Recent Developments in DNA-Based Screening Approaches for Detection of GMO's

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Summary

Quantitative PCR remains the current method of choice for enforcement laboratories for GMO screening and identification. The number of new EU unauthorised and authorised GM events continues to increase on a yearly basis providing a continuing challenge for screening approaches to keep pace. Several new and innovative DNA-based approaches for GMO detection have recently been developed including PCR and amplification-based approaches, microarrays, micro-fluidic bead-based multiplex assays, digital PCR and DNA sequencing. In the case of existing, fully characterised GMO's, methods based on conventional PCR are still appropriate to rapidly detect individual GM targets. However, when the tested matrices contain GMO's for which only partial sequence data is available or known then approaches which rely on DNA sequencing of targeted or enriched libraries may be the only effective way forward. Single point mutations, such as those introduced through synthetic biology or new plant breeding techniques (e.g. by CRISPR genome editing) may only be identified through DNA sequencing and ways to target and screen efficiently for such mutations are required.

Introduction

Labelling policies for Genetically Modified Organisms (GMO's) have been established in numerous countries around the world. The general framework for regulating GM food and feed in Europe is governed by two main regulations: EU Regulation 1829/2003[1] and 1830/2003[2] (and the associated amending Directive 2001/18/EC[3]). These regulations concern the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms, and the placing on the market and traceability/labelling of GMO's, respectively. These regulations encompass a wide range of issues associated with GMO's in general including harmonised procedures for GMO authorisation, implementation of a labelling threshold for adventitious/technically unavoidable contamination, traceability requirements to help facilitate control and verification of labelling claims and the need to ensure clear and mandatory labelling.

A key factor in the enforcement of these regulations has been the development of effective strategies for GMO detection. Although protein approaches for the detection of GMO's do exist these can suffer from differential expression levels, cross reactivity and poor sensitivity in processed foods when the protein undergoes a conformational change. This short review

focuses on DNA-based screening approaches and provides a brief description of some of the current methods being used and developed in this field.

PCR and Real-time PCR-based GMO Detection Approaches

Currently, PCR-based analysis remains the method of choice for routine analysis of GMO content in food and feed samples; Zel *et al*, 2012[4]. In spite of the approach being rapid, flexible and sensitive it suffers from several critical limitations including:

(i) sensitivity to PCR inhibition; Schrader *et al*, 2012[5], Demeke *et al*, 2010[6]
(ii) the need for certified reference materials (CRM's) to perform quantitation; Broeders *et al*, 2012[7], Fraiture *et al*, 2015[8]
(iii) limited throughput (the majority of GMO quantitative real-time PCR assays are currently singleplex)
(iv) the requirement for continual development of new assays to accommodate the introduction of new GMO's

To address these limitations, a number of alternative approaches have been suggested for routine use in analytical testing laboratories.

Real-time PCR Multiplex Strategies

To reduce the limitation of low throughput a number of multiplex strategies have recently been evaluated and which have focused on the screening for characterised transgene motifs including the p35S promoter and tNOS terminator. Chaouachi *et al*, 2014[9] used a four duplex real-time assay system in order to identify GM maize lines (Bt11, Bt176, MON810 and T25). Park *et al*, 2015[10] used three triplicate multiplex real-time PCR systems to enable the tracking of authorised and unauthorised GM soybean events in food and feed. Köppel *et al*, 2014[11] have also reported on the use of a multiplex real-time PCR system for the efficient screening of food products. However, the development of optimised multiplex assays is recognised as being technically challenging, both in terms of primer and probe design, but also with the availability of reporter dyes with emission and absorption spectra of sufficient separation. The majority of validated multiplex real-time PCR GMO assays described in the literature have been limited to the simultaneous detection of two or three targets and to date it has only been possible to combine a maximum of six markers in a single real-time PCR reaction[12].

Pre-spotted Real-time PCR Assay Plates

With the aim of improving screening throughput a number of alternative real-time PCR screening strategies have been evaluated by laboratories within the EU GMO regulatory community. A platform based on the use of multiple singleplex assays, spotted into individual wells of a 96-well PCR plate, has been developed by the European Union Reference Laboratory for GM in Feed and Food (EU-RL GMFF) and used for routine screening purposes; Querci *et al*, 2009[13], Kluga *et al*, 2011[14]. Since these initial studies other

researchers have reported that pre-spotted plates can be used with different legal frameworks; Randhawa *et al*, 2014[15].

PCR Capillary Gel Electrophoresis (PCR-CGE)

Simultaneous detection of multiple targets with the use of fluorescently-labelled primers (e.g. FAM, NED, JOE), has been applied for GMO detection. Vega and Marina, 2014[16] have published an article reviewing the application of capillary and microchip methods for the detection of GMO's. Basak *et al*, 2014[17] have recently published a paper describing the application of PCR-CGE to the detection of transgenic elements in cotton and soybean.

Digital PCR

Digital PCR (dPCR) addresses some of the problems associated with the quantification step of real-time PCR, particularly where assay target numbers are low and/or PCR inhibitors are present. Two approaches of this technology have so far been developed which are referred to as microfluidic chamber dPCR (cdPCR) and droplet dPCR (ddPCR) respectively. In a recent publication Köppel *et al*, 2015[18] described the use of a duplex ddPCR assay to detect and quantify the presence of four transgenic soya traits (MON87769, MON87708, MON87705 and FG72). However, a major limitation in applying the technique to GMO analysis has been the transfer of previously validated real-time PCR assays. The requirement for re-optimisation of primer and probe design, as well as their working concentration, has been reported by Dreo *et al*, 2014[19].

Given current limitations of the technology (e.g. limited multiplex capability, low throughput), dPCR may be best suited towards accurate identification and quantitation and as a tool for value assignment in reference materials as opposed to full utilisation for routine GMO screening[18,20]. However, as the technology continues to rapidly evolve, improve, and develop further, full validation of dPCR may provide evidence of its fitness for purpose for routine testing in this field. Currently, the EU-RL GMFF and the associated European Network of GMO Laboratories (ENGL) are actively assessing dPCR for GMO analysis and seeking to provide published guidance and recommendations for its use including transfer of existing real-time PCR methods into a digital PCR format within the coming months.

Loop-Mediated Isothermal Amplification (LAMP)

Adoption of loop-mediated isothermal amplification (LAMP) for use in the detection of GMO's has been highlighted in several recent scientific publications; Cheng *et al*, 2014[21], Wang *et al*, 2015[22]. LAMP is both sensitive and specific[23] but with the added bonus of being tolerant to many of the inhibitors of PCR; Zhang *et al*, 2012[24]. In their publication, Di *et al*, 2014[25] have reported on the use of LAMP for the rapid detection of GMO ingredients in soybean products with use of MON89788 (maize) and GTS 40-3-2 (soya) event specific assays. Li *et al*, 2014[26] have described the use of element specific LAMP to detect the presence of *cry2Ab* and *cry3A* genetic elements in GMO crops. The potential of LAMP-based technologies for the quantification of GM events in maize has been reported in a number of recent publications; Huang *et al*, 2014[27], Kiddle *et al*, 2012[28], Bhoge *et al*, 2015[29]. One of the

restrictions to official recognition and wider adoption of the LAMP technique appears to be the need for the use of four primers per target which provide both specificity and sensitivity to the technique; Di *et al*, 2014[30].

Micro-fluidic Bead-based Multiplex Assays

Biotinylated targets amplified using PCR methodologies can potentially be analysed with use of Luminex® technology. The manufacturers claim that the system is potentially capable of detecting up to 500 different targets in one sample by using spectrally distinct sets of beads that have been coupled to unique nucleic acid probes. Fu *et al*, 2015 have published a paper on the application of the methodology to identify 13 lines of genetically modified maize by targeting the junction between the plant genome and exogenous gene. They reported that assay sensitivity in the region of 0.1% m/m had been achieved.

Microarray-based Technology

Microarray platforms currently used for the detection of GMO's require the prior amplification of GM targets using PCR followed by the hybridisation of labelled amplicons to an array, an appropriate washing step to remove non-hybridised products and finally detection of the hybridised target[31]. Nucleic acid sequence based amplification implemented microarray approaches (NAIMA), which utilise universal primers, have previously been reported to detect transgenic maize varieties; Dobnik *et al*, 2010[32]. More recently, a multiplex amplification on a chip platform, targeting ninety-one GMO's, has been described in the literature; Shao *et al*, 2014[33]. Currently-available GMO-targeted microarrays therefore represent a higher throughput but lower sensitivity approach compared to current real-time PCR; Kluga *et al*, 2011[34].

DNA Sequencing-based Approaches

The definitive means for confirming the presence of a GMO is to sequence the DNA across the junction between the host genome and the transgenic insert. To obtain relevant DNA sequence a number of different strategies have been described in the recent literature; Leoni *et al*, 2011[35]. However, the implementation of many of these sequencing approaches present considerable difficulties including:

(i) insufficient specificity and sensitivity
(ii) methods are complex and laborious to perform
(iii) the technique is beyond the current scope of some control laboratories; Fraiture *et al*, 2015[8].

However, several approaches which include targeted sequencing and whole genome sequencing have been developed further.

Targeted Sequencing

Targeted sequencing involves the sequencing of either DNA libraries comprised of PCR amplicons or from selected DNA fragments derived from whole genome libraries. Amplicon sequencing allows the characterisation of DNA fragments previously enriched by PCR. Song *et al*, 2014[36] have reported on the use of a cocktail of PCR primers to generate amplicon libraries for both taxon-specific and GMO markers from food samples. The libraries were then sequenced using a variant of the Roche 454 Next Generation Sequencing (NGS) platform; Wu *et al*, 2013[37], and the reads assembled to indicate the presence of GMO's. Although the approach is similar to that of PCR screening it has the added value of providing the sequence of the amplified fragment which is more reliable in proving the presence of a GMO. However, although the analysis of pre-enriched fragments of interest using NGS technology allows the presence of GMO to be categorically confirmed, given its current relatively high cost and prerequisite for bioinformatics expertise, it is difficult to apply to the routine screening of food and feed matrices by analytical laboratories.

Whole Genome Sequencing

A whole genome sequencing (WGS) approach allows the characterisation of samples to be performed without the requirement for any prior knowledge of the transgene inserts that may be present. With this sequencing strategy a DNA library is firstly constructed from sheared genomic DNA extracted from the sample, which is then sequenced in its entirety. The sequencing reads are initially assembled in order to generate contig scaffolds, and then analysed using bioinformatics tools based on prior knowledge of currently characterised GMO's as an aid in confirming the identity of any GMO's; Yang *et al*, 2013[38]. WGS has been used to identify the presence of GM flax FP967; Young *et al*, 2015[39] and transgenic rice TT51-1 and Tlc-19; Yang *et al*, 2013[38]. However, the success of this strategy was acknowledged as being reliant on the availability of a reference genome for the specific varieties and organisms being evaluated; Schatz *et al*, 2012[40]. More recently Willems *et al*, 2016[41] have reported on the use of a statistical framework to analyse WGS data obtained from samples containing low levels of GMO. The study evaluated the performance of the frameworks with processed foods, including GM/non-GM rice mixtures, and concluded that identification of GMO at trace levels could not be easily achieved using the WGS approach. Currently, only targeted sequencing can be used for GM mixtures containing GMO trace levels.

Overall, NGS technologies offer a promising alternative detection method for GMO's, based on potential proof of GMO presence in food/feed matrices via characterisation of their DNA sequences. However, on a technical standpoint, implementation of NGS in routine analysis for GMO's by enforcement laboratories is currently impractical owing to its relatively high cost and requirement for adequate computer infrastructures as well as the services of a bioinformatics specialist to deal with the data generated; Buermans and den Dunnen, 2014[42].

New Challenges facing GMO Screening and Traceability

Recently, the Scientific Committees of the European Commission's Directorate-General have advised that the products of synthetic biology should fall under the legal mandate of pre-

existing GMO risk assessments, including labelling and testing[43]. Furthermore, it is expected that the European Commission will shortly release a document that also recommends that testing and identification of products of new (plant) breeding technologies will also fall under pre-existing GMO legislation.

Products of synthetic biology and new breeding techniques can be produced via a variety of new techniques including: Zinc-finger nucleases (ZFN)[44,45], Transcription Activator-Like Effectors (TALEN)[46,47] and more recently, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) genome editing approach[48,49]. These new techniques allow for small, targeted specific mutations to be introduced into the organism's genome with high efficiency. The changes introduced into the genome, for example by a Single Nucleotide Polymorphism, may be indistinguishable from changes due to natural variation or conventional breeding, providing further technical challenges for screening and traceability.

Only if the sequence polymorphism of the genome that has been modified is known, will detection and identification techniques for products of synthetic biology and new plant breeding approaches be successful. This is dependent upon prior knowledge of the sequence polymorphism and using advanced sequencing methods (e.g. Next Generation Sequencing) in order to make a positive identification, which can be costly and time consuming. However, recent publications; Sullivan *et al*, 2015[50] have suggested using a targeted approach to make the costs associated with such methods more bearable. The authors state that CRISPR technologies may result in changes in chromatin patterns as by-products of the mutation (e.g. by insertion or deletion mutations [indels] or secondary breaks in the double-stranded DNA chain). By using epigenetic-based methods to identify such changes in the chromatin structure the authors suggest that a targeted approach to high-throughput sequencing can be achieved in a cost effective manner in order to identify any DNA modifications.

Conclusion

Quantitative PCR currently remains the method of choice for enforcement laboratories for GMO screening; Fraiture *et al*, 2015[8] despite a number of fundamental limitations inherent with the approach. Several new and innovative DNA-based approaches for GMO detection have recently been developed and evaluated, and include:

(i) PCR and amplification based approaches
(ii) microarrays
(iii) micro-fluidic bead-based multiplex assays
(iv) dPCR
(v) DNA sequencing

In the case of existing, fully characterised GMO's, methods based on conventional PCR are still appropriate to rapidly detect individual GM targets (e.g. by LAMP), multiple targets (CGE, microarray and micro-fluidic bead-based multiplex assays) or to precisely quantitate (dPCR). However, when the tested matrices contain GMO's for which only partial sequence data is available, such as those that produce partial signals with conventional real-time PCR

methods, then approaches which utilise DNA sequencing of targeted or enriched libraries may well yield the most informative results. If no information is available, at this point in time, only WGS is conceivable to identify this category of GMO; Fraiture *et al*, 2015[8]. Equally well, targeted approaches to high-throughput sequencing need to be achievable so that single nucleotide modifications, such as those introduced by using synthetic biology or new plant breeding techniques, can be cost effectively and rapidly identified.

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