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Research Paper

Development of event-specific qPCR detection methods for genetically modified alfalfa events J101, J163 and KK179

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ABSTRACT

Genetically modified alfalfa is authorized for cultivation in several countries since 2005. On the other hand, cultivation in or export to the European Union is not allowed and thus neither certified reference material nor official event-specific detection methods are available. Therefore, based on patent sequence information, event-specific real-time PCR detection methods targeting the junction sequence of the alfalfa genome and the transgenic insert of the respective events J101, J163 and KK179 were developed. Newly developed plasmids were used as reference material for assay optimization and in-house validation. Plasmid standards were quantified using digital droplet PCR and LOD95%, PCR efficiency, robustness and specificity of the assays were determined using real-time PCR. A LOD95% of 10 copies per PCR reaction was observed and PCR efficiencies of 95–97 % were achieved. Different real-time PCR instruments and PCR conditions were applied to test for robustness of the assays using DNA at a concentration of 30 copies per µL for each gm alfalfa event. All replicates were positive independent of the instrument or the PCR condition. DNA from certified reference material of different genetically modified crops as well as reference materials of the three events was used to experimentally test for specificity. No unspecific amplification signal was observed for any of the assays. Validation results were in line with the "Minimum Performance Requirements for Analytical Methods of GMO Testing" of the European Network of GMO Laboratories. Furthermore, an inter-laboratory comparison study was conducted to show the transferability and applicability of the methods and to verify the assay performance parameters.

1. Introduction

Genetically modified (gm) crops were grown on 189.8 million hectares worldwide in 2017 [1], a steady increase since commercialization in 1996. Soybean, maize, cotton and rapeseed still remain the most prominent gm crops. However, other plant species like potato, rice, papaya or tomato have gained importance in recent years. One of those emerging gm plant species is alfalfa (*Medicago sativa*), which is one of the most important forage crops worldwide. Alfalfa has been genetically altered to tolerate drought and salt stress [2], but of higher importance are herbicide tolerant varieties and a low lignin alfalfa variety. By incorporating *CTP2-CP4 epsps* under regulation of an enhanced FMV promoter and an E9 terminator, gm alfalfa event J101 (MON-ØØ 101-8) and J163 (MON-ØØ163-7) gained herbicide tolerance against the total herbicide glyphosate. The RNA interference technique has been applied in event KK-179 (MON-ØØ179-5) in order to block the translation of the *caffeoyl-CoA-3-O-methyltransferase* (*CCOMT*) mRNA. CCOMT is a key enzyme in the lignin pathway and catalyzes the production of Feruloyl-CoA, a precursor of G lignin. Blocking of this enzyme leads to a production shift from G lignin to S lignin and reduces the overall production of lignin in the plant. Thereby, the digestibility of alfalfa KK179 for ruminants will be improved [3]. In 2017, 1.14 million hectares of herbicide tolerant gm alfalfa (J101, J163) and 83'000 ha of lignin modified gm alfalfa event KK179 were grown in the US, whereas Canada planted a total of 1.2 million hectares of gm alfalfa (including 3'000 ha of lignin modified event KK179) [1].

These gm alfalfa varieties are also available as stacked events obtained through conventional breeding. The single events and the stacked events J101 × J163 (MON-ØØ1Ø1-8 × MON-ØØ163-7) and KK179 × J101 (MON-ØØ179-5 × MON-ØØ1Ø1-8) are authorized in several countries (Table 1).

In the European Union (EU), gm crops need to be authorized before being placed on the market [4,5]. An approximal quantity of 9×10^8

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Table 1
Incorporated genetic elements and authorization status of genetically modified alfalfa events J101, J163 and KK179 and their respective stacked events (ISAAA's GM Approval Database. http://www.isaaa.org/gmapprovaldatabase/).

Single event/stacked event	Authorized food and/or feed	Authorized cultivation	Genetic elements of the single event
J101 [#] J101 × J163 [*]	Australia [#] Canada [#] Japan [#,*] Mexico [#,*] New Zealand [#] Philippines [#] Singapore [#] South Korea [#,*] USA [#]	Canada [#] Japan [#,*] Mexico [#] USA [#]	• *P-eFMV* • *I-HSP70* • *CTP2-cp4-epsps* • *T-E9*
J163 [#] J101 × J163 [*]	Australia [#] Canada [#] Japan [#,*] Mexico [#,*] New Zealand [#] Philippines [#] Singapore [#] South Korea [#,*] USA [#]	Canada [#] Japan [#,*] Mexico [#] USA [#]	• *P-enhanced FMV* • *I-HSP70* • *CTP2-cp4-epsps* • *T-E9*
KK179 [#] KK179 × J101 [*]	Argentina [*] Australia [#] Canada [#] Japan [#,*] Mexico [#,*] New Zealand [#] Singapore [#] South Korea [#,*] USA [#]	Argentina [*] Canada [#] Japan [*] USA [#]	• *P-Pal2* • partial *CCOMT* (clockwise) • partial *CCOMT* (anti-clockwise) • *T-nos*

Incorporated genetic elements (P = Promoter; I = Intron; T = Terminator): P-*eFMV* = enhanced Figwort Mosaic Virus promoter, *HSP70* = *heat-shock protein 70* intron; *CTP2* = *chloroplast transit peptide*; *cp4-epsps* = coding sequence for cp4 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium tumefaciens* strain *cp4*; T-*E9* = RuBisCO small subunit *rbcS*; P-*Pal2* = *phenylalanine ammonia-lyase 2* promoter from *Arabidopsis thaliana*; *CCOMT* = *trans-caffeoyl CoA 3-O-methyltransferase* from *Medicago sativa*; T-*nos* = *nopaline synthase* terminator from *Agrobacterium tumefaciens*).

tons of alfalfa (seed, meal, pellets or hay) were imported from the US in 2017 [6]. Alfalfa hay is mainly used as animal feed due to its high nutritional quality. However, alfalfa is also used in biogas facilities due to its high biomass production. As gm alfalfa is not authorized in the EU, neither for cultivation nor for its import as food or feed, it should not enter the European market and has therefore been in the focus of official enforcement authorities.

Detection and identification of gm plants is currently performed by real-time PCR (qPCR) methods using a detection cascade [7]. First, a taxon specific reference gene is amplified to verify the presence of the respective plant species in a sample as well as a successful DNA extraction. Genetic modifications are detected by qPCR screening of commonly incorporated genetic elements (e. g. promoter or terminator). In order to identify a certain gm crop, an event-specific qPCR method is applied targeting the junction sequence of the plant genome and the transgenic DNA insert [8], which is unique for each gm crop. For gm crops authorized in the EU, event-specific detection methods and certified reference material are available as notifiers have to submit detection methods and reference material as part of the authorization process. For non-approved gm crops like gm alfalfa, the situation is totally different.

The aim of this work was to develop an event-specific qPCR detection methods for gm alfalfa events J101, J163 and KK179 based on the available sequence information and to validate these methods according to respective guidelines in the field of GMO testing. To underline the suitability of these methods and as a prerequisite for a national collaborative trial study, an inter-laboratory comparison study was performed using alfalfa reference materials.

2. Material and methods

2.1. Plasmid standards

At the beginning of this project, reference materials for gm alfalfa events J101, J163 and KK179 were not available. Hence, we used sequence information of all three gm alfalfa events, obtained via patent search, to design plasmids for assay development and in-house validation.

For event J101 and J163, sequence information on the junctions of the insert and the respective alfalfa plant genome was obtained from patent WO 2004/070020 [9] (J101: Accession No. CQ857611, CQ857609, CQ857612, CQ857610; J163: Accession No. JA141099, JA141097, JA141100, JA141098). The published data sets contained only details of the junction regions, but not of the whole inserts. Sequence information for event KK179 was obtained from patent WO 2013/003558 [10] (NCBI Accession No. JA901749, JA901748).

Separate plasmids for each event were synthesized by Thermo Fisher Scientific (Waltham, MA, USA) using a pMK-RQ (pJ101, pJ163) or pMA (pKK179) vector backbone. For events J101 and J163, the plasmids contains the 5'-junction (pJ101: 390 bp plant genome and 288 bp insert sequence; pJ163: 223 bp plant genome and 258 bp insert sequence) followed by the 3'-junction (pJ101: 315 bp insert sequence and 266 bp plant genome; pJ163: 358 bp insert sequence and 192 bp plant genome), an *E. coli* ORI and a *kanamycin resistance* gene as a selective marker. The plasmid pKK179 contains the whole KK179 insert, the flanking regions (378 bp plant genome 5', 2'582 bp insert sequence, 869 bp plant genome 3'), an *E. coli* ORI and an *ampicillin resistance* gene as a selective marker. We linearized all plasmids by restriction using a *SfiI* enzyme (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions and successful restriction was checked by capillary electrophoresis (Fragment Analyzer, AATI, Heidelberg, Germany; data not shown).

2.2. Oligonucleotides

Primers and hydrolysis probes spanning the 5' junction (alfalfa genome to transgenic insert) of events J101, J163 and KK179 were designed, as the 3' regions were not suitable for primer and probe design due to high AT content and highly repetitive regions. Position and length of the amplicons are illustrated as small black bars in Fig. 1.

Primers and double quenched probes were synthesized by Integrated DNA Technologies (IDT, Leuven, Belgium). Details on PCR concentration, amplicon size and oligonucleotide sequences are provided in Table 2. Before ordering, all oligo sequences were screened for potential secondary structures [11] and *in silico* specificity by using NCBI Blast [12].

2.3. Optimal oligo concentrations

A 7900 H T qPCR instrument (Applied Biosystems, Foster City, CA, USA) was applied for determination of optimal oligo concentrations for the newly developed event-specific gm alfalfa qPCR methods. PCR primer concentrations varied between 200 nM and 500 nM, whereas PCR probes concentrations varied between 100 nM and 300 nM. The qPCR assays were performed in a 25 µL reaction volume containing 1 × GoTaq Probe qPCR Master Mix (Promega GmbH, Mannheim, Germany), oligos in different concentrations, 5 µL plasmid and nuclease-free water ad 25 µL. Plasmids were diluted 1:10^5 (pJ101 ≅ 2.54 × 10^6 copies) or 1:10^6 (pJ163 ≅ 2.88 × 10^5 copies; pKK179 ≅ 1.5 × 10^5 copies) and analyzed in six replicates for each oligo concentration and combination. Cycling conditions were the following: initial denaturation for 3 min at 95 °C and 45 cycles of 95 °C for 15 s and

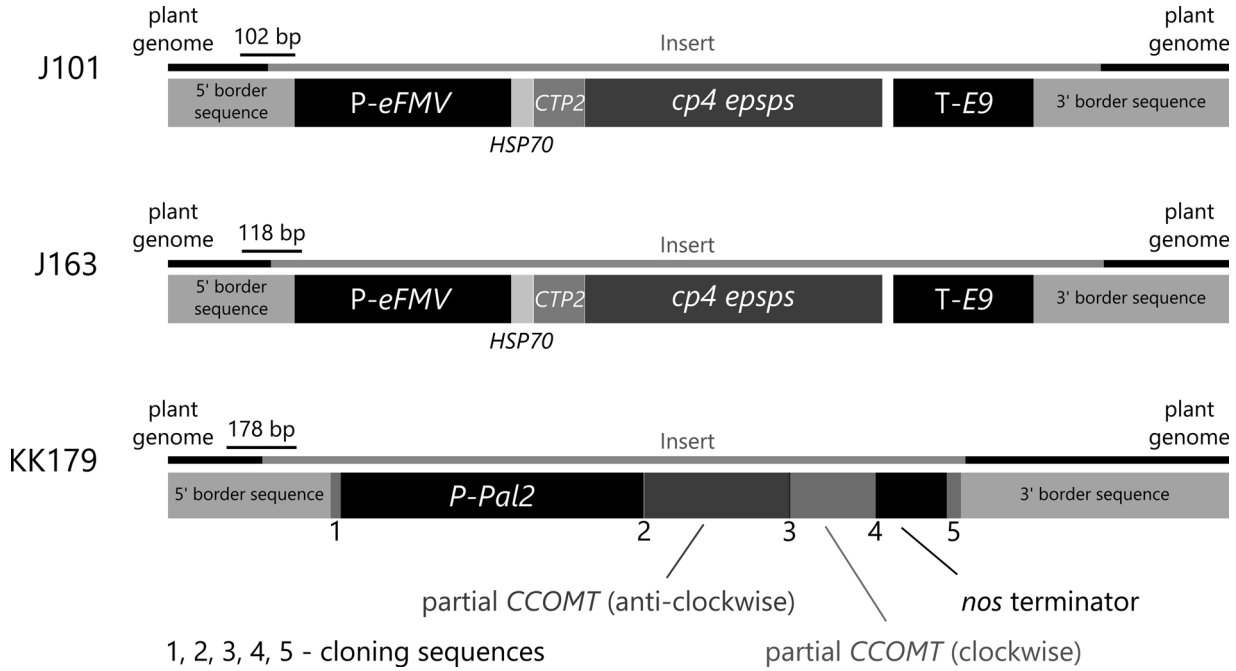

Fig. 1. Schematic genetic structure for genetically modified alfalfa events J101, J163 and KK179. The small black bars indicate the position and length of the amplicon obtained by using the detection systems developed in this work. P-*eFMV* = enhanced Figwort Mosaic Virus promoter; *HSP70* = *heat-shock protein* 70 intron; *CTP2* = *chloroplast transit peptide*; *cp4 epsps* = coding sequence for CP4 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium tumefaciens* strain CP4; T-*E9* = RuBisCO small subunit *rbcS* terminator; P-*Pal2* promoter = *phenylalanine ammonia-lyase 2* promoter from *Arabidopsis thaliana*; CCOMT = *trans-caffeoyl CoA 3-O-methyltransferase* from *Medicago sativa; nos* terminator = *nopaline synthase* terminator from *Agrobacterium tumefaciens*.

Table 2
Primers and probes used for the detection of the alfalfa-specific reference gene *acc1* (*acetyl CoA carboxylase*) and gm alfalfa events J101, J163 and KK179 (FAM = 6-Carboxyfluorescein. /ZEN/ = internal quencher. IBFQ = Iowa Black Fluorescent Quencher; oligo modifications are highlighted in bold letters).

Name	Sequence [5'-3']	Amplicon size	PCR conc.
Acc1-F	gATCAgTgAACTTCgCAAAgTAC	91 bp [13]	150 nM
Acc1-R	CAACgACgTgAACACTACAAC		150 nM
Acc1-P	**FAM**–TgAATgCTC/**ZEN**/CTgTgATCTgCCCATgC–**IBFQ**		50 nM
J101-F	gTCATgTgTTTTgTACTgATCTTgTg	102 bp this work	400 nM
J101-R	gACCTgCAgAAgCTTgATgg		400 nM
J101-P	**FAM**–ACTgAAggC/**ZEN**/gggAAACgACAATCTgATCC–**IBFQ**		200 nM
J163-F	CgggACAAggTCATCCAAACTg	118 bp this work	400 nM
J163-R	ACCTTgTTgAggCTTTggACTg		400 nM
J163-P	**FAM**–TCTgCAggT/**ZEN**/CCTgCTCgAgTggAAGgT–**IBFQ**		200 nM
KK179-F	CTTAgggCACTTgTTAgCATTTTC	178 bp this work	500 nM
KK179-R	CCATATTgACCATCATACTCATTgC		500 nM
KK179-P	**FAM**–TggCTTCAT/**ZEN**/gTCCgggAAATCTACATgg–**IBFQ**		200 nM

60 °C for 60 s. All following qPCR experiments described in this work were performed using the optimized oligo concentrations (see Table 2) and cycling conditions (except for robustness tests; see robustness test section). Optimal concentrations were selected based on PCR efficiency, amplification curves and Cq values.

2.4. Plasmid quantification using droplet digital PCR

For quantification of the plasmid standards, a QX100 droplet digital PCR (ddPCR) system was applied (Bio-Rad, Hercules, USA). A total of 2 µL of each plasmid DNA ($1{:}10^6$ diluted) were added to 18 µL of ddPCR reaction mix containing 1x ddPCR supermix (Bio-Rad, Hercules, USA) and primers and probes dissolved in PCR grade water (final concentrations see Table 2). Water served as non-template control. Droplets were generated using 8-well cartridges in a droplet generator (Bio-Rad, Hercules, USA) and then transferred to a 96-well plate using a multichannel pipette. End-point PCR was performed using a T100 thermal cycler (Bio-Rad, Hercules, USA) under the following conditions: 10 min initial denaturation at 95 °C, 45 cycles of 94 °C for 30 s and 60 °C for 1 min, and finally 10 min at 98 °C A heating ramp rate of 2 °C per second was applied. After amplification, droplet separation, counting and fluorescence measurement were performed in the QX100 Droplet Reader (Bio-Rad, Hercules, USA). The QuantaSoft software (Bio-Rad, Hercules, USA; version 1.7.4) was used for data acquisition and analysis.

2.5. In-house validation

In-house validation was performed according to published guidelines [14,15]. For determination of the 95% limit of detection ($LOD_{95\%}$), PCR efficiency and R^2, serial dilutions of the plasmids were prepared using a 0.2 × TE buffer (100 × TE buffer: 1 M Tris−HCl, 0.1 M EDTA, pH = 8.0; Sigma-Aldrich Chemie GmbH, Munich, Germany); 20 ng μL^{-1} herring sperm DNA (Promega GmbH, Mannheim, Germany) were also added to the 0.2 × TE buffer. Samples were diluted to 1000 copies μL^{-1}, 200 copies μL^{-1}, 20 copies μL^{-1}, 10 copies μL^{-1}, 4 copies μL^{-1}, 2 copies μL^{-1}, 1 copies μL^{-1}, 0.2 copies μL^{-1} and 0.02 copies μL^{-1}. Dilutions between 20 copies μL^{-1} and 0.02 copies μL^{-1} were applied in two runs (12 replicates each dilution) for the determination of $LOD_{95\%}$. The $LOD_{95\%}$ was set as the sample target concentration at which all replicates were tested positive for the respective target [15]. Dilutions between 1000 copies μL^{-1} and 2 copies μL^{-1} were used in a single run (12 replicates each dilution) for PCR efficiency and R^2 determination. Efficiency was calculated using the formula: $E\,[\%] = (10^{\frac{-1}{slope}} - 1) \times 100$. The diluted sample with a concentration of 0.02 copies μL^{-1} was used to verify the initial concentration of the plasmid solution, as a maximum of two replicates out of 12 should be positive in case of a correct initial quantification [15].

Table 3
Results of the digital PCR analysis using the plasmid standards for each gm alfalfa events.

Sample	Dilution	Number of partitions	Individual partition volume	Total partition volume	Final sample concentration
pJ101	1:10^6	9755	0.85 nL	8.29 µL	2.48 × 10^9 copies µL^{-1}
pJ101	1:10^6	9489	0.85 nL	8.07 µL	2.56 × 10^9 copies µL^{-1}
pJ163	1:10^6	11941	0.85 nL	10.15 µL	2.86 × 10^9 copies µL^{-1}
pJ163	1:10^6	12394	0.85 nL	10.54 µL	2.85 × 10^9 copies µL^{-1}
pKK179	1:10^6	13178	0.85 nL	11.20 µL	2.04 × 10^9 copies µL^{-1}
pKK179	1:10^6	13165	0.85 nL	11.19 µL	2.00 × 10^9 copies µL^{-1}

Robustness of the developed assays was tested on three different cyclers: 7900 H T (Applied Biosystems, Foster City, CA, USA), MX3005 P (Agilent Technologies, Waldbronn, Germany) and CFX 96 (Bio-Rad, Munich, Germany). The following parameters were altered and tested in all combinations: two different master mix products [GoTaq Probe qPCR Master Mix (Promega GmbH, Mannheim, Germany), PerfeCta qPCR ToughMix (Quanta Bio, Beverly, MA, USA)], annealing/extension temperature ± 1 °C, oligo concentration −30% and master mix volume (± 1 µL). For each parameter combination, six replicates of a plasmid dilution of 30 cp µL^{-1} (3 times LOD95%) were analyzed. Herring sperm DNA (20 ng µL^{-1} in 0.2 × TE Buffer) was used for plasmid dilution. Due to the use two different master mix products on a single plate, the initial denaturation step was extended to ten minutes, as the PerfeCta qPCR Tough Mix requires a longer initial denaturation step.

2.6. Specificity

An automated DNA extraction method [6] was applied for isolation of genomic DNA from different gm and non-gm reference materials (see Table 3) for specificity testing. These reference materials were purchased from IRMM (Geel, Belgium) or AOCS (Urbana, IL, USA). For non-gm alfalfa, DNA was extracted from leaf material, and for gm alfalfa, the designed plasmids and reference materials (ground flour) of the alfalfa events J101, J163 and KK179 (Forage Genetics, West Salem, USA) were used. Water served as non-template control.

Prior to qPCR, all samples were quantified by ddPCR as described before using the respective event-specific qPCR detection methods as published in the EU Database of Reference Methods for GMO Analysis of the European Union Reference Laboratory for GM food and feed [17]. The non-gm alfalfa material was quantified using a taxon-specific method [13]. PCR conditions were the same as described in 2.4. Measured copy number concentrations were used to adjust all non-target samples to 500 copies µL^{-1}. A buffer consisting of 0.2 × TE buffer and 20 ng µL^{-1} herring sperm DNA was used for dilution. All samples were analyzed in six replicates. The target DNA was diluted to 3× $LOD_{95\%}$ per PCR reaction.

2.7. Inter-laboratory comparison study

Seven laboratories participated in a comparative laboratory study conducted by the Federal Office of Consumer Protection and Food Safety (BVL). Each laboratory received the oligonucleotides (synthesized by Integrated DNA Technologies, Belgium) and the GoTaq Probe qPCR Master Mix. Single quenched probes were used for the inter-laboratory comparison study, labelled with FAM and BHQ1, in order to be independent of a certain manufacturer. Further, the laboratories received J101, J163 and KK179 genomic DNA solutions (adjusted to 500 copies µl-^{1} of the target sequence of each event in alfalfa genomic DNA adjusted to 22,752 copies µl^{-1} of the *acc1* reference gene) and plasmid DNA (500 copies µl^{-1} of the target sequence of each event) as calibration standard. These samples were used to determine the qPCR efficiency, LOD95% and the coefficient of determination.

In order to verify the false positive/false negative rate for each method, ground seed samples (2.5 g) with a relative gm content of 0.1% (mass/mass) of each of the three gm alfalfa events (J101, J163, KK179) were prepared by mixing non-gm alfalfa with gm alfalfa material and provided as coded samples to all participating laboratories. For extraction of DNA from the two coded flour samples, a manual CTAB-based DNA extraction method was recommended (CTAB = cetyltrimetylammoniumbromide; http://gmo-crl.jrc.ec.europa.eu). Successful DNA extraction was evaluated by amplification of the *acc1* alfalfa-specific reference gene (see Table 2).

2.8. Routine alfalfa seed samples

DNA from routine alfalfa seed samples from 2016 and 2017 (3 samples each year) and from ground seed samples of all three gm alfalfa events (kindly provided by Forage Genetics, West Salem USA) was isolated using an automated DNA extraction procedure [16] and analyzed for the potential presence of gm alfalfa using the newly developed event-specific detection methods. Further, presence of alfalfa DNA and absence of PCR inhibitors were tested by amplifying an alfalfa-specific reference (*acc1*) gene [13]. Samples were analyzed in duplicates (undiluted and 1:4; in reference gene qPCR also 1:16 and 1:64) using an ABI 7900 H T qPCR instrument. The final PCR reaction mix contained 1× GoTaq Probe qPCR Master Mix (Promega GmbH), primers and probes as outlined in Table 2, 5 µL DNA and water ad 25 µL. Cycling conditions were the following: initial denaturation for 3 min at 95 °C and 45 cycles of 95 °C for 15 s and 60 °C for 60 s. DNA from events J-101, J-163 and KK-179 served as positive controls, whereas PCR grade water served as a non-template control.

3. Results

All experiments were carried out with optimized oligo concentrations as outlined in Table 2.

3.1. Plasmid quantification

Plasmids used for in-house validation were quantified using ddPCR. Quantification results as outlined in Table 3 were used for serial dilution of the plasmids.

3.2. In-house validation

A dilution series of the respective plasmid solution of events J101, J163 and KK179 was analyzed in 24 replicates to determine the $LOD_{95\%}$. When using plasmid concentrations of 100 cp µL^{-1} to 10 cp µL^{-1} all 24 replicates were positive. At a concentration of 5 cp µL^{-1}, 23 out of 24 replicates were positive. So the $LOD_{95\%}$ was set to 10 copies per PCR reaction for all events. The calibration curve revealed R^2 values of 0.9985 (J-101), 0.999 (J-163, KK-179) and PCR efficiencies of 95.1% (J-101), 97.3% (J-163) and 95.6% (KK-179). These performance parameters are in line with the "Minimum Performance Requirements for Analytical Methods of GMO Testing" of the European Network of GMO Laboratories (ENGL) [18].

Results of the robustness tests underlined that changing oligo concentrations (−30%), annealing/extension temperature (± 1 °C), master mix product or master mix volume (± 1 µL) does not influence

Table 4
Specificity tests of the event-specific detection assays for gm alfalfa J-101, J-163 and KK-179 using qPCR. A "+" indicates a successful amplification, whereas a "- "indicates a negative result. AOCS = American Oil Chemists' Society; ERM = European Reference Materials.

Species	Event	Reference material	J101	J163	KK179
Alfalfa	J-101	plasmid pJ101	+	–	–
Alfalfa	J-163	plasmid pJ163	–	+	–
Alfalfa	KK-179	plasmid pKK179	–	–	+
Alfalfa	J-101	genomic DNA	+	–	–
Alfalfa	J-163	genomic DNA	–	+	–
Alfalfa	KK-179	genomic DNA	–	–	+
Alfalfa	non-gm	Alfalfa leaf DNA	–	–	–
Maize	MON810	ERM-BF413gk	–	–	–
Maize	Bt11	ERM-BF412f	–	–	–
Maize	NK603	ERM-BF415e	–	–	–
Soybean	MON87701	AOCS 0809-A	–	–	–
Soybean	MON87708	AOCS 0311-A	–	–	–
Soybean	MON87769	AOCS 0809-B	–	–	–
Soybean	GTS 40-3-2	ERM-BF410gk	–	–	–
Rapeseed	GT73	AOCS 0304-B	–	–	–
Rapeseed	MON88302	AOCS 1011-A	–	–	–
Sugar beet	H7-1	ERM-BF419b	–	–	–
Negative control	–	–	–	–	–

the qPCR results. However, it seems that the choice of the qPCR instrument has an impact on the results of event J163 as a mean ΔCq of 2.3 was observed when using the ABI 7900 H T and the Bio-Rad CFX96. For event KK-179, results using the ABI 7900 H T and the Bio-Rad CFX96 were similar, however a mean ΔCq of 3.2 was observed when using the ABI 7900 H T and the Agilent MX3005 P. For event J101, no differences were monitored when using different qPCR instruments. All replicates throughout the robustness tests resulted in positive amplification signals, thus fulfilling the validation acceptance criterion [15].

3.3. Specificity

Specifitiy tests revealed no unspecific qPCR signals for events J101, J163 and KK179. This also complies with the *in silico* specificity screening of the oligos using the NBCI blast tool. The results of the specificity testing are depicted in Table 4.

None of the tested reference materials resulted in a false positive amplification signal underlining the high specificity of the developed event-specific assays. Positive amplification signals originate only from the respective gm alfalfa plasmid standards or gm alfalfa genomic DNA materials.

3.4. Inter-laboratory comparison study

Seven laboratories participated in this inter-laboratory comparison study. All participants serially diluted a plasmid solution for each gm alfalfa event and used it as calibration standard to verify the qPCR performance characteristics. The results are depicted in Table 5. For event J101, all laboratories obtained qPCR efficiencies between 95.4% and 109.6%. For event J163, qPCR efficiencies varied between 95.9% and 106.9%. Laboratory 1 and 7, however, have exceeded the prescribed value of 110%. For KK179, qPCR efficiency values between 97.3% and 106.7% were observed. Though, Lab 4 and 6 obtained values fallen below the prescribed value of 90%.

The same serial dilutions were analyzed using the alfalfa-specific reference gene *acc1* and resulted in qPCR efficiencies ranging from 96.6% to 108.3%, with slope values between -3.14 and -3.41 and a coefficient of determination (R^2) of 0.99 for all laboratories.

For determination of the LOD95%, genomic DNA was serially diluted and analyzed in twelve replicates. The results are shown in Table 6. All laboratories were able to detect 10 nominal copies of J101 or J163 in all twelve replicates and 6 out of 7 laboratories detected 5 nominal copies in all twelve replicates.

Two laboratories observed only 9 or 11 positive replicates for KK179 respectively, underlining that using this method with a single quenched probe leads to a reduced sensitivity compared to the use of a double quenched probe. This is confirmed when comparing the calculated LOD95% values using the QuoData web-tool (Table 7). We observed a strong fluctuation of the values when applying the KK179

Table 6
Inter-laboratory qPCR results of the developed event-specific detection methods using serially diluted genomic DNA with regard to limit of detection; bold numbers indicate deviating values. The total number of positive replicates (out of 12 replicates) is given.

	nominal copy number of target sequence in qPCR					
	0.1	0.5	1	2	5	10
			J101			
Lab 1	1	5	7	9	12	12
Lab 2	**4**	7	8	11	12	12
Lab 3	1	4	7	8	12	12
Lab 4	1	4	6	11	12	12
Lab 5	0	4	7	10	12	12
Lab 6	1	5	6	8	12	12
Lab 7	0	3	5	10	11	12
J163						
Lab 1	1	5	9	11	12	12
Lab 2	2	7	8	11	12	12
Lab 3	**4**	5	11	10	12	12
Lab 4	1	4	12	12	12	12
Lab 5	lang = "DE" > 1	4	8	10	12	12
Lab 6	1	4	9	10	11	12
Lab 7	0	7	8	9	12	12
KK179						
Lab 1	0	4	3	7	10	12
Lab 2	0	3	6	9	12	12
Lab 3	0	0	0	3	4	**9**
Lab 4	1	3	4	10	11	12
Lab 5	0	0	5	7	10	12
Lab 6	0	5	lang = "DE" > 6	7	11	12
Lab 7	0	1	7	10	9	**11**

Table 5
Interlaboratory qPCR results of the developed event-specific detection methods using serially diluted genomic DNA with regard to qPCR efficiency, slope and coefficient of determination (R^2).

Lab No.	PCR instrument	J101			J163			KK179		
		Slope	R^2	qPCR efficiency [%]	Slope	R^2	qPCR efficiency [%]	Slope	R^2	qPCR efficiency [%]
1	Roche LC96	−3.44	0.99	95.4	−3.04	0.99	113.1	−3.17	0.96	106.7
2	AriaMx	−3.13	0.99	108.9	−3.17	0.98	106.9	−3.23	0.99	103.8
3	ABI 7900 H T	−3.42	0.99	96.1	−3.43	0.99	95.9	−3.39	0.99	97.3
4	ABI 7500	−3.19	0.97	105,6	−3.24	0.98	103.5	−3.69	0.95	86.7
5	CFX BioRad	−3.11	0.99	109.6	−3.25	0.99	103.2	−3.31	0.97	100.3
6	CFX96 Touch	−3.31	0.99	100,3	−3.19	0.99	105.9	−3.66	0.98	87.6
7	LC 480 II	−3.17	0.99	106.7	−2.74	0.99	131.7	−3.13	0.98	109.7

Table 7
Calculation of the LOD95% for the three event-specific qPCR methods for gm alfalfa J101, J163 and KK179 using the QuoData web-tool (https://quodata.de/en).

	J101 $LOD_{95\ \%}$ [copies]	J163 $LOD_{95\ \%}$ [copies]	KK179 $LOD_{95\ \%}$ [copies]
Lab 1	3.5	2.4	7.2
Lab 2	3.0	2.2	4.3
Lab 3	4.0	2.0	> 10
Lab 4	3.2	1.7	4.99
Lab 5	3.5	3.1	7.8
Lab 6	4.0	3.6	5.3
Lab 7	4.9	3.0	7.6

Table 8
Inter-laboratory qPCR results of the developed event-specific detection methods using DNA isolated from ground alfalfa samples (0.1 % gm (mass/mass) or non-gm); "+" indicates a positive amplification signal, "−" indicates a negative qPCR result; bold markings indicate deviating values. All samples were analyzed in duplicates.

		J101 0.1 % gm	J101 non-gm	J163 0.1 %	J163 non-gm	KK179 0.1 %	KK179 non-gm
Lab 1	undiluted	+/+	−/−	+/+	−/−	+/+	−/−
	1:10 diluted	+/+	−/−	+/+	−/−	−/−	−/−
Lab 2	undiluted	+/+	−/−	+/+	−/−	+/+	−/−
	1:10 diluted	+/−	−/−	+/+	−/−	+/−	−/−
Lab 3	undiluted	+/+	−/−	+/+	−/−	+/+	−/−
	1:10 diluted	+/+	−/−	+/+	−/−	+/−	−/−
Lab 4	undiluted	+/+	−/−	+/+	−/−	+/+	−/−
	1:10 diluted	+/−	−/−	+/+	−/−	+/+	−/−
Lab 5	undiluted	+/+	−/−	+/+	−/−	+/+	−/−
	1:10 diluted	+/+	−/−	+/+	−/−	+/+	−/−
Lab 6	undiluted	+/+	−/−	+/+	−/−	+/+	−/−
	1:10 diluted	+/+	−/−	+/+	−/−	+/−	−/−
Lab 7	undiluted	+/+	−/−	+/+	−/−	+/+	−/−
	1:10 diluted	+/+	−/−	+/+	−/−	+/−	−/−

qPCR method and very consistent values when applying the other two methods (J101/J163).

Each of the participating laboratories analyzed 12 samples. None of them reported false positive or false negative results, except Lab 1, which did not detect KK179 in a 1:10 dilution of the sample with a gm content of 0.1% (Table 8). However, Lab 1 also detected KK179 in the undiluted sample. All samples were tested positive for the presence of the alfalfa-specific reference gene *acc1* (data not shown).

In this inter-laboratory comparison study, the false positive/false negative rate was evaluated using data from seven participating laboratories. This is below the recommendation by the Federal Office Of Consumer Protection and Food Safety [19]. The number of participating laboratories will be adjusted accordingly in the context of the forthcoming ring trial.

3.5. Routine alfalfa seed samples

Alfalfa event J101, J163 and KK179 could not be detected in 2016/2017 routine alfalfa seed samples. All samples were positive for the alfalfa-specific reference gene *acc1* and no PCR inhibition was observed.

4. Discussion

In recent years, the number of gm crops being commercialized worldwide has increased yearly. One of the newer gm crops is alfalfa, which is used to supplement soybean in feed products. None of the gm alfalfa events is currently authorized for cultivation or its use as food or feed in Europe, which is a prerequisite before being legally placed on the European market. Analysis of food, feed or seed for the presence of gm crops is accomplished using qPCR methods targeting the junction sequence between the transgenic insert and the plant genome of the respective gm event. Those event-specific detection methods were not available for any gm alfalfa event. So we developed and in-house validated qPCR-based detection methods for gm alfalfa J101, J163 and KK179. As reference material was not available at the beginning of this project, we designed plasmids containing the 3'- and 5'-junction sequences of J101 and J163, and the complete insert including the junction sequences of event KK179. These plasmids were used for in-house validation of the developed methods. The obtained assay performance parameters were in line with guidelines for the validation o [14,15]. PCR efficiencies were in between the acceptance criteria values of 90% and 110% with a dynamic range of 5 to 5'000 copies per reaction. The in-house validation revealed a LOD95% of 10 copies per reactions using the designed plasmids. The calculated LOD95% obtained in the comparative laboratory study using genomic DNA and the QuoData tool was even lower as outlined in Table 7.

Double quenched probes were used during the in-house validation and single quenched probes in the inter-laboratory comparison study, in order to be independent of a specific oligonucleotide manufacturer. However, the comparative study showed, that using single quenched probes in the KK179 detection method leads to lower qPCR efficiencies and a higher LOD95%. This is supported by Produnikov et al. [20] who reported a up to 30-fold increase in sensitivity when moving the quencher to an internal position in a probe. Based on our results, we recommend using double quenched probes for the KK179 detection method. Applying the other two detection methods (J101 and J163) resulted in similar performance values regardless of whether single or double quenched probes were used.

All detection methods showed a high specificity and no unspecific amplification signals were observed using certified reference materials of different gm crops.

Even though our routine samples were tested negative for the presence of gm alfalfa, we are sure that our developed methods can help to detect and identify admixtures or contaminations of gm alfalfa in food, feed and seed. The common qPCR screening approach detecting one or more of the incorporated genetic elements can be replaced by our event-specific methods. Alfalfa events J101 and J163 cannot be distinguished using a qPCR screening approach, as both events contain the same genetic elements, however, incorporated at different sites in the genome. This problem can be solved by using our event-specific detection methods targeting the 5' junction sequences.

DNA extraction from alfalfa can be difficult, as alfalfa has a high content of proteins and carbohydrates, which can lead to a reduced DNA yield or PCR inhibition. A comparison of two improved DNA extraction procedures is part of the second inter-laboratory comparison study, the results of which are currently being evaluated.

Finally, an official full collaborative-trial study for validation of the methods including the DNA extraction step will be performed in near future.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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