occurred. This would allow conclusions to be drawn regarding the likelihood of a particular mutation arising as a result of traditional processes or precision breeding. If such a link does exist, then this could potentially be exploited further in recommending pragmatic threshold levels for PBO determination and classification.

Experimental based evidence

Several scientific papers on detection of genome edited products have been published, many which are based on theoretical principles only. It is therefore important that further practical experimental work be conducted in the laboratory environment, to qualify these theoretical assumptions using evidenced based research.
This review recommends that further research is conducted, supported through practical laboratory work, in order to provide a more evidenced based approach to inform whether different potential methods could be used as possible options for unequivocal identification of PBOs.

Additional recommendations to augment an infrastructure towards the design, development and implementation of analytical methods for the detection of PBO products

Target analyte for potential detection of genome editing: DNA

There is general scientific consensus between experts in the field, that molecular biology based techniques afford the best potential for detecting minute changes in the genome of an organism. It is therefore recommended that methods based on modern molecular biology techniques, inclusive of qPCR, dPCR, and NGS, currently offer the best analytical potential for also detecting PBOs and their products.

Interpretive elements from the current Genetic Technology Act

The Genetic Technology (Precision Breeding) Act enacts primary legislation to amend the regulatory definition of a GMO, to exclude those organisms which exhibit genetic changes which could have occurred through traditional processes. The Genetic Technology Act (1) may provide challenges in terms of an interpretative context, with concerns raised regarding any requirement for products containing PBOs to be traceable and labelled.
Included in its definition, the Act describes a precision bred organism as an organism having features of its genome which could have resulted from traditional processes. It is unclear at the current stage how up-regulation or over-expression of a gene may be interpreted. Additionally, mutations which occur in regulatory sequences, non-coding RNA genes, introns, telo/centromeres, satellite DNA, STRs, pseudogenes, etc., many of which do not result in a functional protein per se, may also have interpretive difficulties according to the current text of the Act.

There does not appear to be a definitive requirement for traceability and labelling of PBOs, where the Act states that “Regulations . . . may, in particular . . . impose requirements for the purpose of securing traceability in relation to food or feed produced from precision bred organisms that is placed on the market in England.” Should there be no traceability requirements, it will be very

challenging to prevent food and feed containing a precision bred organism from being subject to fraud and adulteration.

Classification of genome editing approaches

Interpretative elements regarding classification of genome editing approaches (e.g. intragenesis) between the UK and other trading regions of the world may provide further regulatory challenges in the future. It is recommended that policy makers continue to remain aware of any complementary or contrasting definitions regarding genome editing classification, should this have an impact upon UK trade.

Screening for PBOs

Genome editing is an umbrella term, encompassing a range of techniques which can differ in their mode of action and their end result. Such diversity, coupled with the precision changes afforded by the techniques, often means that there are little to no common elements or motifs which can be used to design general screening strategies for all PBOs.

However, some limited screening may be appropriate for PBOs as part of a triage system, should a priori information be known regarding the sequences of interest. Methods detecting short DNA alterations (such as qPCR, dPCR and NGS), without necessarily identifying the specific PBO, could be used as effective screening tools. Such a screening approach is not likely to inform the analyst if the targeted mutation is as a result of genome editing, as opposed to traditional processes. However, they could provide some confidence whether further analytical work is necessary, as the result of the test will fall into one of two camps.

The first outcome would be that the sample does not possess a genetic variation consistent with the profile of the PBO, and therefore no further analysis is required. The second outcome would be that the test sample does possess a profile consistent with that of the PBO, but also potentially consistent with genetic variation that could have occurred as a result of traditional processes. In these instances, further investigations would need to be conducted to identify the source of the genetic change, and confirm whether or not the sample is a PBO.

It is recommended that a specialist literature review be conducted, potentially informed by departments within the UK and EU governments, to assess the availability of sequences of interest associated with genome edited products and potential PBOs, in order to inform a decision on the utility of possible screening approaches and associated decision support systems.

Quantification of PBOs

It is theoretically possible that quantification of PBOs could be achieved, but this is dependent upon a priori information on the sequence of interest, evidence that the sequence is specific to a particular PBO, a validated detection method and availability of an appropriate reference material. As effective methods for the detection and identification of PBOs have yet to be developed, this presents a barrier for further progress towards developing quantitative approaches.

It is recommended that agreed and validated methods are developed and made available for the specific detection of particular PBO lines, prior to addressing the potential quantitative assessment of such PBOs.

Reference materials

The section entitled “Supportive Tools” provides details on reference materials as a supportive tool, including one of the main challenges regarding the potential transient nature of PBO reference materials due to segregation of multiple on- and off-target mutations.

It is recommended that further research be conducted regarding the likelihood of multiple mutations segregating out in a PBO, to help inform the possibility of developing appropriate PBO reference materials.

Off-target mutations

It has been suggested that the presence of off-target mutations may provide an additional tool to augment methods for detection for PBOs. However, this strategy will only be effective should the off-target mutation exhibit complete genetic linkage to the mutation of interest, and therefore will be inherited as one discrete unit, otherwise they are likely to segregate independently in subsequent generations. In synergy with the previous recommendation, this report recommends that further research be conducted regarding the likelihood of multiple mutations segregating out in a PBO, to help inform the likelihood of using off-target mutations as an additional tool for traceability of PBOs.

Practicability

Practicability can be defined as “The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. cost/sample) of the module” (7). It is important to note that most control and analytical laboratories are commonly equipped with real-time PCR instrumentation as well as trained personnel to use these, but may not necessarily have immediate access to dPCR or NGS. As digital PCR and NGS are being proposed as methods for the potential detection of genome edited products, where the skill base and associated costs may be prohibitive, they may be less practicable solutions.

It is likely that the analytical strategy for detection of PBOs may be different from current conventional detection methods for GMOs, due to a PBO possessing more than one intended or beneficial mutation in its genome as a result of genome editing. Unless these mutations exhibit strong genetic linkage, they are not likely to be inherited as coherent single units in subsequent generations following sexual crosses, and the individual mutations are likely to segregate. It may be necessary to have an individual validated detection method available for each separate mutation, to ensure full traceability. The application of multiple methods for one sample is both costly and time consuming, meaning that detection of a PBO line may not be practicable.

It is recommended that, where practical, development of PBO detection methods should focus on the real-time PCR technique, to make the methods more accessible to analytical laboratories. However, this review also recognises that it may not be possible to develop fit for purpose methods of detection for PBOs without using dPCR or NGS, and these techniques should not be excluded purely on the basis of practicability alone.

(Pan-genomic) databases

The section on “Supportive Tools” provides information on the main practical challenges of implementing a pan-genomic database for each taxon, but also acknowledges the unparalleled resource and opportunity such a database would provide to the research and controls community.

Whilst recognising the technical challenges of developing and maintaining such a pan-genomic data base, it is recommended that the UK continues to consider development of such a resource, either as a UK centric version for controls within the UK, or through engagement in parallel EU and international activities, to better achieve harmonisation and shared responsibilities.

Discussion

Common terms and definitions associated with genome editing in plants and animals have been reviewed, considering key published texts from the EU and UK, with the aim of harmonising vocabulary and providing further clarity. A focus has been provided on more UK centric terms and definitions as outlined in the Genetic Technology “Precision Breeding” Act and key supportive UK expert groups. Harmonisation of this terminology helps contribute towards removing ambiguity and promoting further understanding in relation to common terms used in the genome editing process.

A review was provided on the current state of the art of methods for the potential detection, identification and quantification of precision bred products in the UK. Using synergistic parallels drawn from the development of methods for the detection and quantification of GMOs, informed discussions and conclusions on the scope and limitations of potential methods have been drawn.

Modern DNA based molecular biology approaches, inclusive of qPCR, dPCR and NGS, remain the best techniques for detection of alterations in an organism’s genome. Microarrays, protein based methods and metabolite methods are not well suited for detection of small genome alterations, suffering from general issues inclusive of sensitivity/specificity, requirement for further confirmation, and responses being dependent upon developmental and environmental conditions.

Technically, modern molecular biology based approaches are capable of detecting minute changes in the sequence of an organism’s genome, given certain requirements have been met (including a priori information on the sequence of interest and its flanking regions). What these techniques are not necessarily capable of, in isolation and just targeting single alterations, is providing unequivocal identification of the source of that mutation, be that as a result of genome editing, or traditional processes.

In specific instances, detection and identification may be technically possible, if enough additional information is available to prove that a DNA sequence is unique to a specific genome edited line. Genome editing and new mutagenesis techniques are umbrella terms, incorporating a diversity of modes of action and resultant mutations, and each PBO may have to be treated on a case-by-case basis.

The current deficit in methods for the detection of PBOs precludes their effective quantification, and it is likely traditional screening approaches will have limited use due to the lack of common control elements incorporated into the genome of an organism following genome editing. Real-time PCR remains the most practicable technology for use in analytical laboratories, and detection methods based on dPCR or NGS, which may have to be used, could provide a further hurdle in terms of accessibility and affordability. The potential transient nature of genomes associated with PBOs may preclude generation of suitable reference materials, due to likely segregation of on- and off-target mutations. Those same off-target mutations could help provide additional confidence in the identity of a PBO, but only if they are stable and heritable alongside the mutation of interest. Weight of evidence approaches may help augment successful identification of PBOs, but a number of these heritable markers have only been postulated in theory and further R&D and experimental led evidence is required to support this.

Recommendations are made in order to provide the infrastructure for working towards a toolkit for the design, development and implementation of analytical methods for detection of PBO products. These are in full recognition that, currently, fully effective methods for PBO detection have yet to be demonstrated.

The five main recommendations are as follows. Firstly, because of the nature of PBOs and the diversity of techniques used to produce these, it may be wrong to generalise upon the efficacy of a technique for detection of all PBOs. It is recommended that PBOs should be assessed on a case-by-case basis to determine the extent of the genetic change, which will then inform the likelihood of a method for detection.

Linked with this, is the second recommendation that a specialist review be conducted, potentially informed by UK government departments, to assess the nature, number and extent of mutational changes associated with potential PBOs which may be submitted as part of the authorisation process, to enable an informed decision on the likelihood of developing successful detection methods. This could be further informed as part of the official authorisation process and housing of DNA sequence information in a public register.

Thirdly, a weight of evidence approach, considering other heritable markers associated with a PBO as well as supporting material from potential epigenetic/epitranscriptomic and documentary traceability, may provide further confidence in confirmation of a PBO product. Further specialist research and development, allied with laboratory-based evidence, is required to explore the hypothetical issues for the detection of PBOs.

Fourthly, it is recommended that an additional specialist review is conducted, assessing the extent/type/nature of the genetic changes, and exploring the feasibility of associating causation factors to these. A probabilistic approach supported with bioinformatics to determine the likelihood of a particular changes occurring through genome editing or traditional processes, could facilitate rapid classification and pragmatic labelling of products and organisms containing specific mutations more readily.

Finally, several scientific publications on detection of genome edited products have been mainly based on theoretical principles. It is therefore recommended to further qualify these using evidenced based research, ideally with practical experimental work in the laboratory environment.

These five main recommendations are augmented with supplementary ones. Prior to considering a quantitative approach for PBOs, methods for detection need to be agreed and developed. Further research needs to be conducted on the segregation of off-target mutations, so as to inform development of appropriate reference materials as well as the potential utility of off-target mutations as additional tools for traceability. As an aid in practicability and implementation in control laboratories, and where feasible, it is recommended that real-time PCR technique be the method of choice for PBO detection. Finally, the feasibility of developing and maintaining appropriate pan-genomic databases per taxon should be considered, optimally as a shared resource.

Conclusions

The main implications of the findings are summarised below:

1. Annex I “Terminology” has been provided to help harmonise the terms being used with reference to genome editing in plants and animals, as an aid to promoting further clarification and understanding of the technical terminology used.
2. Consensus scientific opinion supports that modern molecular biology techniques (i.e., qPCR, dPCR and NGS) can technically detect small alterations in an organism’s genome, given specific prerequisites of a priori information on the sequence of interest and the associated flanking regions.
3. Modern molecular biology techniques (qPCR, dPCR, and NGS), continue to offer the best analytical potential for designing and developing methods for detecting PBOs. The current skill base and costs required for dPCR and NGS means that traditional qPCR, now embedded in most analytical laboratories, remains the most practicable solution.
4. Should sufficient information be known regarding a sequence alteration and there is confidence that the sequence alteration is specific to a PBO, then detection, identification and potentially quantification can theoretically be achieved.
5. Genome editing and new mutagenesis techniques are umbrella terms, incorporating a plethora of approaches with diverse modes of action and resultant mutational changes.

Generalisations regarding the use of one technique for the traceability of all such products cannot be made. Each genome edited product may have to be assessed on a case-by-case basis.

6. The application of modern molecular biology techniques, in isolation and by targeting just a single alteration, are unlikely to provide unequivocal identification of the source of a mutation, as these may arise through genome editing or traditional processes.
7. Traditional screening approaches, similar to those used for testing for conventional transgenic GMOs, cannot easily be applied for PBOs due to the deficit in common control elements incorporated into the host genome following genome editing. However, some limited screening may be appropriate for PBOs as part of a triage system, should a priori information be known regarding the sequences of interest.
8. Several scientific papers support the use of “minimum qualifying information” and weight of evidence approaches: the unique combinations of the site of interest, flanking regions, genetic background, off-target mutations and possible epigenetic and epitranscriptomic changes, which collectively may produce a unique “signature” or “footprint” to provide unequivocal identification of a particular genome edited line. Additional supplementary and supporting information such as supplier, origin, pedigree and other appropriate documentation, as well as blockchain, may be useful. Further specialist research and development, allied with laboratory based evidence, is required to evaluate the likelihood of success of using a weight of evidence approach for the design and development of detection methods for PBOs.
9. It is recommended that PBOs submitted for authorisation be assessed on a case-by-case basis in terms of the extent, type and number of genetic changes they contain in order to make a more informed decision of the likelihood of a molecular biology method (or methods) being designed and developed to detect a PBO. This could be further informed as part of the official authorisation process and housing of DNA sequence information in a public register.
10. An approach to model the likelihood of the extent, type, nature, amount and numbers of mutations occurring as a result of traditional processes, based on historical datasets, is recommended. Probabilistic modelling based on historical data sets using bioinformatics and statistical approaches may help infer the likelihood of a particular change occurring because of genome editing. This approach may provide a more pragmatic solution to the labelling and traceability issue, whereby agreed thresholds are assigned to whether a defined change is likely to be as result of genome editing.
11. Several scientific publications on detection of genome edited products have been based on theoretical principles only. It is recommended that further research is conducted, supported through practical laboratory work, to provide a more evidenced based approach to inform whether different potential detection methods could be developed for PBOs. There is a need to further explore the hypothetical issues for the detection, identification and quantification of food/feed containing precision bred organisms with objective experimental evidence through R&D.
12. Quantification of PBOs will remain challenging, and the current deficit of suitable methods to detect and identify PBOs precludes accurate quantification.
13. Development of suitable reference materials to aid in the traceability of PBOs remains an issue, particularly for those PBOs which house on- and off-target mutations which can potentially segregate out in the resulting progeny. Off-target mutations may provide an additional tool to augment methods for detection, but unless these exhibit strong genetic linkage to the mutation of interest, these are likely to segregate out in resulting generations. Further research needs to be conducted regarding the likelihood of multiple mutations segregating out in a PBO, to help inform the possibility of developing appropriate PBO reference materials, as well as the likelihood of using off-target mutations as an additional tool for traceability of PBOs.
14. Whilst recognising the technical challenges of developing and maintaining a pan-genomic data base, it is a recommendation from this report that the UK continues to consider

development of such a resource, either as a UK centric version for controls within the UK, or through engagement in parallel EU and international activities to better achieve harmonisation and shared responsibilities. Such databases would provide an unparalleled scientific resource and would be central to the design of reliable detection methods, as well as affording positive confirmation that a mutation is as a result of genome editing.

Key recommendations and future work

As part of this report, key recommendations are made to provide the infrastructure for working towards a toolkit for the design, development and implementation of analytical methods for detection of PBO products. These have been provided on the basis that the current state of the art regarding the inability/limitations of differentiating between traditional processes and precision bred events currently precludes the establishment of fully effective detection methods.

Recommendations have been fully described in the “Challenges and recommendations” section, but the five key recommendations for future work have been summarised below:

Diversity of techniques encompassed by the term “genome editing”

PBOs may be generated as result of the multitude of techniques incorporated by the umbrella term of genome editing, all of which may differ in their mode of action and the resultant mutational changes. Because of the diversity of techniques and their effects, generalisations regarding the efficacy of detection methods appropriate to all PBOs are not possible. For example, developing methods for the specific detection of a PBO which contains just one SNV will be extremely challenging. However, SDN-3 activity which results in insertion of larger DNA sequences at a predefined genomic locus, may potentially be more suitable for the design of appropriate methods of detection.

It is therefore recommended that each PBO be treated on a case-by-case basis to evaluate the likely success of a method providing unequivocal detection of a particular PBO.

Assessment of PBOs intended for submission for authorisation

It is important to assess the extent, type, nature and number of mutations associated with PBOs intended for submission as part of the authorisation procedure. Should the majority of these consist of larger sequence mutations present at unique points on the genome of an organism (e.g. following certain types of SDN-3 type activity), then there is increased confidence that molecular biology methods could be used to provide unequivocal identification of a specific PBO.

This report recommends additional work be conducted, in parallel with UK and international authorities, to monitor those PBOs potentially destined for authorisation, and actively assess the extent of the genetic variability and mutations, in order to make an informed decision on the type and complexity of detection methods that may be effective. This could be further informed as part of the official authorisation process and valuable data, such as DNA sequence information, be held in a publicly accessible register.

Weight of evidence approaches

It has been suggested by some scientists that a weight of evidence approach be used as an aid to traceability of genome edited products. This concept centres on using other indicators, aside from the single mutation of interest, in order to increase the likelihood of providing a unique signature or footprint. This includes taking into account the genetic background, flanking regions, off-target mutations, potential CRISPR/Cas activity, feasibility of heritable epigenetic and epitranscriptomic changes, as well as supplementary material from supplier, origin, pedigree,

blockchain and other documentation.

The theory behind using a weight of evidence approach is currently hypothetical only, and opponents to this approach cite that not all changes are heritable, and those that are may also segregate out in subsequent generations. It is recommended that further research and development, allied with laboratory-based evidence, be conducted to evaluate the likelihood of success of weight of evidence approaches for the detection of PBOs.

Probabilistic based approaches on genetic changes and causation factors

An approach to model the likelihood of the extent, type, nature, amount and numbers of mutations occurring as a result of traditional processes, based on historical datasets, could prove useful. Probabilistic modelling using bioinformatics and statistical approaches may help infer the likelihood of a particular change occurring because of genome editing. This approach may provide a more pragmatic solution to the labelling issue, whereby agreed thresholds are assigned to whether a defined change is more likely to be as a result of genome editing as opposed to traditional processes.

It was outside the mandate of the current review to provide a specialist literature review to make an informed decision regarding any correlation between number/type of genetic change and the source of the mutation. Further research and development need to be conducted in order to determine whether this approach is likely to be successful.

Experimental based evidence

The range of techniques encompassed within the umbrella term of genome editing is diverse, as is their mode of action and resultant mutations. Should sufficient information be known regarding the sequence alteration and confidence can be attributed to that sequence alteration being specific to a PBO, then detection, identification and quantification can potentially be achieved.

Several scientific publications on detection of genome edited products have been mainly based on theoretical principles. These need to be further qualified using evidenced based research, ideally with practical experimental work in the laboratory environment. This review recommends that further work is conducted, supported through practical laboratory exercises, to provide a more evidenced based approach to inform whether different methods could be developed for unequivocal identification of PBOs.

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Annex 1

Summary of key terms related to genome editing

Gene editing: techniques for genome engineering that involve nucleic acid damage, repair mechanisms, replication and/or recombination for incorporating site-specific modification(s) into a

gene or genes. Gene editing is a subclass of **genome editing** (39).

Genome editing: techniques for genome engineering that involve nucleic acid damage, repair mechanisms, replication and/or recombination for incorporating site-specific modification(s) into a genomic DNA (39).

Edit: modification to nucleic acid sequence resulting from the application of genome editing components. Example edits include insertions, deletions, substitutions, deamination, methylation and demethylation (39).

Target: nucleic acid sequence subject to intentional binding, modification and/or cleavage during a genome editing process (39).

Off-target: refers to genome position and/or nucleic acid sequence distinct from the target. An off-target edit is an example of an unintended edit (39).

InDel mutation: sequence change caused by the insertion and/or deletion of nucleotides (39).

Genome editing can encompass a plethora of techniques that can generate different kinds of alterations in the genome, varying from single nucleotide variations (SNVs) to deletions and insertions of many base pairs. Example key approaches and components of applied genome editing approaches, alongside their relevant definitions, are provided below:

Site-directed nuclease: (sequence specific nuclease): enzyme capable of cleaving the phosphodiester bond between adjacent nucleotides in a nucleic acid polymer at a specific sequence (39).

SDN-1: site-directed nuclease type 1 introduces random mutations (e.g. substitutions, insertions, and deletions) at the target plant genomic site (40). SDN-1 are derived from bacteria and includes genome-editing systems such as CRISPR (41). SDN-1 approaches include DNA repair mechanisms involving non-homologous end joining, which can result in InDels. However, should the lesions occur at two spatially separated locations within a chromosome, SDNs can result in larger DNA deletions as well (41).
SDN-2 and SDN-3 type changes: Produces targeted changes in the DNA sequence based on homologous end joining. Typically, a DNA template is introduced into the cell to help direct the DNA repair mechanism to generate the precise sequence change that is required. SDN-2 and -3 mediated changes can also result in small InDels, but also larger changes inclusive of partial/whole gene and allele replacement, or insertion of cisgenes. (41). SDN specific details:

- **SDN-2** (site-directed nuclease type 2) makes use of template DNA to generate a predicted modification (i.e. intended sequence modification) at the target plant genomic site (40).
- **SDN-3 (**site-directed nuclease type 3) can introduce a larger DNA sequence change (up to several kilobases) into a predefined insertion region of a genomic locus (40).

Cas nuclease: (CRISPR associated nuclease): enzyme that is a component of CRISPR systems that is capable of breaking the phosphodiester bonds between nucleotides (39).

Epigenetic changes: Changes to the DNA structure, including methylation status, which can result in a change in gene expression and heritable phenotypic changes (41)

Site directed nuclease (SDN): an enzyme that recognises a specific sequence and cleaves the DNA usually creating a double-strand break (40). Three types of SDN approaches are commonly used.

New Genomic Techniques (NGTs)

New Genomic Techniques (NGTs) can be defined as “techniques that are capable of altering the genetic material of an organism and that emerged or have been developed since 2001” (42), the latter date corresponding to when the current legislation on genetically modified organisms (GMOs) was adopted (Directive 2001/81/EC).

In this context, additional terminology has also been defined. **Established Genomic Techniques** (EGTs) refers to genomic techniques developed prior to 2001, when the Directive 2001/18/EC was officially adopted (40). As well as transgenic approaches, this also includes chemical and irradiation approaches which have a historical record of being safe. In some instances, EGTs are also referred to as **Established Techniques of Genetic Modification** (ETGM) (17), and can be characterised as transgenic organisms (GMOs) produced via biotechnological applications prevalent in the late 1970’s/early 1980’s. **Conventional GMOs** refers to organisms produced using recombinant DNA technology, typically containing DNA sequences randomly introduced from the same (or other species) (3). Conventional GMOs refer to organisms produced using EGTs prior to the adoption of Directive 2001/18/EC.

New Genomic Techniques can refer to a range of genetic engineering techniques inclusive of targeted mutagenesis, cisgenesis and intragenesis (43). In some publications, New Genomic Techniques are synonymous with **New Plant Breeding Techniques** (NPBTs) (17).

Targeted mutagenesis: is often used as an umbrella term to describe techniques that introduce specific mutations in selected target regions of the genome. These changes typically occur without inserting additional genetic material (43). Examples of targeted mutagenesis include nucleotide substitutions, deletions and insertions (one or several nucleotides long), deletions resulting from targeting two sequences in close vicinity, e.g. at two sites within a single gene. Targeted mutagenesis is mainly achieved through the cell’s natural DNA recombination/repair mechanism, mediated through the use of a site-directed nuclease (SDN).

Cisgenesis: modifying the genetic material of an organism with another sequence, typically from the same species or a sexually compatible one. Results in an exact copy (same orientation) of the DNA sequence already present in the breeder’s gene pool (19, 41, 43). A cisgenic product generally also includes introns and flanking promoter/terminators from the native species in the same sense orientation. Cisgenesis refers to the insertion of DNA from a related species that is sexually compatible (41).
Intragenesis: modifying the genetic material of an organism with combinations of different sequences from the same species or sexually compatible ones. Results in a re-arranged copy (different orientation) of the sequences present in the breeder’s gene pool. (19, 41, 43). The insert usually comprises full/partial coding region of gene(s) combined with promoters/terminators from different genes of the same species or a sexually compatible one (41).

Note that further definitions for general molecular biology terms can be found in (44) and (45), which this report adheres to.

Summary of key terms related to Precision Breeding

A number of concepts and terms are introduced in the “Genetic Technology (Precision Breeding) Act” (1).

In Part 1 “Precision breeding: definitions” of the Act, **a precision bred organism (PBO)** arises from a **precision bred plant** or animal. To be classified as **precision bred**, an organism must meet the following three criteria:

- Any feature of its genome results from the application of **modern biotechnology**;
- Every such feature is stable (capable of propagation and therefore heritable);

- Every feature of its genome could have resulted from **traditional processes** (whether or not in conjunction with selection techniques)

In relation to the above, the Act also defines the following:

- **“Modern biotechnology”** is any technique mentioned in regulation 5(1)(a) or (b) of the Genetically Modified Organisms (Deliberate Release) Regulations 2002 (S.I. 2002/2443).
- In relation to plants, **“traditional processes”** include sexual fertilisation, spontaneous mutation, in vitro fertilisation, polyploidy induction, embryo rescue, grafting, induced mutagenesis, and somatic hybridisation or cell fusion of plant cells of organisms which are capable of exchanging genetic material.
- In relation to animals, these **“traditional processes”** include sexual fertilisation, spontaneous mutation, artificial insemination, in vitro fertilisation, embryo transfer, polyploidy induction, and recovery and transfer of primordial germ cells.

As part of the assessment of these traditional processes in the context of the Act, no account is to be made of copy numbers, epigenetic status or location on the genome.

Prior to the Act being published, ACRE (Defra’s Advisory Committee on Releases into the Environment), released additional guidance and definitions related to plant products as a result of modern technologies (41).

They stated that a GM plant whose genetic makeup could have been produced by traditional breeding techniques or could have arisen through natural processes could be termed a **Qualifying Higher Plant** (QHP) (41), which could therefore qualify as a precursor to PBOs described in the Act.
To qualify as a QHP, plants subject to SDN cannot contain any transgene elements (DNA from outside the gene pool (sexually compatible) (41).

Although rare in nature in its natural state, SDN-mediated epigenetic changes rely on the same biochemical mechanisms that occur naturally, and hence plants altered in this way are likely to be QHPs (41).

Transgenesis generally refers to the in vitro (outside the cell) manipulation of exogenous DNA from a sexually incompatible species, followed by insertion into the host genome using recombinant DNA technology (41). ACRE qualified that such transgenic changes were likely to be classified as GMOs and not as QHPs.

Plants obtained with targeted mutagenesis techniques which contain inserted recombinant DNA (or parts of transformation vectors used), would likely also be classified as GMOs (41).

Cisgenic plants (insertion of DNA from a related species that is sexually compatible, in the same orientation) are likely to be considered QHPs as their genetic composition is consistent with the genetic variation that could occur naturally within that species, or as a result of traditional techniques and selection (41).

Intragenic plants (insertion of DNA from a related species that is sexually compatible, in a different orientation) are unlikely to result from traditional breeding techniques or natural processes, and therefore are unlikely to be regarded as QHPs (41).