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ORIGINAL PAPER

Novel TaqMan PCR screening methods for element *cry3A* and construct *gat*/T-*pinII* to support detection of both known and unknown GMOs

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Abstract The import and use of genetically modified organisms (GMOs) is strictly regulated in the European Union. In order to maintain the legislation on GMOs, a genetic element screening is generally applied as a first step to detect authorised as well as unauthorised GMOs. Subsequent identification of GMOs that relate to the detected elements is performed by the application of event-specific detection methods. However, as the diversity of GMOs on the world market is increasing, there is an ongoing need for methods for additional informative screening elements. Genes that are increasingly applied in GMOs are *cry3A* (including variants *mcry3A* and *eCry3.1Ab*) conferring resistance to Bt toxins, and *gat*, detoxifying glyphosate. Novel TaqMan PCR detection methods for element *cry3A* and construct *gat*/T-*pinII* were developed to support the identification of maize MIR604, 98140, 5307, canola 61061 and 73496, and soybean 356043. Also, other unknown (unauthorised) GMOs containing *cry3A* and/or *gat*/T-*pinII* can potentially be detected. Specificity, efficiency and sensitivity of the methods were evaluated.

Keywords Screening · qPCR · Real-time · PCR · Detection · Identification

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Introduction

In the European Union (EU), the import and use of genetically modified organisms (GMOs) is strictly regulated. Currently, March 2016, 66 GM crop plant varieties are authorised in the EU (10 × cotton, 37 × maize, 6 × canola, 12 × soybean and 1 × sugar beet) [19]. EU-authorised GMOs and derived materials have to be labelled as such [19]. Some additional rules apply for GMO-derived feed materials. Under Regulation (EC) No 619/2011 (European [17], GMOs that are in the process of being authorised (currently 20 GM plants) are allowed up to 0.1 % in feed if they have already been authorised in another country and if reference material and a detection method are available. When a product or ingredient is not labelled as GMO-derived, EU-authorised GMOs are allowed to be present in the ingredient up to 0.9 %, if this presence is unintended and cannot be technically avoided. Some GMOs that are withdrawn from the market or where the market approval has expired are allowed up to 0.1 % in feed (European Commission, [17–19]. All other GMOs are not authorised for the EU market (0 % tolerance).

Specific detection methods are needed for enforcement of these labelling requirements. TaqMan detection methods that target the authorised GM events are delivered by the respective applicants, verified by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF) and interlaboratory validated by the EURL-GMFF together with the European Network of GMO Laboratories (ENGL) [7]. Specific targets for detection strategies are GMO-related genetic elements (coding sequences (CS-), promoters (P-) or terminators (T-)), constructs (the junction sequences bridging adjacent elements) and events (area spanning the 5′ or 3′ insertion site and the flanking plant region). Because already many GMOs are

allowed on the EU market, screening strategies targeting GMO elements and constructs are applied to limit the number of event-specific methods that need to be performed [14, 37, 43]. Also, pre-spotted screening and event plates have been developed [34, 36] to facilitate the screening and identification of GMOs in food and feed products.

Depending on the type of samples to be tested, more or fewer elements and constructs can be selected for screening. Screening with, for example, five detection methods as described by Waiblinger et al. [43] would be a good option to cover the majority of known GMOs in samples for which the absence of known GMOs has to be confirmed. For samples that are labelled as GMO and that may contain several different GMOs (e.g. feed), an extended element and construct screening can be more informative, as here the focus will be primarily on the detection and identification of unauthorised GMOs [13, 37]. If specific detected elements cannot be explained by the subsequently identified events, this can be an indication for the presence of unauthorised GMOs in the sample. The EUginius GMO database (www.euginius.eu) provides an analysis tool that can aid the analyses based on the GMO elements that have been identified and those that have been found negative in the screening. The JRC GMO-Matrix event finder [1] provides a comparable identification strategy. Screening strategies are also described in a CEN Technical Standard NPR-CEN/TS 16707:2014 en [12], and a review on this subject has been written by Fraiture et al. [20].

Where the detection of promoter P-35S from Cauliflower mosaic virus (CaMV) or terminator T-*nos* from *Rhizobium radiobacter* was once sufficient to detect most authorised GMOs, there now is a tendency to use a wide variety of promoters, coding genes and terminators in more recent GMOs. Also, the use of plant-derived GMO elements requires the use of construct-specific methods instead of element-specific methods to avoid false positives in feed samples containing multiple species. Therefore, additional detection methods are sometimes necessary in order to target specific (groups of) GMOs. Some recent examples of additional qPCR screening methods are the element *bar* and construct *ctp2/cp4epsps* [21], the elements *cry1A.105* and *cry2Ab2* [15], promoters P-FMV, P-*nos*, P-*SSuAra*, P-*TA29*, P-*ubi*, P-*rice actin* and terminators T-35S, T-*E9*, T-*ocs*, T-*g7* [14], element *vip3A* [29], and element *cry1Ab/Ac* and construct P-*ubi/cry* [22]. Recent examples of SYBR® Green detection methods are P-*nos* and P-FMV [9], *cry3Bb* and *gat*/T-*pinII* [10], and the application of Combinatory SYBR® Green PCR Screening (CoSYPS) [4, 30, 42]. Detection and identification of GMOs is not static. Progressive insight, changes in GMO authorisations, development of novel GMOs with sometimes (plant-derived) elements for which no suitable detection methods are available, regularly require adjusted adequate detection strategies. Nevertheless, there are still GMOs for which no suitable screening elements are available.

Here, we describe the development of TaqMan PCR detection methods for the coding sequence *cry3A* and the construct gat/T-*pinII* to facilitate the detection of present and future GMOs containing these elements. The element *cry3A* and construct *gat*/T-*pinII* were selected as targets since they are present in GMOs that harbour zero or one element that can be detected in the element screening, which is not sufficient for the identification of the GMO(s) present. The *cry3A* method was initially intended for the screening for MIR604 maize (SYN-IR6Ø4-5) and 5307 maize (SYN-Ø53Ø7-1), but may also detect other GMOs containing *cry3A*. At least 27 potato events that are deregulated in the USA [2], the Russian 2904/1 kgs (ISAAA's GM Approval Database [24]), a GM potato developed in Bulgaria [26] and two GM potatoes developed in China [23, 31] contain *cry3A* or variants thereof. For other GMOs like the currently unauthorised MZIR098 (SYN-ØØØ98-3) containing *ecry3.1Ab* (chimaera of *mCry3A* and *cry1Ab*), the *cry3A* detection method may be suitable, although this GMO also contains the CaMV 35S promoter and the *nos* terminator that will already be used in most screening approaches. Also, in experimental lines of tobacco [35], poplar [16], alfalfa [41], Norway spruce [8] and rice [27], *cry3A* was applied, conferring resistance against Coleoptera. The *gat*/T-*pinII* method can be used to screen for canola 61061 (DP-Ø61Ø61-7) and 73496 (DP-Ø73496-4), maize 98140 (DP-Ø9814Ø-6) for which events no GMO element screening methods are available yet, and soybean 356043 (DP-356Ø43-5) that can be screened for only using the promoter element P-35S. In the present paper, we describe the newly developed methods and evaluate these in the light of established ENGL method performance requirements [25].

Materials and methods

Samples

MIR604 and Event 5307 were used as positive reference materials for the *cry3A* detection method. A range of GMOs (including those containing variants of the *cry* gene) were used as negative control. For the construct *gat*/T-*pinII*, maize 98140 and soybean 356043 were used as positive controls. A range of GMOs (including those containing either *gat* or *pinII*) were used as negative control. In Table 1, all reference materials are described.

Table 1 GMO reference materials obtained from the ERM (European Reference Materials: IRMM, Geel, Belgium) or AOCS (American Oil Chemist's Society: Urbana, IL, USA)

Crop	GMO	Reference	Potential target(s) *cry*/*gat*/T-*pinII*	*cry3A*	*gat*/T-*pinII*
Soybean	A2704-12	AOCS 0707-B 100 %		−	−
	A5547-127	AOCS 0707-C 100 %		−	−
	CV127	AOCS 0911-C > 96.32 %		−	−
	DAS44406	ERM-BF436e 10 %		−	−
	DAS68416	ERM-BF432d 10 %		−	−
	DAS81419	ERM-BF437e 10 %		−	−
	DP305423	ERM-BF426d 10 %		−	−
	DP356043	ERM-BF425d 10 %	CS-*gat*4601, T-*pinII*	−	+
	FG72	AOCS 0610-A2 100 %		−	−
	GTS40-3-2	ERM-BF410f 5 %		−	−
	MON87701	AOCS 0809-A 100 %	CS-*cry1Ac*	−	−
	MON87705	AOCS 0210-A > 99.4 %		−	−
	MON87708	AOCS 0311-A 100 %		−	−
	MON87769	AOCS 0809-B 100 %		−	−
	MON89788	AOCS 0906-B > 99.4 %		−	−
Maize	Event 3272	ERM-BF420c 9.8 %		−	−
	5307	AOCS 0411-D > 99.88 %	CS-*ecry3.1Ab*	+	−
	Bt11	ERM-BF412f 4.89 %	CS-*cry1Ab*	−	−
	Bt176	ERM-BF411f 5 %	CS-*cry1Ab*	−	−
	DAS40278	ERM-BF433d 10 %		−	−
	DAS59122	ERM-BF424d 9.87 %	CS-*cry34Ab1*, CS-*cry35Ab1*, T-*pinII*	−	−
	98140	ERM-BF427d 10 %	CS-*gat*4621, T-*pinII*	−	+
	1981	ERM-BF438e 10 %		−	−
	4114	ERM-BF439b > 98.6 %	CS-*cry34Ab1*, CS-*cry35Ab1*, T-*pinII*, CS-*cry1F*	−	−
	GA21	ERM-BF414f 4.29 %		−	−
	MIR162	AOCS 1208A > 99.98 %	CS-*vip3Aa20*	−	−
	MIR604	ERM-BF423d 9.85 %	CS-*cry3A*	+	−
	MON810	ERM-BF413f 5 %	CS-*cry1Ab*	−	−
	MON863	ERM-BF416d 9.85 %	CS-*cry3Bb1*	−	−
	MON87427	AOCS 0512-A > 99.94 %		−	−
	MON87460	AOCS 0709-A > 99.05 %		−	−
	MON88017	AOCS 0406-D > 99.05 %	CS-*cry3Bb1*	−	−
	MON89034	AOCS 0906-E > 99.42 %	CS-*cry1A.105*, CS-*cry2Ab2*	−	−
	NK603	ERM-BF415f 4.91 %		−	−
	TC1507	ERM-BF418d 9.86 %	CS-*cry1Fa2*	−	−
Canola	73496	ERM-BF434e 10 %	CS-*gat*4621, T-*pinII*	−	+
	MON88302	AOCS 1011-A > 99.94 %		−	−
	Topas 19/2	AOCS 0711-D 100 %		−	−
Cotton	281-24-236 * 3006-210-23	ERM-BF422d 10 %	CS-*cry1Fa2*, CS-*cry1Ac*	−	−
	GHB119	ERM-BF428c 10 %	CS-*cry2Ae*	−	−
	GHB614	AOCS 1108-A4 > 99.99 %		−	−
	MON1445	AOCS 0804-B > 99.4 %		−	−
	MON15985	AOCS 0804-D > 98.45 %	CS-*cry1Ac*, CS-*cry2Ab2*	−	−
	MON531	AOCS 0804-C > 97.39 %	CS-*cry1Ac*	−	−

Table 1 continued

Crop	GMO	Reference	Potential target(s) *cry*/*gat*/T-*pinII*	*cry3A*	*gat*/T-*pinII*
	MON88913	AOCS 0906-D		–	–
	T304-40	ERM-BF429c 10 %	CS-*cry1Ab*	–	–
Potato	EH92-527-1	ERM-BF421b 100 %	P-*pinII*, CS-*pinII*, T-*pinII*	–	–
Tomato	Wild type	*Solanum lycopersicum*	P-*pinII*, CS-*pinII*, T-*pinII*	–	–

Targets and non-targets that could cross-react (like *cry*-genes for *cry3A*) are depicted. The last two columns give the result of the specificity testing where '+' is detected and '−' is not detected. *CS* coding sequence, *T* terminator

DNA extraction

DNA from reference material was isolated from 100 mg material using a CTAB DNA isolation method [33], the Maxwell® 16 Tissue DNA Purification Kit (Promega) used with the Maxwell® 16 machine (Promega), or with CTAB/Qiagen (CTAB lysis step, followed by the DNeasy Plant Mini Kit (Qiagen) isolation. Optical density was measured on a Nanodrop spectrophotometer (ND-1000, Montchanin, DE, USA), which was used to quantify and assess the purity of the DNA. DNAs (extracted from reference materials, or provided as DNA) were diluted with water (Gibco distilled water, DNase/RNase free, Life Technologies, Grand Island NY, USA) to 10 ng/µl final concentration and stored at 4 °C, or at −20 °C for long-term storage.

Primers and probes

Primers and probe for *cry3A* were designed using Beacon Designer 7.0 (PREMIER Biosoft, Palo Alto, CA, USA). Settings for the amplicons were Ta = 60 °C ± 2 °C; amplicon length 70–120 bp (base pairs).

Primers and probe for *gat*/T-*pinII* were designed using Beacon Designer 8.14 (PREMIER Biosoft, Palo Alto, CA, USA). Settings for the amplicons were Ta = 60 °C ± 5 °C; amplicon length 60–120 bp. The detection method was partially based on the SYBR Green qPCR CoSYPS method described by Broeders et al. [10]. Primers and probes were synthesised by Biolegio (Nijmegen, Netherlands) with a high-purity method. Probes were purified by HPLC.

Sequence analysis was performed at the GenBank database by using the BLASTn tool (Basic Local Alignment Search Tools Nucleotide) of the National Center for Biotechnology Information (NCBI). The alignments among similar sequences were performed by using the software Clustal Omega [38] version 1.2.1.

Mastermix and PCR programme

The following mastermix was used in 25-µl final volume: 1 × Diagenode mastermix with UNG (Diagenode, Belgium) to prevent amplification of previously amplified products, 400 nM forward and reverse primer each, 200 nM probe, 5 µl water, 5 µl DNA (10 ng/µl, or dilutions thereof). PCR programme in Bio-Rad CFX96: initial decontamination with UNG (uracil-N-glycosylase) 120 s at 50 °C, denaturation and activation 600 s at 95 °C, then 45 cycles with 15 s at 95 °C, 60 s at 60 °C (hold at 20 °C indefinitely). qPCR trace files were analysed with the Bio-Rad CFX manager software version 3.1.

Limit of detection and PCR efficiency

Limit of detection and PCR efficiency were determined according to the minimum performance requirements for analytical methods of GMO testing [25] and the guidelines published by Broeders et al. [11]. The detection limit should at least be 20 haploid genome copies. The TaqMan PCR analysis was evaluated for each primer/probe pair by the serial dilution of CRM for and in water. MIR604 maize was used for *cry3A*. DP073496 canola and DP356043 soybean were used for *gat*/T-*pinII*. The copy numbers in the calibration curve samples are obtained by dividing the amount of sample DNA (picograms) by the published average 1*C* value for the maize (2.725 pg), canola (1.15 pg) and soybean (1.13 pg) nuclear genome [3].

Results and discussion

TaqMan PCR development

The *cry3A* gene (BCH abbreviation CS-cry3A-BACTU) is derived from *Bacillus thuringiensis* subsp. *tenebrionis*, strain BI 256-82. It codes for a Bt-toxin, conferring resistance to Western corn rootworm (*Diabrotica virgifera virgifera*), Northern corn rootworm (*D. longicornis barberi*) and other related Coleopteran species [5]. The gene is often codon-optimised [40] for the target species (*mcry3A*), or fused to cry1Ab (eCry3.1Ab). At least 30 known potato events contain *cry3A* or variants thereof. For *cry3A* in potato, a conventional PCR detection method was already developed [39], but to reduce chances of contamination,

Table 2 Primers and probe designed for the detection of element CS-*cry3A* and construct *gat*/T-*pinII*

Target	Name	Sequence 5′–3’	Amplicon size (bp)	Reference
cry3A	cry3A-F	CAGCAACATCGAGAACTA	117	MIR604 (GenBank DJ437707.1) 5307 (GenBank GU327680.1)
	cry3A-R	CTCCAGTAGTTGAAGCTG		
	cry3A-P	FAM-TGTTCGACTACCTGCACCGC-BHQ1		
gat/T-*pinII*	gat/T-pinII-F	GGACCTCACATCCTGATGTATAA	111	356043 (GenBank FB741980.1)
	gat/T-pinII-R	GCATCCTTTTATTTCATACATTA		
	gat/T-pinII-P	FAM-CCATCTTCTGGATTGGCCAACTTAA-BHQ1		

Table 3 CLUSTAL Omega (1.2.1) multiple sequence alignment with 9 100 % identical amplicon entries A (represented by 98140: KP784699, containing *gat*/T-*pinII*) and 8 100 % identical amplicon entries B (represented by patent WO2010077890, containing *cry3A* with one difference in the amplicon) shows the difference (at nt 24 in bold) between entries A and B

```
Fw-probe-Rev GGACCTCACATCCTGATGTATAA                                CCATCTTCTGGATTGGCCAACTTAA TAATGTATGAAATAAAAGGATGC
Entry A      ......................GAGGATCACATAACTAGCTAGTCAGTTAACCTAGACTTGT.....................T.....................
Entry B      ......................AAGGATCACATAACTAGCTAGTCAGTTAACCTAGACTTGT.....................T.....................
```

Dots represent identity with the primers and probe binding sites

qPCR in closed vials is preferred. A loop-mediated isothermal amplification (LAMP) assay for detection of cry3A is also available [28], but for applicability in a TaqMan PCR screening strategy, this is not suitable. The gene is also applied in experimental lines of GM tobacco, poplar, alfalfa, Norway spruce and rice, and maize Event 5307, MIR604 and MZIR098. The latter one is currently pending for the determination of non-regulated status in the USA [2].

The construct gat/T-*pinII* is a synthetic fusion sequence between the coding sequence of the glyphosate-N-acetyltransferase (*gat*) gene and the terminator from the proteinase inhibitor II (*pinII*) gene. The *gat* gene (CS-gat-BACLI) is derived from *Bacillus licheniformis*. It codes for glyphosate-N-acetyltransferase, which is able to detoxify glyphosate [6]. There are at least two modified genes present in known GMOs: GAT4621 and GAT4601. The *pinII* terminator (T-pinII-SOLTU) is derived from *Solanum tuberosum* [5]. The construct *gat*/T-*pinII* is at least present in canola 61061 and 73496, maize 98140 and soybean 356043.

Table 2 describes the primers and probes that were developed for the two TaqMan PCR detection methods, based on the GenBank entries in the reference.

Specificity

The specificity of the *gat*/T-*pinII* detection method was checked by similarity searches in silico by BLAST analysis against GenBank. BLASTn against the patent database with the *gat*/T-*pinII* amplicon from maize 98140 returned 17 different patents in the first 100 hits, of which 4 belonged to either canola 61061 and 73496, or maize 98140. The other hits were GMOs containing the *gat*/T-*pinII* construct (Table 3). Patent US 8901377 is a patent on GM sunflower. The last eight patents showed a single difference in the amplicon, between the forward primer and the probe. This difference will not hinder a correct amplification of the amplicon.

BLASTn against the nucleotide collection (nr/nt) database with the *gat*/T-*pinII* amplicon from maize 98140 returned two entries (both maize 98140).

The specificity of the *cry3A* detection method was checked in silico by BLAST analysis against GenBank. BLASTn against the patent database with the *cry3A* amplicon from maize MIR604 returned eleven different patents in the first 100 hits, of which three belonged to either MIR604 or Event 5307. The other hits were GMOs containing the *cry3A* gene.

BLASTn against the nucleotide collection (nr/nt) database with the *cry3A* amplicon from maize MIR604 returned three entries. A CLUSTAL Omega multiple sequence alignment returned the alignment shown in Table 4. Amplicons for MIR604 and Event 5307 were included from the patent search.

The synthetic *cry3A* genes GenBank: JN989558.1 [32] and GenBank: M84650.1, and the two potato *cry3A* genes are not likely to be amplified with the designed primer combination. *eCry3.1Ab* (GenBank: GU327680.1) is the *cry3A* gene from Event 5307.

Summarising, the BLASTn analyses against nr/nt and patent databases show that the element-specific method for *cry3A* is specific for *cry3A*, and only returns hits with MIR604, Event 5307 and some other entries that contain *cry3A*. BLASTn analyses against nr/nt and patent databases show that the construct-specific method

Table 4 CLUSTAL Omega (1.2.1) multiple sequence alignment of the *cry3A* amplicon

```
Primers/probe cry3A       CAGCAACATCGAGAACTA                  TGTTCGACTACCTGCACCGC
JN989558.1|cry3A_synt     .........T........CATTAGGAAGCCACACC.......T........TA.GATTCA
DJ437707.1|MIR604         ..................CATCCGCAAGCCCCACC...................ATCCA
HJ236403.1|Event5307      ..................CATCCGCAAGCCCCACC...................ATCCA
GU327680.1|eCry3.1Ab      ..................CATCCGCAAGCCCCACC...................ATCCA
M84650.1|cryIIIA_synt     .TCT..T..T........CATTCGCAAGCCACACC....T............A.AATCCA
X73600.1|potato_cry3A     .TCT.....A..A.....CATTCGTAAACCACATC.A........T......A.AATTCA
X70979.1|potato_cry3A     ......T.....A.....CATTAGGAAACCACATC.C........T..T...A.AATTCA

Primers/probe cry3A                                                 CAGCTTCAACTACTGGAG
JN989558.1|cry3A_synt     GTTCCATACCGGTTTCCAGCCTGGATATTACGGGAACGAT........T......TC
DJ437707.1|MIR604         GTTCCACACGCGTTTCCAGCCCGGCTACTACGGCAACGA..................
HJ236403.1|Event5307      GTTCCACACGCGTTTCCAGCCCGGCTACTACGGCAACGA..................
GU327680.1|eCry3.1Ab      GTTCCACACGCGTTTCCAGCCCGGCTACTACGGCAACGA..................
M84650.1|cryIIIA_synt     ATTCCACACGCGTTTCCAACCAGGATACTACGGTAACGA.TCT............TC
X73600.1|potato_cry3A     GTTTCACACGCGGTTCCAACCAGGATACTATGGAAATGA.TCT........T...TC
X70979.1|potato_cry3A     ATTCCACACAAGGTTTCAACCAGGATACTATGGTAACGA.TC.........T...TC
```

Dots represent identity with the primers and probe binding sites

for *gat*/T-*pinII* is specific for the *gat*/T-*pinII* construct, and only returns hits with canola 61061 (DP-Ø61Ø61-7) and 73496 (DP-Ø73496-4), and maize 98140 (DP-Ø9814Ø-6). No hit with soybean 356043 (DP-356Ø43-5) could be found that confirmed the entry as soybean 356043. Some other entries that contain *gat*/T-*pinII* were found, including a GM sunflower. One group of entries (all from Du Pont de Nemours and Company) from GenBank had one difference in the amplicon, without affecting its amplification.

Experimental results from testing the methods with non-target transgenic events, non-transgenic material and target material showed that all reference materials described in Table 1 were negative for the *cry3A* detection method, except MIR 604 and Event 5307. For the construct-specific method *gat*/T-*pinII*, all reference materials described in Table 1 were negative for *gat*/T-*pinII*, except canola 73496 (50 ng 100 %: Cq = 23), maize 98140 (50 ng 10 %: Cq = 28) and soybean 356043 (50 ng 10 %: Cq = 30). Since the *pinII* terminator originates from *S. tuberosum*, both *S. tuberosum* and *S. lycopersicum* were tested as well. GMOs containing T-*pinII* (maize DAS59122 and 4114) were also included. All were found negative for the *gat*/T-*pinII* construct-specific detection method. All results were in agreement with the expected outcome and confirmed the specificity of the two methods with the reference materials tested.

Efficiency and correlation coefficient

The efficiency and correlation coefficient R^2 were calculated with CFX software using five serial dilutions in duplicate (two reactions per concentration). In Table 5, the average values are given. Efficiency and R^2 are in accordance with ENGL minimum performance requirements that specify an efficiency range between 90 and 110 % and an R^2 higher than or equal to 0.98, in order for a PCR method to be used for quantification. The method performed in a linear quantitative manner in the full range DNA concentrations tested.

Table 5 Average values for efficiency (%) and R^2 of the *cry3A* and *gat*/T-*pinII* detection methods

Method and target DNA	Efficiency (%)	R^2	DNA range tested (ng)
cry3A with MIR604 maize	102.3	0.9900	150–0.24
gat/T-*pinII* with 73496 canola	98.7	0.9964	150–1.20
gat/T-*pinII* with 356043 soybean	101.0	0.9983	50–0.005

Limit of detection

For *gat*/T-*pinII*, a first estimate of the LOD_{10} (10 repeats of different copy levels where the LOD is at the amount of copies that were detected 10/10) was performed, ranging from 40 (10/10), 20 (10/10), 10 (10/10), 5 (7/10) and 1 copies (6/10) per reaction in 10 repetitions (with 73496 canola and 356043 soybean). The estimated LOD_{10} was below 10 copies. The 10 copies dilution was repeated 60 times to obtain an estimate of the $LOD_{95\ \%}$. At 10 copies, 93 % (57/60) of the reactions were positive. Next, 20 copies were repeated 60 times, resulting in 60/60 positives (100 %) for 73496 canola and 356043 soybean. The estimated $LOD_{95\ \%}$ is below 20 copies.

For *cry3A*, the initial LOD estimation was performed with 10 reactions per concentration, ranging from 25 (10/10), 10 (10/10), 5 (9/10), 2.5 (7/10) and 0.5 copies (2/10) (MIR604) per reaction. LOD_{10} was below 10 copies.

The 10 copies level was repeated 60 times to verify the $LOD_{95\,\%}$ at 10 copies, resulting in 59/60 positives (98.3 %). The estimated $LOD_{95\,\%}$ is below 10 copies.

Conclusion

Two new methods were developed to improve the screening approach for known and unknown GMOs containing either the element *cry3A* or the construct *gat*/T-*pinII*. The cry3A detection method was specific for the cry3A element. No false positives were found with the *cry3A* detection method, including those GMOs containing *cry*-related genes, and no false negatives were found since all the GMOs containing *cry3A* were positive. The *gat*/T-*pinII* detection method was specific for the *gat*/T-*pinII* construct. No false positives and no false negatives were found with the *gat*/T-*pinII* detection method, because only the GMOs with *gat4621*/T-*pinII* and *gat4601*/T-*pinII* constructs were positive. Both methods were found satisfactory with regard to their specificity, PCR efficiency, linearity and sensitivity in the light of criteria for such methods as have been established by the European Network of GMO Laboratories. The applicability and practicability is expected to be similar as for other TaqMan screening methods. After full validation, the methods can be used in GMO element screening approaches to facilitate the detection and identification of maize 5307, MIR604 and 98140, soybean 356043, canola 73496 and potentially present currently unknown GMOs containing *cry3A* or *gat*/T-*pinII*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with animals performed by any of the authors.

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