



Assessment of the Real-Time PCR Method Claiming to be Specific for Detection and Quantification of the First Commercialised Genome-Edited Plant

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Abstract

A real-time PCR method was recently published with a claim to be specific for the detection and identification of some genome-edited oilseed rape (OSR) lines commercialised in North America. The method was designed to detect a single base mutation in the AHAS1C gene, which confers herbicide tolerance. The authors claim that the method is event-specific for the genome-edited OSR line 5715 and fulfils all requirements for GMO analytical methods according to EU regulations. We have thoroughly assessed the method in relation to the minimum performance requirements (MPR) established by the European Network of GMO Laboratories (ENGL). The method was found to be sufficiently sensitive and robust when tested with pure genomic DNA of the OSR line 40 K. However, our results show that the method is not event-specific and detects also OSR lines carrying the same point mutation caused by somaclonal variation. Moreover, impaired robustness was observed using non-modified genomic DNA at the amount specified in the original protocol. Significant non-specific PCR amplifications with PCR products as non-target template DNA and with genomic DNA from numerous OSR varieties as well as from wild radish were found by three ISO/IEC 17025 accredited reference laboratories in tests using different master mixes and PCR cyclers models. The assessment shows that the method does not meet the MPR for qualitative PCR methods and therefore is not fit-for-purpose for official controls of genetically modified products in the EU. Suggestions are provided for conditions under which analytical methods for genome-edited organisms should be validated.

Keywords Genome editing · Official control · SU canola · Oilseed rape (OSR) · GMO · Detection

Introduction

For marketing authorisation of genetically modified organisms (GMO) and derived food and feed products, the European legislation demands event-specific methods for their detection, identification and quantification (Regulation (EC) No 1829/2003 of the European Parliament and the Council of 22 September 2003 on genetically modified food and

feed 2003). Organisms generated by random mutagenesis (resulting from, e.g. irradiation or mutagenic chemicals) are exempt from the obligations of Directive 2001/18/EC on the deliberate release of GMOs (European Parliament and the Council 2013). In contrast, as ruled by the Court of Justice of the European Union on July 25th 2018 (European Court of Justice 2018), organisms developed by using new genomic techniques (NGT) are not exempt from the legal obligations. Consequently, genome-edited organisms and derived food and feed products require the development of methods by the applicants for product detection, identification and quantification before authorization in the European Union.

NGT enable site-specific alterations of the DNA sequence, including the generation of single nucleotide variants (SNVs), sequence deletions or insertions. The development of methods for sensitive, event-specific and robust detection or even quantification of GMOs with, in

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the extreme, only one SNV is analytically challenging and may particularly be difficult to be applied to food and feed products with a complex composition (European Network of GMO Laboratories (ENGL) 2019; Grohmann et al. 2019). Important prerequisites for method development are the prior knowledge on the altered genome sequence(s) and the availability of appropriate reference materials. Methods suitable for detecting a SNV may require advanced techniques to allow the specific detection, e.g. high affinity DNA analogues, RNaseH-dependent real-time PCR (rhPCR), digital PCR (dPCR) or targeted next-generation sequencing (NGS) (Ribarits et al. 2021; Zhang et al. 2021).

The acetohydroxyacid synthase (AHAS) multigene family, which is involved in branched-chain amino acid biosynthesis, has been identified as target for conferring tolerance against sulfonylurea (SU) and imidazolinone herbicides (Kolkman et al. 2004). Several OSR varieties with SU tolerance were developed and placed on the North American market. The company Cibus US LLC has developed OSR varieties carrying a SNV in the AHAS1C and AHAS3A genes, a G to T transition leading to amino acid substitution from tryptophan to leucine and conferring to SU herbicide tolerance (Health Canada 2016). The developer generated the SNV either by using conventional breeding methods or by the use of NGT (oligonucleotide directed mutagenesis) (Canadian Food Inspection Agency 2020; European Network of GMO Laboratories (ENGL) 2019; Schopke et al. 2012). The same SNV, but present only in AHAS3A, was derived using conventional breeding and are commercialised by the company BASF as Clearfield OSR in several countries for many years (Health Canada 2016; Tan et al. 2005).

A recent article (Chhalliyil et al. 2020) described the development of a real-time PCR method for the detection of the SU-tolerant OSR varieties 40 K, C1511 and C5507 placed on the North American market, which according to the authors are collectively derived from line 5715. They describe that they have generated a pair of primers, with the forward primer being designed to differentiate between AHAS1C and AHAS3A, and the reverse primer to discriminate between the wild type and altered AHAS sequences, putatively restricting amplification to AHAS1C alleles possessing the SNV in question.

The authors claim that the method “fully meets the requirements for GMO testing methods laid down in European Union law and regulations”, and they suggest that “qPCR-based method development may be applicable to virtually any genome-edited organism” (Chhalliyil et al. 2020). They further underline that the method is event-specific for OSR line 5715 and would have been shown to fulfil the minimum performance requirements (MPR) of the European Network of GMO laboratories (ENGL) including those for GMO quantification. Shortly after the article was published, non-governmental organisations requested EU regulatory bodies to carry out official controls to prevent illegal imports, as the “first

open source detection test for a gene-edited GM crop” would be now available (Greenpeace European Unit 2020; Verband Lebensmittel ohne Gentechnik e.V. (VLOG) 2020). Afterwards, the ENGL published a first theoretical evaluation of the method, which underlined the need for an experimental study (European Network of GMO Laboratories (ENGL) 2020).

According to EU legislation regarding method validation in the frame of official controls, Art. 34(1) of Regulation (EU) 2017/625 prescribes that “Methods used for sampling and for laboratory analyses, tests and diagnoses during official controls and other official activities shall comply with Union rules establishing those methods or the performance criteria for those methods” (European Parliament and the Council 2017). In accordance with the latter provision, the performance criteria for GMO detection methods have been established in the ENGL document (European Network of GMO Laboratories (ENGL) 2015). However, Chhalliyil et al. (2020) have only applied the limited approach of the ENGL guidance document for method verification (Hougs et al. 2017) and the guidelines described for validation of qualitative real-time PCR methods (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL) 2016).

To assess the performance characteristics of the method, we used the criteria of the MPR document that apply for qualitative real-time PCR methods (European Network of GMO Laboratories (ENGL) 2015). Accordingly, the method was tested in terms of sensitivity, specificity and robustness with various reference materials, including PCR amplicons. When assessing the method, the analytical conditions used corresponded exactly to those described by the method developer.

In this study, the method was carefully examined independently by three accredited reference laboratories and we addressed the question, whether the method is fit-for-purpose for official controls of genetically modified food, feed and seed in the EU.

Materials and Methods

OSR Samples and Reference Materials

Ground material from the OSR varieties 40 K, CC9014, CC9016 and the non-modified variety CC9018 were provided by Cibus US LLC (San Diego, CA, USA). Seeds of *Raphanus raphanistrum* were provided by Templiner Kräutergarten (Templin, Germany). Certified reference materials were purchased from the European Commission’s Joint Research Centre (JRC) or from the American Oil Chemists’ Society (AOCS). Further materials and corresponding providers are described in Table S1, Table S2 and Table S3.

Different lots of reference materials (140 in total) were used for the specificity tests, including materials of 30 OSR

varieties (11 transgenic GM events), 21 maize varieties (19 GM events), 16 soy varieties (15 GM events), 10 cotton varieties (9 GM events), three potato varieties (two GM events), two sugar beet varieties (one GM event), one GM alfalfa variety, one GM rice variety, one non-modified tomato variety, one non-modified linseed variety, one non-modified wheat variety, and one *R. raphanistrum* weed variety.

DNA Extraction

At the German National Reference Laboratory for Genetically Modified Organisms (NRL GMO) of the Federal Office of Consumer Protection and Food Safety (BVL) seeds were ground using an Ultra Centrifugal Mill (ZM 200, Retsch, Haan, Germany) with a ring sieve of 500 µm. Extraction of genomic DNA from ground materials was performed with the Quick-DNA Plant/Seed Miniprep Kit (Zymo Research Europe GmbH, Freiburg, Germany), the Fast ID Genomic DNA Extraction Kit (Genetic ID Europe AG, Augsburg, Germany), the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) or the GENESpin Kit (Eurofins GeneScan Technologies GmbH, Freiburg, Germany) according to the manufacturer's instructions, or by use of the CTAB method (Mazzara et al. 2005). DNA solutions were stored at -20 °C until use.

At the Swiss Federal Food Safety and Veterinary Office (FSVO) seeds were ground using a ball mixer mill (Tissue-Lyser II, Qiagen/Retsch, Haan, Germany) with steel grinding jars. Extraction of genomic DNA from ground materials was performed with the NucleoSpin Food Mini Kit (Macherey–Nagel AG, Oensingen, Switzerland) according to the manufacturer's instructions. DNA solutions were stored at -20 °C until use.

At the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) of the European Commission's Joint Research Centre (JRC) powder reference materials were extracted with the Food proof sample preparation kit III (BIOTECON Diagnostics GmbH, Potsdam, Germany). For sugar beet DNA was extracted by use of CTAB (Mazzara et al. 2005), for potato a CTAB/microspin method was used (European Commission's Joint Research Centre 2009). DNA solutions were stored at -20 °C until use.

Further details can be found in Table S1, Table S2 and Table S3.

DNA Quantification and Quality Analysis

At the German NRL GMO (BVL), all DNA solutions were quantified by the use of the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Wilmington, DE, USA) according to the manufacturer's instructions. Fluorescence was measured with the Infinite F200 Pro (Tecan, Männedorf, Switzerland).

At the FSVO DNA concentrations were measured using a NanoDrop One micro-UV/Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

At the EURL GMFF, the quality of the DNA solutions was assessed considering the ratio of UV absorbance at 260 nm and 280 nm with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The DNA concentrations were determined with the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Wilmington, DE, USA) as the arithmetic mean of two readings on the basis of a five-point standard curve using a VersaFluor fluorometer (Bio-Rad Laboratories S.r.l., Milan, Italy) as fluorescence detector.

DNA concentrations in copy numbers per microliter were converted from DNA mass concentrations by using the haploid genome weight of the respective species (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL) 2016), namely 1.15 pg per haploid genome for rapeseed. The copy number of PCR products as DNA templates was calculated in a comparable way, taking into account the amplicon length and molecular weight.

Tests to verify the absence of inhibition in DNA extracted from materials were conducted according to the ENGL document on method verification (Hougs et al. 2017). They were carried out by the use of the respective validated reference gene method, which was either the FatA(A) (Jacchia et al. 2014) or CruA method (Savini et al. 2007) (Table 1) in case of rapeseed samples.

Oligonucleotide Primers and Probes

Sequences and chemical modifications of primers and probes for the SU rapeseed method were as described in Chhalliyil et al. (2020). All oligonucleotides were purified by HPLC by the manufacturers. For the measurements at the NRL GMO (BVL) DNA primers and probes were synthesised by metabion GmbH (Planegg/Steinkirchen, Germany), and the LNA-containing SU-Reverse Primer was synthesised by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). For the FSVO DNA primers and probes were synthesised by Microsynth AG (Balgach, Switzerland). For the EURL GMFF primers and probes were purchased from Thermo Fisher Scientific (Wilmington, DE, USA), and the LNA SU-Reverse Primer was purchased from Integrated DNA Technologies (Coralville, IA, USA).

As taxon-specific reference gene for rapeseed either the acyl-ACP thioesterase gene (FatA(A)) (Jacchia et al. 2014) or the cruciferin A gene (CruA) system (Savini et al. 2007) was used. Corresponding sequences are shown in Table 1. For *R. raphanistrum* the actin gene (Laube et al. 2010) was used.

Table 1 Sequences and concentrations of oligonucleotides used. + A denotes the locked nucleic acid (LNA) modification of desoxyadenosin; 6-FAM, 6-carboxyfluorescein; BHQ1, Black Hole Quencher 1; MGBNfq, Minor Groove Binder Non-Fluorescent Quencher

Target/genotype	Name	Sequence (5' to 3')	Amplicon Length	Final concentration	Reference
AHAS3A & AHAS3A	AHAS-Forward	CTC CgC AgT ACg CgA TTC A	384 bp	0.4 µM	modified from Chhalliyil et al. 2020
	AHAS-Reverse	ggT CCC CgA gAT AAg TgT gA		0.4 µM	
AHASIC-WT	SU-SNV-G-Forward	TCC TCg ACg AgC TAA CCg AAg ggA Agg CAA TTA TCA gTA	363 bp	0.4 µM	this study
	AHAS-Reverse	ggT CCC CgA gAT AAg TgT gA		0.4 µM	
AHASIC-SU	SU-SNV-G-Forward	TCC TCg ACg AgC TAA CCg AAg ggA Agg CAA TTA TCA gTA	339 bp	0.4 µM	this study
	SU-SNV-Reverse	TgT TAG CTT TgT AgA ACC gAT CTT CCA ATT gCA TgA CCA TCC CAA gAT gC		0.4 µM	
AHAS3A-WT	SU-SNV-C-Forward	TCC TCg ACg AgC TAA CCC AAg ggA Agg CAA TTA TCA gTA	363 bp	0.4 µM	this study
	AHAS-Reverse	ggT CCC CgA gAT AAg TgT gA		0.4 µM	
AHAS3A-SU&CL	SU-SNV-C-Forward	TCC TCg ACg AgC TAA CCC AAg ggA Agg CAA TTA TCA gTA	339 bp	0.4 µM	this study
	SU-SNV-Reverse	TgT TAG CTT TgT AgA ACC gAT CTT CCA ATT gCA TgA CCA TCC CAA gAT gC		0.4 µM	
SU rapeseed	SU-Forward Primer	TCC TCg ACg AgC TAA CCg	334 bp	1.6 µM	Chhalliyil et al. 2020
	SU-Reverse Primer	gCT TTg TA g AAC CgA TCT TCC [+A]		1.6 µM	
FatA(A)	SU-Probe	6-FAM-ACA ACC AgC ATC TTg ggA Tgg TCA-BHQ1		0.8 µM	
	Forward Primer 09–0–3249	ACA gAT gAA gTT Cgg gAC gAg TAC	126 bp / 129 bp (depending on rapeseed variety)	0.3 µM	Jacchia et al. 2014
CruA	Reverse Primer 09–0–3251	CAG gTT gAg ATC CAC ATg CTT AAA TAT		0.9 µM	
	Probe 09-QP-87	6-FAM-AAg AA g AAT CAT CAT gCT TC-MGBNfq		0.15 µM	
CruA	Forward Primer MDB510	ggC CAg ggT TTC CgT gAT	101 bp	0.2 µM	Savini et al. 2007
	Reverse Primer MDB511	CCg TCg TTg TA g AAC CAT Tgg VIC-AgT CCT TAT gTg CTC CAC TTT CTg gTg CA-TAMRA		0.2 µM	

Preparation of and Tests with PCR DNA Templates

Four amplicons were prepared by PCR amplification of OSR genomic DNA (AHAS1C-WT, AHAS1C-SU, AHAS3A-WT, and AHAS3A-SU&CL). First, a PCR product was amplified as starting material for subsequent introduction of the SNVs with mismatched primers, amplifying the AHAS1C as well as the AHAS3A sequence. Therefore, 100 ng of conventional (non-genetically modified) OSR DNA (AOCS 0304-A) was amplified with the AHAS forward and reverse primers described in Chhalliyil et al. (2020), but with 2 and 6 nucleotide shortened 3'-termini, respectively, reverting the specificity for the AHAS1C gene. Sequences and final concentrations of all primers are shown in Table 1. PCR was performed in a volume of 50 µl, using 1 unit of Hot Diamond Taq DNA Polymerase (Eurogentec, Seraing, Belgium), 2.5 mM MgCl₂ and 0.2 mM of each dNTPs. The following PCR programme was used: 3 min at 95 °C, followed by 40 cycles of (30 s at 95 °C, 30 s at 52 °C and 1 min at 72 °C) and 3 min at 72 °C. The PCR product was purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey–Nagel, Oensingen, Switzerland) and diluted to 100,000 copies/µl in 0.2×TE buffer (2 mM Tris, 0.2 mM EDTA, pH 8). Then the four DNA PCR templates containing the desired SNVs were amplified by using 50,000 copies of the diluted PCR template and the respective mismatched forward and reverse primers (SU-SNV-C-Forward, SU-SNV-G-Forward, SU-SNV-Reverse; see Table 1) with identical reaction conditions for amplification. The resulting PCR products were purified and both DNA strands were sequenced by Sanger sequencing with the same primers as used for PCR amplification (Microsynth AG, Balgach, Switzerland).

The AHAS1C-WT has a G at position 1676 that leads to a mismatch with the LNA-coupled A at the 3'-end of the SU-reverse primer. AHAS1C-SU represents the genetic sequence of variety 40 K, for which this primer has no mismatch. AHAS3A-WT has a C at position 1372 that mismatches with the G at the 3'-end of the SU-forward primer, and further a G at position 1667 so that the SU-reverse primer has a mismatch with the LNA-A at the 3'-end. Finally, AHAS3A-SU&CL has a C at position 1372 that mismatches with the G at the 3'-end of the SU-forward primer, but matches with the LNA-modified A at the 3'-end of the SU-reverse primer.

Tenfold serial dilutions of the DNA PCR templates, from 500,000 down to 5 copies per PCR, were amplified with the real-time PCR SU rapeseed method by the use of the Kapa Probe *Fast* and *Force* mix on the two real-time PCR thermal cyclers Rotor-Gene Q and LightCycler 480 Instrument II, respectively.

Real-Time PCR Analysis

The assay was conducted according to the protocol of Chhalliyil et al. (2020) in a final volume of 25 µl containing up to 300 ng of a DNA template and 12.5 µl Master Mix (Kapa

Probe *Fast* and Kapa Probe *Force* qPCR Master Mix, respectively, Sigma-Aldrich), with final concentrations of 1.6 µM of both primers and 0.8 µM of the probe. The following PCR programme was used: 10 min at 95 °C, followed by 45 cycles of 15 s or 30 s at 95 °C and 1 min at 60 °C. For analyses with Kapa Probe *Fast*, denaturation was conducted for 30 s, whereas with Kapa Probe *Force* the denaturation was performed for 15 s or 30 s as given in the text to exclude any relevant influence on the method performance.

At the German NRL GMO (BVL) sensitivity tests for the SU rapeseed method were performed on the ABI 7500 Fast System (Thermo Fisher Scientific). Specificity was assessed on different PCR machines, including the ABI 7500 System and the ABI 7500 Fast System (Thermo Fisher Scientific), the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Feldkirchen, Germany) and the LightCycler 480 Instrument II (Roche Life Science, Mannheim, Germany).

Robustness was tested on the ABI 7500 Fast System and the LightCycler 480 Instrument II. In order to allow comparisons between PCR runs on the ABI 7500 and ABI 7500 Fast Systems, the fluorescence threshold was always set to 0.2. The heating rates used were as follows: 100% for the ABI 7500 System and the ABI 7500 Fast System; 3.3 °C/s for the CFX96; 4.4 °C/s and 2.2 °C/s for amplification, respectively, for the LightCycler 480 Instrument II. At the FSVO specificity was tested on the Rotor-Gene Q (QIAGEN, Hilden, Germany) and on the LightCycler 480 Instrument II (Roche Life Science, Mannheim, Germany). The heating rates used were as follows: 4.4 °C/s and 2.2 °C/s for amplification, respectively, for the LightCycler 480 Instrument II, > 15 °C/s for the Rotor-Gene Q. At the EURL GMFF specificity tests were run on the ABI 7500 System (Thermo Fisher Scientific) with a heating rate of 100%.

For verifying the amount of amplifiable rapeseed DNA, the taxon-specific reference genes *FatA(A)* (Jacchia et al. 2014) or *CruA* (Savini et al. 2007) were analysed by real-time PCR. Therefore, at the NRL GMO (BVL) the *FatA(A)* assay was conducted in a final volume of 25 µl, containing 12.5 µl Master Mix (TaqMan Universal PCR Master Mix No AmpErase UNG, Thermo Fisher Scientific) and final concentrations of 300 nM and 900 nM for primer 09–0–3249 and primer 09–0–3251, respectively, and 150 nM for the probe 09–QP–87. The following PCR programme was used: 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Analyses were done with the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). At the FSVO *FatA(A)* was determined under the same conditions by the use of the Kapa Probe *Force* qPCR Master Mix on the Rotor-Gene Q system. The *CruA* method was run at the EURL GMFF according to the setup indicated in Chhalliyil et al. (2020) that is modified

from the previously validated protocol (Savini et al. 2007) as follows: final volume of 25 μl , containing 12.5 μl Master Mix (Kapa Probe *Force*) and final concentrations of 200 nM for each primer MDB510 and MDB511 and 200 nM for the probe TM003 (Table 1, Table S4, Table S5 and Table S6).

Gel Electrophoresis

PCR products from real-time PCR analysis on the Rotor-Gene Q were verified for correct fragment size on a 2% agarose gel (Agarose A6877, Sigma-Aldrich). As DNA stain HDGreen Plus (Elchrom Scientific, Switzerland) was used. The gel was run in 1 \times TBE buffer (89 mM Tris, 89 mM boric acid, 1 mM EDTA, pH 8). DNA bands were visualised using a Quantum CX5 Edge Gel-Imaging System (Vilber Lourmat SAS, France) with UV pad.

In Silico Specificity Analyses

The EURL GMFF carried out in silico analyses by similarity searches against public NCBI and local copies of the NCBI non-redundant nucleotide sequence database (nt) and the patent nucleotide database downloaded from NCBI (National Center for Biotechnology Information), as well as the Central Core Sequence Information System (CCSIS) database (European Commission's Joint Research Centre, 2021) of the European Commission's Joint Research Centre (JRC). The latter database is storing all GMO related sequence data, i.e. the inserted sequences and their flanking genomic regions, submitted in the frame of applications for EU authorisations by applicants as part of their legal obligations, in addition to public GMO sequence data from other sources.

The similarity search was carried out using BLASTN tool (Altschul et al. 1990) version 2.2.31 (parameters: -dust no -soft-masking no). Moreover, an ePCR amplification was performed against the CCSIS database using the e-PCR tool, version 2.3.12 (Schuler 1997), (parameters: -n 2, -g 2, -f 3, -t 4, -m 1000, -d 20–1000).

Experimental Specificity Analyses

For the four DNA PCR templates (AHAS1C-WT, AHAS1C-SU, AHAS3A-WT, and AHAS3A SU&CL), representing the sequences of the wild type or modified AHAS1C and AHAS3A variants, the specificity was tested with template amounts in the range of 500,000 to 5 copies per PCR.

For genomic DNA from rapeseed as well as from *R. raphanistrum*, 100 ng and up to 300 ng DNA per PCR as described in Chhalliyil et al. (2020) were tested. For the OSR varieties 40 K, CC9014 and CC9016, 2,500 and 100 genomic copies, i.e. 2.88 ng/PCR and 0.12 ng/PCR, respectively, were additionally tested. For DNA solutions

with concentrations of less than 60 ng/ μl (i.e. 300 ng/PCR), specificity was tested with the maximum DNA amount possible (exact DNA amounts are indicated in the text). For genomic DNA of non-rapeseed species specificity analyses were performed using 2,500 genomic copies or up to 300 ng per PCR. All samples were tested at least in duplicates.

As positive control 10 or 100 genomic copies or up to 300 ng per PCR of the OSR variety 40 K were used. PCR conditions were as described in the “Real-Time PCR Analysis” section.

Robustness Analyses

The conditions for robustness testing were as follows: real-time cycler (ABI 7500 Fast vs. LightCycler 480 Instrument II), reaction volume (26 μl vs. 24 μl), primer concentrations (unchanged vs. 30% less), probe concentration (unchanged vs. 30% less) and annealing temperature (59 $^{\circ}\text{C}$ vs. 61 $^{\circ}\text{C}$). Since the master mix used for that method is a ready-to-use mix prepared by the manufacturer, its final concentration was not changed in this study. To take all of these factors into account, a multifactorial design approach as described in the MPR (European Network of GMO Laboratories (ENGL) 2015) was applied (Table 2).

Robustness was assessed at a DNA concentration of three times the LOD_{abs} using 40 K DNA (i.e. 30 genomic copies), either without adding background DNA or with 100 ng background DNA of Clearfield (LG Conrad CL) or non-GMO rapeseed per PCR, respectively. Furthermore, the specificity of the PCR method was assessed by the use of 300 ng per PCR of Rf2 rapeseed DNA under robustness conditions.

Results

Sensitivity Tests

We examined the sensitivity of the real-time PCR method according to the MPR document of the ENGL (European Network of GMO Laboratories (ENGL) 2015). In that context, the limit of detection (LOD) is the lowest amount or concentration of an analyte that can be reliably detected in a sample. The absolute LOD (LOD_{abs}) should be below 25 copies per PCR reaction with a level of confidence of 95%, ensuring $\leq 5\%$ false negative results. In order to reach the required level of confidence, a suitable number of replicates has to be tested. The method developer (Chhalliyil et al. 2020) applied a procedure for determining the LOD on the basis of modelling the probability of detection. We decided to assess the LOD_{abs} with an experimental approach that is generally used for the validation of the LOD_{abs} in the frame of applications for the authorisation of genetically modified

Table 2 Multifactorial experimental design for the robustness analysis. ABI, ABI 7500 Fast System; LC480II, LightCycler 480 Instrument II

Factor	Combination							
	1	2	3	4	5	6	7	8
Annealing Temperature	59 °C	61 °C	59 °C	61 °C	61 °C	59 °C	61 °C	59 °C
Final Reaction Volume	24 µl	24 µl	26 µl	26 µl	26 µl	26 µl	24 µl	24 µl
Primer Concentrations	1600 nM	1120 nM	1600 nM	1120 nM	1600 nM	1120 nM	1600 nM	1120 nM
Probe Concentration	800 nM	560 nM	560 nM	800 nM	560 nM	800 nM	800 nM	560 nM
Real-time Cycler	ABI	ABI	ABI	ABI	LC480II	LC480II	LC480II	LC480II

food and feed (European Commission 2013). The number of replicates tested per concentration was 60, and the LOD_{abs} has been set at the lowest concentration yielding at least 59 positive results (Cochran 1977; Zar 1999). We aimed to verify that the LOD_{abs} of the method is in line with the MPR, and thus we tested only 10 genomic copies of OSR variety 40 K without background DNA. All 60 replicates yielded positive PCR amplifications (Fig. S1, Table S7). Therefore, the LOD_{abs} of the method is below or equal to 10 genomic copies and in line with the MPR regarding sensitivity of real-time PCR methods.

In Silico Specificity Tests

According to the MPR, specificity is the property of a method to respond exclusively to the characteristic or analyte of interest (European Network of GMO Laboratories (ENGL) 2015). The PCR method should only produce amplification products with the target sequence for which the method was developed. This should be demonstrated by similarity searches against sequence databases.

The Primer-BLAST algorithm was used to investigate in silico the specificity of the PCR primers against the NCBI non-redundant sequence (nt) database. These oligonucleotides are designed for a gene region that is highly conserved among Brassicaceae. The in silico analyses highlighted the presence of GenBank entries carrying an identical annealing site for the SU forward primer and almost identical—if not for the last nucleotide at position 22—to the SU reverse primer, which is the SNV on which the method relies for its specificity to the AHAS1C allele in SU-tolerant OSR varieties (Online Resource 1). Interestingly, a sequence from the acetolactate synthase 1 gene of *R. raphanistrum* (AJ344991.1) has also a perfect match with the SU reverse primer and only one mismatch with the forward primer. We therefore aligned the probe to this predicted amplicon, and verified that there was a 100% match (Online Resource 2).

The 334 bp amplicon sequence of the PCR product obtained by the method was analysed by BLASTN (NCBI) against the CCSIS database. Nucleotide identity between 86.5 and 86.2 percent identity was found for GMO sequences. However, further analyses and alignments of

these sequences to the primers and probe of the method revealed no significant similarity. The retrieved sequences are mostly annotated as acetolactate synthase 1 genes (data not shown).

In the similarity search (BLASTN analysis) with the amplicon sequence against the patent nt database, around 500 hits were found. The first 48 hits were examined and a high degree of similarity (> 99.4 percent identity) was found to the method's amplicon (Online Resource 1). These sequences are recorded as “Mutated acetohydroxyacid synthase genes in *Brassica*”, or “Herbicide-resistant *Brassica* plants and methods of use”, or “Herbicide tolerant plants” or as sequences from patents.

The amplicon sequence of the SU method was then used for similarity search (BLASTN analysis) against the non-redundant nucleotide database of NCBI. Results were similar to those obtained with the Primer-BLAST tool.

As it could be expected, wild-type and GMO OSR lines (*Brassica napus*) as well as wild cabbage (*Brassica oleracea*) and Ethiopian rape (*Brassica carinata*) have almost identical sequences to the PCR target sequence. The results of the in silico specificity tests suggests that non-specific PCR amplifications are possible due to this minimal difference and a careful experimental check of the method's specificity is necessary.

Experimental Specificity Tests

For assessing the method specificity, three different ISO/IEC 17025 accredited reference laboratories examined the certified reference materials they had in stock using independently purchased reagents. Seeds of Clearfield varieties and *R. raphanistrum* were provided by the NRL GMO (BVL), but independent DNA extractions were performed. For all extractions and PCR measurements negative controls were included. Thus, any risk of contamination during this study was reduced to a minimum.

Specificity should be tested experimentally with non-target GMO events, non-GMO materials and the target material (European Network of GMO Laboratories (ENGL) 2015). The method was actually developed with the Kapa Probe

Fast Master Mix for the real-time PCR, but it was further tested with the Kapa Probe *Force* Master Mix by the Laboratory for GMO Analysis of the Umweltbundesamt GmbH (Environment Agency Austria) (Chhalliyil et al. 2020). Since a change in the enzyme mix might have an influence on specificity of a genotyping method, we included both master mixes in our study.

First, we analysed if the method specifically discriminates between the wild type AHAS1C and AHAS3A sequences, and between the SNV and the wild type AHAS1C gene sequences, respectively. Four DNA PCR templates for the wild type or the relevant SNVs primer binding sites in the AHAS1C and AHAS3A gene sequences were prepared, which represented the different OSR variants in order to investigate the binding of the primers to the individual sequences (Chhalliyil et al. 2020). The correctness of the amplicon sequences was verified by Sanger sequencing of both strands (data not shown). The experiments with the AHAS1C-SU template, representing the genetic sequence of Cibus SU OSR variety 40 K, showed amplification over the whole dilution range (Fig. 1; Table S8). The delta-Cq value between tenfold dilution levels was around 3.5, reflecting the theoretical expectations. The results obtained with the two different master mixes and instruments were similar (Fig. S2, Fig. S3 and Fig. S4; Table S9, Table S10 and Table S11). The three other DNA PCR templates, AHAS1C-WT, AHAS3A-WT and AHAS3A-SU&CL, should not give any significant amplification signal. However, these three non-target DNA PCR templates led to clear amplification signals with resulting Cq values between 30 and 40, even at concentrations of around 50 copies per PCR. For the DNA PCR templates AHAS1C-WT and AHAS3A-SU&CL, each having one mismatch in either the forward or reverse primer binding site, the increase of Cq values corresponded to higher template dilutions for the three highest concentration levels. Further dilution of templates led to Cq values above 37. With the DNA PCR template AHAS3A-WT, which has two mismatching nucleotides, non-specific amplification was measured as well, but the Cq values did not show any concentration dependency. The tested combinations of PCR master mixes and instruments gave similar results. The lowest levels of non-specific amplification signals were observed using the Kapa Probe *Fast* mix on the LightCycler 480 Instrument II (Fig. S4; Table S11) and the highest ones when applying the Kapa Probe *Force* mix on the Rotor-Gene Q (Fig. 1; Table S8).

The G to T mutation in the AHAS1C gene is not only present in the OSR varieties (40 K, C1511, C5507) analysed by Chhalliyil et al. (2020), but also in other SU-tolerant OSR varieties. We further tested two other non-commercialised varieties (CC9014, CC9016) that contain the identical SNV in the AHAS1C gene and an additional SNV in AHAS3A, all resulting from somaclonal variation during plant tissue

culture or by targeted mutagenesis using NGT (Table S12), respectively. The published SU rapeseed method clearly showed positive signals with 300 ng (approx. 260,000 genomic copies), 2.88 ng (approx. 2,500 genomic copies) and 0.12 ng (approx. 100 genomic copies) DNA per PCR for 40 K, CC9014 and CC9016, independently from the origin of the SNV (Fig. 2). Thus, the method is not event-specific for the supposedly genome-edited OSR line 5715 as it has been claimed (Chhalliyil et al. 2020).

We also examined the specificity against species other than rapeseed, that are commonly found in food and feed, using the Kapa Probe *Force* master mix. According to the MPR document methods submitted within an application for EU authorisation (European Parliament and the Council 2003) have to be tested with all GM events for which reference materials are available. Furthermore, non-authorised GM event and conventional plant varieties were tested. None of these reference materials showed any amplification with 2,500 genomic copies per PCR (Fig. S5). Seven materials from different species other than rapeseed were additionally tested with higher DNA concentrations (150 or 300 ng DNA per PCR). However, none of these showed PCR amplifications with that method (Fig. S6; Table S13).

Moreover, as a consequence of the *in silico* specificity analysis, the common weed variety *R. raphanistrum* was tested. Whereas no clear amplification was measured with the Kapa Probe *Force* master mix up to 300 ng DNA per PCR (Fig. S7), the method showed considerable non-specific amplification with these concentrations, when the Kapa Probe *Fast* master mix was used (Fig. 3; Table S14).

The specificity was then tested against all available reference materials for GM rapeseed events and a wide range of non-GM as well as Clearfield rapeseed varieties using 100 ng or up to 300 ng genomic DNA per PCR, which is in accordance with the amount specified in the original protocol (Chhalliyil et al. 2020). Both types of master mixes and independently performed specificity analyses in three different reference laboratories were taken into account for these tests. By the use of the Kapa Probe *Force* mix, most of the Clearfield materials showed flat PCR amplification curves with late detection (Cq > 37) with 300 ng DNA in at least one replicate, but no clear positive amplification signals were detected for Clearfield (Fig. S8). However, by using the Kapa Probe *Fast* master mix, all Clearfield materials showed significant non-specific amplification curves with 300 and 100 ng DNA, respectively (Fig. S9, Fig. S10; Table S14, Table S15).

Similarly, most of the seven non-modified OSR varieties showed with the Kapa Probe *Force* master mix flat PCR amplification curves with late detection. However, for two of them significant non-specific amplification curves could be observed in all replicates, most apparently with one certified reference material (AOCS 0306-B) (Fig. S11).

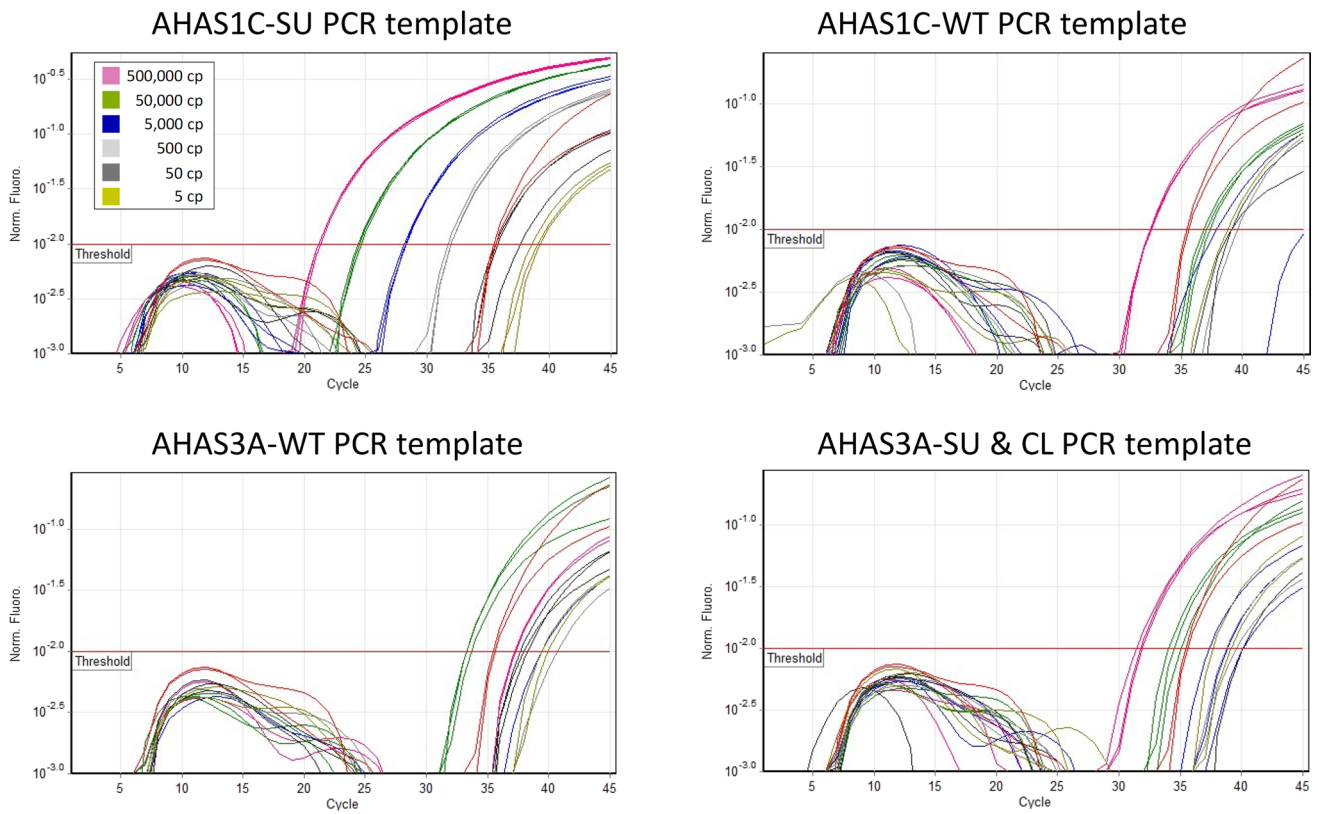


Fig. 1 PCR amplification of tenfold serial dilutions of 4 DNA PCR templates, representing the sequences of the wild type or modified AHAS1C and AHAS3A variants. Samples were analysed with the Kapa Probe *Force* mix on the Rotor-Gene Q system. The range of

template concentration was 500,000 to 5 copies per PCR. As positive control 10 genomic copies of 40 K were amplified (red). Denaturation 15 s

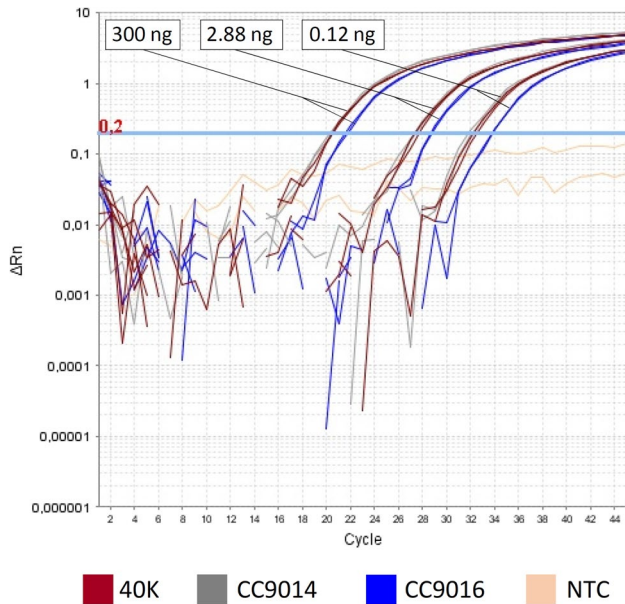


Fig. 2 PCR amplification of different rapeseed varieties with the SU rapeseed method. Each of the pure rapeseed sample was analysed in three different concentrations in duplicate with the Kapa Probe *Force* mix on the ABI 7500 Fast System. Denaturation 30 s

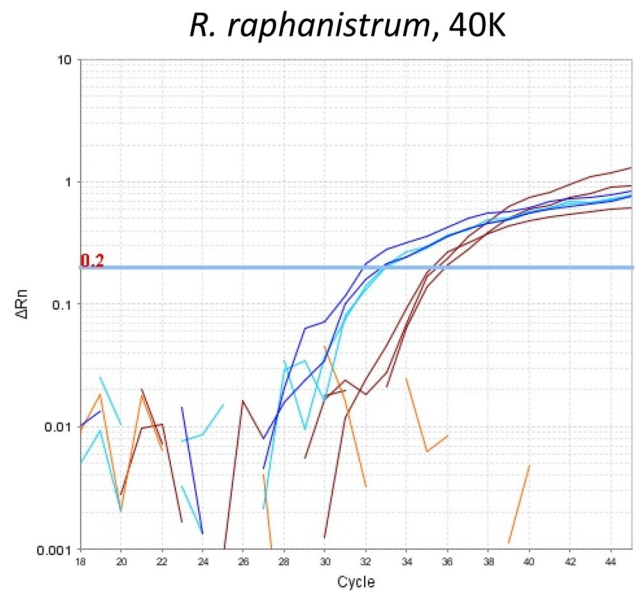


Fig. 3 PCR amplification of DNA from *R. raphanistrum* weed with the SU rapeseed method using the Kapa Probe *Fast* mix on the ABI 7500 Fast System. *R. raphanistrum* was analysed in 100 ng (light blue) and 300 ng DNA per PCR (dark blue) in duplicate. As positive control 10 genomic copies of 40 K DNA (red) were amplified. Orange, NTC. Denaturation 30 s

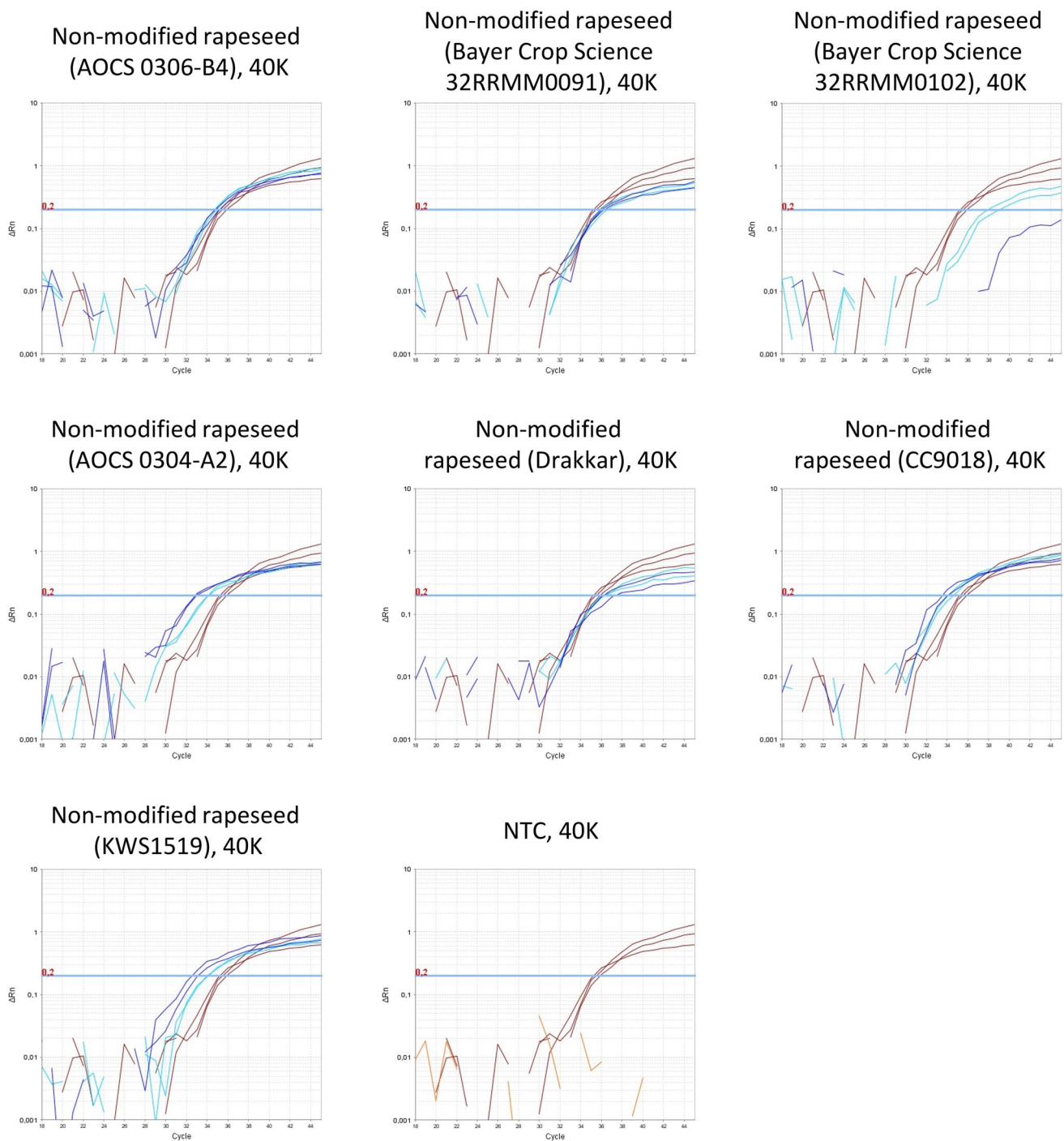


Fig. 4 PCR amplification of DNA from 7 non-modified OSR lines with the SU rapeseed method using the Kapa Probe *Fast* mix on the ABI 7500 Fast System at the BVL. Samples were analysed in 100 ng

(light blue) and 300 ng DNA (dark blue) per PCR in duplicate. As positive control 10 genomic copies of 40 K DNA were amplified (red). Orange, NTC

Therefore, the latter non-GM OSR material was used for further analyses and reproducibly revealed non-specific amplification at all concentrations and for all bottled aliquots tested (Fig. S12; Table S16, Table S17). Again, the use of the Kapa Probe *Fast* master mix led to more significant

non-specific amplifications with all non-GM OSR materials tested at 300 and 100 ng DNA level, respectively (Fig. 4, Fig. S13; Table S14, Table S15, Table S18).

We then analysed the specificity of the SU rapeseed method (Chhalliyil et al. 2020) against 11 GM rapeseed

events with 100 ng and up to 300 ng DNA per PCR. If available, three different batches or bottled aliquots of the certified reference materials were used. By use of the Kapa Probe *Force* master mix, nine out of 11 GM rapeseed materials reproducibly revealed non-specific amplification on different PCR cyclers at all concentrations with DNAs from all tested batches and aliquots (Fig. 5, Fig. S14, Fig. S15, Fig. S16, Fig. S17; Table S16, Table S17, Table S19, Table S20, Table S21). The Kapa Probe *Fast* master mix showed again more obvious non-specific and reproducible amplification signals with all 11 GM rapeseed materials (Fig. S18, Fig. S19, Fig. S20; Table S14, Table S15, Table S18).

Slightly different PCR machines and block geometries as well as denaturation times were used and discussed by the method developer (Chhalliyil et al. 2020). To exclude that these small experimental changes may affect the specificity of the method, we repeated the tests with the Kapa Probe *Force* master mix for the PCR-positive OSR varieties using the ABI 7500 Fast System and a period of 15 s for the denaturation step (Fig. S21; Table S22) and the ABI 7500 System with 30 s of denaturation (Fig. S22; Table S23). Neither the cycler type nor the denaturation time had any significant effect on the non-specific amplifications.

Robustness of the SU Rapeseed Method

The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure (European Network of GMO Laboratories (ENGL) 2015). First, the robustness was assessed with 30 genomic copies of the 40 K DNA without adding background DNA. The method yielded positive amplification signals in all replicates in all combinations tested (Fig. 6a; Table S24), showing that it is sufficiently robust under these conditions. Since the method specificity is based solely on a single 3' terminal mismatch of the reverse primer, the detection of the SNV will be much more challenging in the presence of wild type DNA as a background. Therefore, we measured 30 genomic copies of 40 K in a background of 100 ng per PCR of Clearfield DNA (LG Conrad CL) and non-modified rapeseed, respectively, under all experimental conditions. Noteworthy, the amplification curve was flattened compared to tests without DNA background (Fig. 6b), and the analysis software of one real-time PCR cycler did not reveal a detection under all conditions tested (Table S24). Differences were observable depending on the analysis mode used leading to failed detection, which was confirmed in a repeat analysis (Table S24).

Additionally, we performed the robustness test of the method with DNA from Clearfield (LG Conrad CL), non-GM rapeseed and Rf2 rapeseed in order to assess the specificity under slightly changed conditions. Non-specific

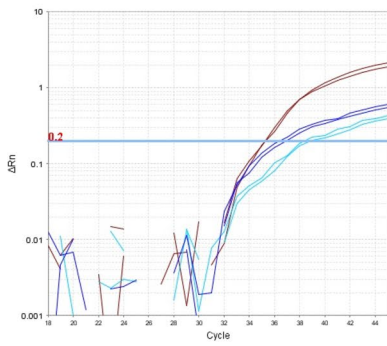
amplification remained clearly visible with some experimental changes (Fig. 6a, combinations 2 and 4). Notably, the non-specific amplification could be reduced (but not completely prevented) by annealing at 59 °C instead of 60 °C (Fig. 6a, combinations 1 and 3). However, this also led to a flattening of the PCR amplification curve for 40 K in a background of non GM rapeseed DNA (Fig. 6b, combinations 1 and 3). The non-specific amplification at the LightCycler 480 Instrument II (Fig. 6a and b, combinations 5–8) was reduced (but not completely prevented) in comparison to experiments with the ABI 7500 Fast (Fig. 6a and b, combinations 1–4), and the LightCycler software automatically detected amplification only in the *high sensitivity mode*, but not in the *high confidence mode* (Table S24). However, the *high confidence mode* failed to robustly detect 40 K in background DNA.

Discussion

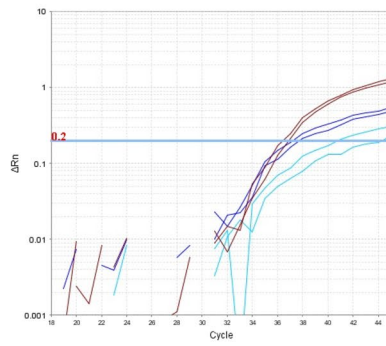
Detecting and monitoring the presence of genome-edited crops is a challenge for EU authorities and official control, because a crop plant with, for example, a single SNV in its genome may not be distinguishable from plants that have acquired the same point mutation naturally or by means of classical mutagenesis techniques and would, without previous knowledge, remain unidentified (European Network of GMO Laboratories (ENGL) 2019). The published method for the detection of a specific herbicide-tolerant OSR variety, which was assumed to be modified at a single base in the AHASIC gene by NGT, promised to be event-specific and “a game-changer at various levels” by demonstrating its applicability for official control and as general procedure “to develop further detection methods for most, if not all, genome-edited crops” (Greenpeace European Unit 2020). To evaluate these statements, we thoroughly assessed the method with a focus on its analytical performance characteristics, particularly its specificity, sensitivity and robustness. We followed exactly the published experimental protocol and DNA concentrations and compared the recommended genotyping reagents and PCR conditions independently in three different reference laboratories.

The method developer reported data on sensitivity, robustness and partly specificity, but only limited validation data concerning quantification (Chhalliyil et al. 2020). Indeed, no quantification is required under the current legal circumstances, since to date no application for authorisation as food, feed or seed for genome-edited rapeseed has been submitted to the European Commission. Consequently, only validation criteria for qualitative GMO detection methods have been examined. However, no complete validation according to the mandatory MPR guidance document for

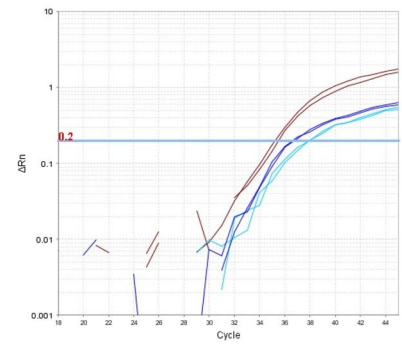
73496, 40K



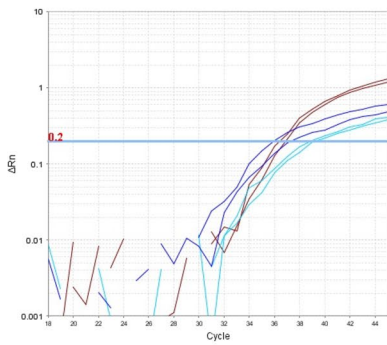
Ms1, 40K



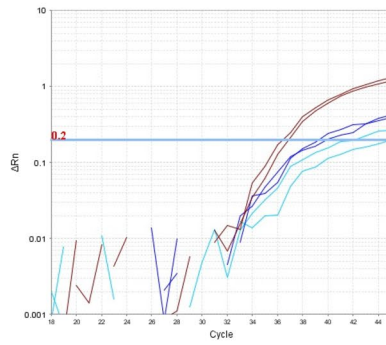
Ms11, 40K



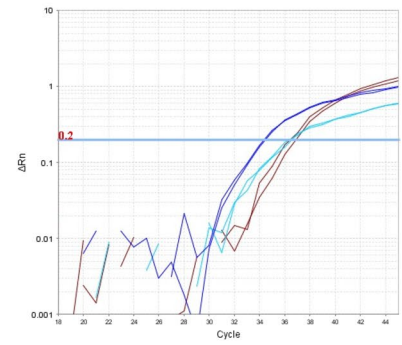
Ms8, 40K



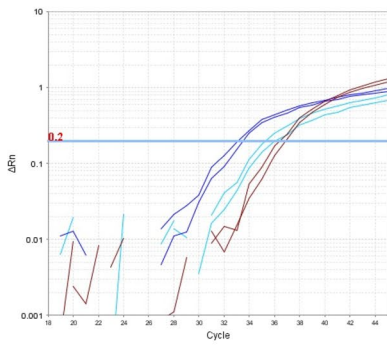
Rf1, 40K



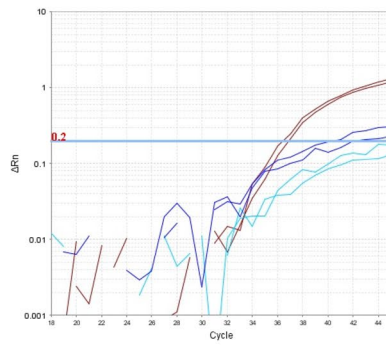
Rf2, 40K



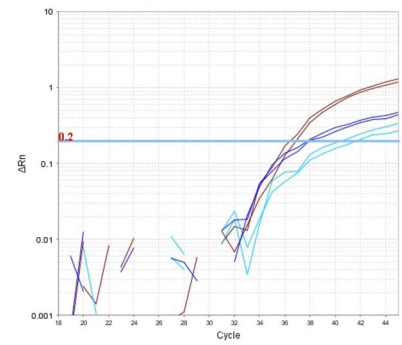
Rf3, 40K



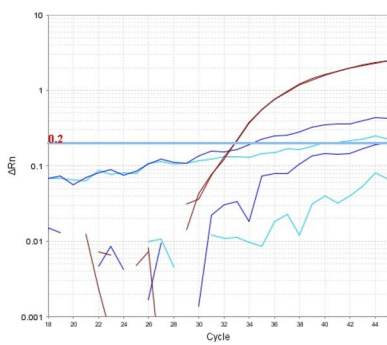
T45, 40K



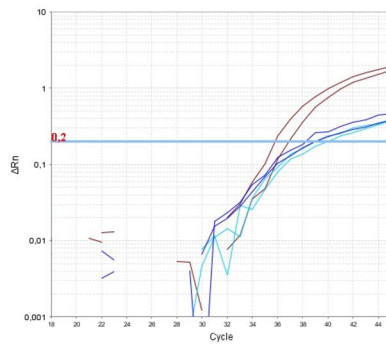
Topas 19/2, 40K



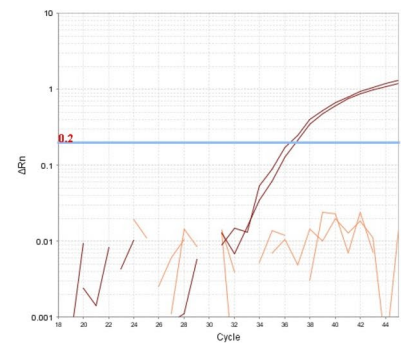
MON88302, 40K



GT73, 40K



NTC, 40K



◀**Fig. 5** PCR amplification of DNA from 11 GM rapeseed lines with the SU rapeseed method. Samples were analysed in duplicate with the Kapa Probe *Force* mix on the ABI 7500 Fast System at the BVL. If available, three different batches or bottled aliquots of certified reference materials were analysed (only one representative is shown, see Table S19 for further details). Dark blue, 205–300 ng DNA; light blue, 100 ng rapeseed DNA per PCR; red, 10 genomic copies of 40 K DNA (100 cp for 40 K DNA in MON88302 experiment). Denaturation 30 s

methods applied in official control of genetically modified food, feed and seed has been performed by the original authors, particularly in terms of method specificity and applicability.

Sensitivity and Robustness Assessment

In our sensitivity analysis for qualitative methods, performed with genomic 40 K DNA from non-processed material in the absence of non-GMO background DNA, we could confirm a LOD_{abs} of the method equal to or below 10 genomic copies, which is in accordance with the current MPR document (European Network of GMO Laboratories (ENGL) 2015).

A putative limitation for applicability is the amplicon size of 334 base pairs. According to the MPR (European Network of GMO Laboratories (ENGL) 2015), the amplicons used for the PCR must be smaller than the DNA fragments of the sample isolated during the sample purification. In the case of highly fragmented DNA, e.g. from processed food or feed samples, an effective reduction in the amount of amplifiable PCR template is expected which would thus lead to an impairment of the sample-specific practical LOD. The applicability of the method in processed food and feed has not yet been demonstrated. It currently remains unclear, whether the method is sufficiently sensitive to detect the target sequence in samples with severely degraded DNA. When analysing (non-processed) rapeseed, however, an impaired LOD is not expected.

Sufficiently sensitive detection with 40 K DNA in the absence of non-modified rapeseed background DNA could be observed with slightly changed experimental conditions in the robustness tests. However, it was not always possible to detect 30 copies of 40 K DNA in the presence of 100 ng non-GM rapeseed DNA. The failure to detect 40 K DNA was associated with a flattening of the amplification curve, presumably due to a competition in primer binding between modified and non-modified DNA sequences. Consequently, the method is not considered as robust enough to be used for analysing the presence of the SNV and a corresponding OSR event in routine samples taken from imported bulk commodities. The current version of the MPR document does not explicitly require a LOD or robustness testing in the presence of high amounts of non-modified background DNA as a competitor. However, as demonstrated by our results, it

is recommended to take this assessment into account during the validation of methods for the detection of SNVs derived by NGT, as it would reflect routine situations where the unmodified DNA is present in significant excess.

Consequently, although the SU rapeseed method might formally fulfil the sensitivity and robustness requirements according to the current MPR document, it showed a notable impairment of robustness when the analyte is measured in a background of DNA with non-modified (wild type) sequences.

Specificity Assessment

Sequencing the AHAS1C and AHAS3A genes using synthetic DNA templates confirmed the expected DNA sequences. Specificity studies with these materials were performed in order to investigate the reactivity of the primer system to well-known individual DNA sequences. Although the method clearly revealed the lowest Cq values for the modified AHAS1C DNA sequence present in the Cibus lines at high to medium DNA concentrations, the non-targeted DNA sequences (representing wild type and Clearfield rapeseed) generally showed PCR amplification signals, with similar Cq values in the low copy number range. Consequently, the SU rapeseed method cannot unequivocally distinguish between AHAS1C and AHAS3A as well as between the G or T nucleotides at the intended SNV position when using synthetic templates. These initial observations already suggested that the method cannot sufficiently differentiate between the Cibus SU, wild-type and Clearfield rapeseed.

Numerous OSR reference materials were considered for method specificity testing and significant non-specific amplification occurred with DNA amounts specified in the original protocol (100 or 300 ng per PCR). The non-specific amplification with any of these materials could simulate the presence of (lower amounts from) Cibus varieties 40 K, C1511 and C5507, leading to false follow-up measures for that consignment. From an analytical point of view, non-specific amplification of DNA from non-modified OSR is particularly problematic, since in case of positive amplification during analysis of a sample it is not possible to unambiguously assign it to traces of SU modified rapeseed material.

The observed non-specific amplification curves with DNA from non-SU rapeseed lines were in several cases lower compared to the fluorescence intensity during the plateau phase with 40 K DNA as positive control material (30 copies per reaction). However, the reduced plateau of fluorescence intensity in amplifications of 40 K DNA samples with background non-SU rapeseed DNA samples suggests the presence of interferences of DNA amplification in mixed samples, as expected under routine control situations. Furthermore, differences in the fluorescence intensity between specific and non-specific amplification have not

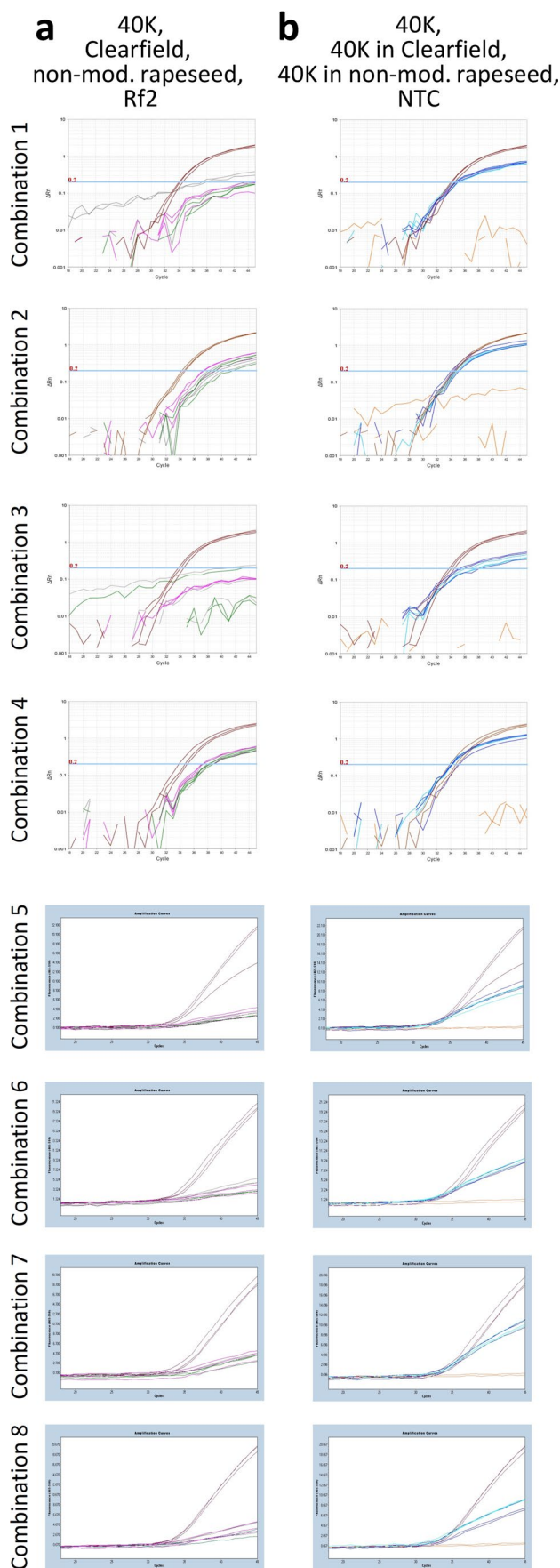
Fig. 6 PCR amplification for the robustness analysis with the SU rapeseed method. Samples were analysed according to a multifactorial design approach in triplicate per condition with the Kapa Probe Force mix on the ABI 7500 Fast System (combinations 1–4), and on the LightCycler 480 Instrument II (combinations 5–8, high sensitivity mode), respectively. **a**, 30 genomic copies of 40 K DNA in TE buffer (red), 100 ng DNA of LG Conrad CL Clearfield DNA (green), 100 ng DNA per PCR of non-modified rapeseed (AOCS 0306-B4, grey) and 300 ng DNA per PCR of Rf2 GM rapeseed (purple), respectively. **b**, 30 genomic copies of 40 K DNA either in TE buffer (red), in background of 100 ng per PCR of Clearfield DNA (LG Conrad CL, dark blue) or in background of 100 ng per PCR of non-modified rapeseed DNA (AOCS 0306-B4, light blue). Orange, NTC; Denaturation 30 s

been observed for all rapeseed materials and were further prone to small changes in conditions during the robustness test. Thus, the fluorescence intensity is not a robust measurement parameter to differentiate between specific and non-specific amplification.

Considerations for Method Development

For genotyping PCR methods that aim to specifically detect SNVs on the basis of the primer design, the specificity is highly dependent on 1) the ability of the primer(s) to favour the binding to the intended, perfectly matched DNA sequence in the presence of the non-modified sequence, and 2) to allow the elongation preferentially of the perfectly matched DNA duplex by the DNA-polymerase. Small variations of the test conditions in routine use, which are addressed by testing the robustness, may critically change the balance between primer binding to the perfectly matched and to the single-base mismatched target sequence and could impair the polymerase specificity.

The modified AHAS1C target sequence conferring the herbicide tolerance in Cibus varieties (40 K, C1511, C5507) differs from wild type sequences by one SNV. Herbicide-tolerant Clearfield OSR varieties carry the same, conventionally bred point mutation, but only in AHAS3A. Therefore, a PCR method for specifically detecting the DNA sequence present in Cibus OSR varieties has to perform very efficiently favouring the amplification of the intended SNV over the unintended sequence. For such a purpose, several genotyping strategies have previously been reported, including the use of probes coupled to minor groove binder (MGB), probes and primer modified with locked nucleic acids (LNAs) or peptide nucleic acids (PNAs) (Fouz and Appella 2020) as well as DNA polymerases efficiently excluding an extension of primer mismatches. These nucleotide modifications significantly increase the thermodynamic stability of oligonucleotide and complementary DNA duplexes, leading to higher melting temperatures, which is more effective for perfectly matched duplexes than for mismatched duplexes (You 2006). The differences in melting temperatures can be used for mismatch discrimination and thus for specific



SNV detection. During method development, combinations of oligonucleotides, modifications and experimental conditions (i.e. annealing temperature, oligonucleotide concentration) should be identified that efficiently make use of the difference in melting temperatures between matched and mismatched annealing needed for SNV genotyping. Non-specific amplification should not occur or has to be discriminated by additional specificity effects contributed by, e.g. a hydrolysis probe. We observed for the assessed PCR method non-specific amplification with many OSR varieties within the detection range. This non-specific amplification would falsely indicate the detection and presence of SU tolerant OSR.

The SU rapeseed method (Chhalliyil et al. 2020) makes use of 1) a (non-modified) forward primer that perfectly matches the AHAS1C gene sequence, but that has only one single mismatch at the 3' position for discrimination against the AHAS3A gene, and 2) a reverse primer that perfectly matches the Cibus SU rapeseed AHAS1C and as well the Clearfield AHAS3A gene sequence, respectively. This reverse primer has only one single mismatch at the 3' terminal position for discrimination against the wild type sequence and is modified with one LNA at this position. The length and nucleotide composition of a primer predominantly determine the melting temperature and hence the mismatch discrimination efficiency. For the LNA modified reverse primer (22 base pairs) the theoretical difference in melting temperature for perfectly matched duplex (A-T, amplifying the modified sequence) versus a single mismatch (A-G, amplifying the wild type sequence) is in the range between 2 and 5 °C (You 2006). Since the melting temperature only defines the temperature at which half of the DNA molecules form a stable double helix, a small fraction of the primer population can still anneal at temperatures 2 to 5 °C above the nominal melting temperature. The exact relationship between oligonucleotide binding and temperature is dependent, among other factors, on salt conditions, oligonucleotide sequence, and complementary binding effects. Since the SU rapeseed method (Chhalliyil et al. 2020) is amplifying non-specifically unintended targets differing in a single base, it is assumed that such duplexes with a single base-pair mismatch are still formed at the chosen annealing temperature in the presence of high copy numbers of the wild-type sequence. Studies on the effectiveness of single mismatches at the 3' end of primers show that particularly G/T mismatches are more prone than others to prime an extension by the DNA polymerase in PCR (Huang et al. 1992; Kwok et al. 1990; Newton et al. 1989; Simsek and Adnan 2000). In addition, small changes in PCR conditions, as demonstrated in the robustness tests, could affect the vulnerable primer binding equilibrium resulting in different levels of

non-specific amplification depending on the experimental factor combination.

In order to develop a PCR method for the detection of genome-edited DNA sequences with SNVs, one possible approach could be to use more LNA-modified nucleotides or other genotyping strategies such as LNA clamps or blocks (Dominguez and Kolodney 2005; Fouz and Appella 2020) and inhibition or even exclusion of the 3' degradation by an exonuclease activity of the polymerase. The rhPCR could be as well an alternative approach (Dobosy et al. 2011). Furthermore, emphasis should be placed on the choice of the DNA polymerase and the corresponding master mix, which should not have any proof-reading activity degrading the 3' end of an oligonucleotide, but rather a high discrimination efficiency. Although both master mixes tested in this study are recommended for genotyping applications by the manufacturer, in our study the Kapa Probe *Fast* qPCR Master Mix more frequently produced non-specific amplifications than the Kapa Probe *Force* master mix.

During validation of a method for SNV detection and GMO testing, the confirmation of LOD and robustness should be performed in the presence of DNA of the non-modified species at the maximum working concentration of the PCR method, representing the routine situation in bulk commodity testing. Furthermore, additional specificity tests with DNA of the unmodified variety should be done under robustness testing conditions, since small modifications as occurring in routine method applications might favour a non-specific amplification.

Routine Application of SNV Detection Methods

Contrary to the claims in the article (Chhalliyil et al. 2020), the results obtained in our study suggest that the published method is not fit-for-purpose for the identification of genome-edited OSR lines developed by Cibus US LLC. As shown by the amplifications with OSR lines having the identical SNV, but generated by somaclonal variation, the method could not distinguish between DNA from genome-edited and conventionally bred organisms. Since for this specific trait several OSR varieties carrying the identical SNV exist, its detection alone is not sufficient to conclude that the tested sample contains a genome-edited product. In addition, it has already been shown that the use of herbicides on agricultural areas can induce a selection pressure on plants, leading to the identical SNV in the ALS gene (Nandula et al. 2020; Tan and Medd 2002).

Methods for SNV detection fulfilling the MPR criteria may be applicable for a screening of the presence of known genome-edited products. A negative screening result would exclude the presence of the genome-edited product; a

positive screening result would be an indication requiring follow-up analyses with event-specific methods or non-analytical tracking and document checking. In order to develop such potential screening methods, detailed information on the DNA sequence modifications and the availability of suitable reference materials are required (European Network of GMO Laboratories (ENGL) 2019; Kolkman et al. 2004). In case of applications for authorisation of genetically modified food and feed in the EU, applicants are responsible for the development of event-specific methods and the availability of reference materials. So far, no application has been filed for a genome-edited GMO free of any transgenic elements.

Conclusions

Although the method for the detection of specific herbicide-tolerant OSR varieties published by Chhalliyil et al. (2020) meets the minimum performance requirements for sensitivity, it does not comply with the minimum performance requirements for qualitative GMO testing methods according to European legislation regarding specificity and robustness. Unspecific reactivity with closely resembling DNA sequences has been observed. Therefore, the method cannot unambiguously detect the SNV and may amplify non-genetically modified rapeseed and other variants under routine working conditions. Furthermore, the method does not allow the event-specific identification of OSR line 5715. In summary, the method is not fit-for-purpose for use in official control of genetically modified food, feed and seed.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12161-022-02237-y>.

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Declarations

Ethics Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Conflict of Interest Christopher Weidner declares that he has no conflict of interest. Sophia Edelmann declares that she has no conflict of interest. Dominik Moor declares that he has no conflict of interest. Kathrin Lieske declares that she has no conflict of interest. Christian Savini declares that he has no conflict of interest. Sara Jacchia declares that she has no conflict of interest. Maria Grazia Sacco declares that she has no conflict of interest. Marco Mazzara declares that he has no conflict of interest. Jörn Lämke declares that he has no conflict of interest. Kolja Neil Eckermann declares that he has no conflict of interest. Hendrik Emons declares that he has no conflict of interest. Joachim Mankertz declares that he has no conflict of interest. Lutz Grohmann declares that he has no conflict of interest.

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