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Identification of single target taxon-specific reference assays for the most commonly genetically transformed crops using digital droplet PCR

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ABSTRACT

Knowledge of the number of DNA sequences targeted by the taxon-specific reference assays is essential for correct GM quantification and is key to the harmonisation of measurement results. In the present study droplet digital PCR (ddPCR) was used to determine the number of DNA target copies of taxon-specific assays validated for real-time PCR for the four main genetically modified (GM) crops. The transferability of experimental conditions from real-time PCR to ddPCR was also explored, as well as the effect of DNA digestion. The results of this study indicate that for each crop at least one taxon-specific assay can be identified as having a single DNA target. A short list of taxon-specific reference assays is proposed as best candidates for the relative quantification of GM events for soybean, maize, cotton and oilseed rape. The investigated assays could be in most cases transferred to ddPCR without further optimisation. The use of DNA digestion did not improve ddPCR characteristics such as rain and resolution at the conditions tested.

1. Introduction

Key to the development of a harmonised monitoring system for genetically modified organisms (GMOs) in food and feed is that results expressing the GM content are reliable, comparable across laboratories and import/export regions, and that they comply with the regulations. A labelling threshold of 0.9% for adventitious or technically unavoidable presence of authorised GMOs is in force in the European Union for which the GMO content is measured relatively to the ingredient (species) (European Parliament and the Council of the European Union, 2003, 2004a, 2004b, 2014; European Commission, 2011; European and Parliament the Council of the European Union, 2013). The European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF), managed by the European Commission's Joint Research Centre, has validated so far in collaborative trials more than sixty event-specific methods using real-time PCR for the relative quantification of GMOs. These methods are used in routine monitoring for official controls. Appropriate quantification depends on the correct amplification and counting of two different targets: the GM event-specific DNA sequence and the taxon-specific sequence. The event-specific assay (an assay is herewith defined as the set of primers and probe, validated with known reaction mix and reaction conditions) is designed to target the insert-to-host genome sequence, which has been selected to be unique (single copy) in the plant genome, due to the random integration process of the DNA insert that occurs with the transformation technologies used so far. For the design of a taxon-specific assay there is in principle a wide selection of host genome sequences that comply with the demands for a standard real-time PCR assay. However, strict requirements have been set for quantitative real-time PCR (qPCR) methods submitted in the context of the authorisation of a new GMO in the European Union: the taxon-specific assay has to be specific to the crop of interest, stable across varieties and should address a single copy in the plant genome, if possible (EURL GMFF, 2015b). Thus, the design of a taxon-specific assay requires sufficient information about the genetics of the plant and necessitates proper optimisation and validation of the whole analytical procedure (Debode et al., 2017; Jacchia et al., 2015b, 2015a). The exercise can be particularly demanding when it comes to allopolyploid species such as cotton and oilseed rape, in which multiple sets of chromosomes (sub-genomes) are present, deriving from distinct species. However, the knowledge of the number of DNA targets per genome for a taxon-specific assay is essential for the correct quantification of a GM event and for the conversion of results of a GM event quantification performed in copy numbers into mass fractions (ratio of the measured GM mass to the total mass of the ingredient). Such conversion is ultimately required when it comes to the measurement of the so called 'low level presence' of GMOs in feed consignments (European

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Commission, 2011).

The European Network of GMO Laboratories (ENGL) has recently elaborated an approach on how to achieve this conversion (Corbisier et al., 2017). The determination of the corresponding conversion factor would employ digital PCR (dPCR), another technology exploiting DNA amplification and detection by using PCR chemistry (Vogelstein & Kinzler, 1999), which has the potential to become the next gold standard in GMO quantification (Corbisier, Bhat, Partis, Rui Dan Xie, & Emslie, 2010; Deprez et al., 2016; Dobnik, Štebih, Blejec, Morisset, & Žel, 2016; Fraiture et al., 2015; Iwobi, Gerdes, Busch, & Pecoraro, 2016; Lievens, Jacchia, Kagkli, Savini, & Querci, 2016). Unlike real-time PCR, where quantification requires a calibration system with standards at known GM DNA concentrations, dPCR does not necessitate a calibration with DNA and estimates the number of targeted copies per reaction, under certain assumptions directly (Sykes et al., 1992; Vogelstein & Kinzler, 1999).

In the present study this feature of dPCR was used to determine the number of DNA targets addressed by the taxon-specific assays for soybean, cotton, maize and oilseed rape listed below. The experimental data were backed by detailed bioinformatics analyses. The purpose of this work was to compile a short list of best candidate taxon-specific reference assays for the relative quantification of GMOs in the most commonly transformed crops (Parisi, Tillie, & Rodríguez-Cerezo, 2016). The taxon-specific assays for the following DNA targets validated in the frame of Regulation (EC) No 1829/2003 (2003) were tested: for soybean (*Glycine max*): *Le1 A* (EURL GMFF, 2012b) and *Le1 B* (EURL GMFF, 2015a); for cotton (*Gossypium hirsutum*): *AdhC* (EURL GMFF, 2012a), *SAH7* (EURL GMFF, 2006) and *acp1* (EURL GMFF, 2009); for maize (*Zea mays*): *hmg* (EURL GMFF, 2005), *ZmAdh1* (EURL GMFF, 2014) and *aldolase* (EURL GMFF, 2016); and for oilseed rape (*Brassica napus*): *ccf* (EURL GMFF, 2013b), *cruA* (EURL GMFF, 2007) and *FatA(A)* (EURL GMFF, 2013a), plus *FatA* (Monsanto Biotechnology Regulatory Sciences, 2004). In this context the transferability of these real-time PCR assays to the QX200 droplet dPCR platform was investigated. The effect of DNA digestion on measurement results was also explored.

2. Materials and methods

2.1. Plant materials

Certified reference materials (CRMs) were used for DNA extraction: maize MON810 level 2, nominal 2% GMO in mass fraction (ERM-BF413ek) and NK603 level 4, nominal 2% GMO in mass fraction (ERM-BF415e); soybean 356043 level 3, nominal 10% GMO in mass fraction (ERM-BF425d) and DAS-68416 blank, nominal 0% GMO (ERM-BF432a); cotton GHB119 level 2, nominal 10% GMO in mass fraction (ERM-BF428c) and T304-40 blank, nominal 0% GMO (ERM-BF429a); oilseed rape 73496, nominal 10% GMO in mass fraction (ERM-BF434e), non-modified canola whole seed (AOCS 0304-A). Except for AOCS 0304-A, produced by the American Oil Chemists' Society, all other CRMs are from the European Commission's Joint Research Centre (JRC). DNA from *B. napus*, *Brassica rapa*, *Brassica juncea*, *Brassica nigra*, *Brassica oleracea*, *Brassica carinata* was prepared by the EURL GMFF.

2.2. Genomic DNA extraction and quality check

The genomic DNA used in this study was extracted with a CTAB DNA extraction method modified from ISO 21571 (International Organization for Standardization, 2005) (for soybean, oilseed rape and with an additional phenol-chloroform purification step for cotton when necessary) or a NucleoSpin® Food kit (Macherey-Nagel, for maize). In cases of suboptimal recovery or inhibition, the NucleoSpin® Food kit (for oilseed rape) or the foodproof® Sample Preparation kit III (Biotecon Diagnostics, for cotton) were used. The integrity of the extracted genomic DNA was tested by electrophoresis on a 1% [w/v] agarose gel stained with ethidium bromide, while the absence of PCR inhibitors was assessed through a real-time PCR inhibition run as described by Zel et al. (Žel et al., 2008). Only pure, non-inhibited, high molecular weight DNA was used. The extractions were done at least in duplicate with CRMs containing two different GM events per each crop; DNA samples were maintained at 4 °C for the duration of the experiments, then stored at −20 °C.

2.3. DNA quantification

Extracted genomic DNA was quantified fluorometrically with the Qubit® Fluorometer (Thermo Fisher Scientific) and the Qubit dsDNA BR assay Kit (Molecular Probes, Life Technologies) prior to its quality assessment for calculating the amount of DNA to be used in restriction digestion reactions. Before diluting for the ddPCR runs all DNA samples (digested and non-digested) were re-quantified together by using a Biorad VersaFluor fluorometer and the Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes) with a five point standard curve ranging from 1 to 500 ng/mL.

2.4. DNA digestion

The required DNA extracted from each CRM was enzymatically digested. For maize, soybean and oilseed rape, *Eco*RI (New England Biolabs) was used, while for cotton *Dra*I (New England Biolabs) was used instead. These two restriction enzymes were chosen because they do not cut inside tested amplicons. *Tsp*RI (New England Biolabs) was also used in a limited set of cotton experiments. Its restriction site is present in the secondary target (herewith defined as the DNA target of an assay that presents mismatches in the primers and/or probe annealing sites) of the assay for *acp1*. The enzymatic digestions were performed in a final volume of 250 µL according to the manufacturer's conditions and inactivated appropriately. After digestion, 5 µL of the digestion solution was loaded on a 1% agarose gel, stained with ethidium bromide and visualised under UV light. If partial or incomplete digestion occurred, the digestion was repeated. Subsequently, samples were precipitated with ethanol (except for T304-40 digested with *Dra*I which was not precipitated because of the limited amount available) and re-quantified. Since it was not possible to achieve complete DNA digestion with *TspRI*, the presence of undigested DNA was verified by comparing the λ values (average number of targets per droplet) measured with ddPCR for the primary and secondary targets of the assay for *acp1*. The amplification yield of the secondary *acp1* target relative to the primary target decreased to 5% in digested samples, compared to 35% in non-digested samples.

2.5. Sample preparation

The total DNA content in samples for in-house testing of all the taxon-specific assays was quantified by PicoGreen (see above) and subsequently diluted to the concentration of interest in 0.1 × TE. The final concentration of the samples to be used in ddPCR was determined by taking into consideration the haploid genome masses of the different species [1.13 pg for soybean; 2.33 pg for cotton; 2.73 pg for maize and 1.15 pg for oilseed rape (Bennett & Leitch, 2012)] and by calculating the concentration needed to theoretically have 1 target copy/droplet in ddPCR, with a droplet size of 0.85 nL, as used by the QuantaSoft software (version 1.6.6.0320) (Corbisier et al., 2015). The DNA amount added to each ddPCR reaction was: 26.6 ng for soybean, 27.1 ng for oilseed rape, 54.8 ng for cotton and 64.2 ng for maize.

2.6. Real-time PCR

The inhibition runs for all materials and the qPCR for GHB119 cotton were performed following the validated methods (EURL GMFF, 2005, 2007, 2009, 2012a, 2012b) with an ABI 7900 platform (Life Technologies) or an ABI 7500 platform (Applied Biosystems).