Eur Food Res Technol (2012) 234:13–23
DOI 10.1007/s00217-011-1605-7

ORIGINAL PAPER

Four new SYBR®Green qPCR screening methods for the detection of Roundup Ready®, LibertyLink®, and CryIAb traits in genetically modified products

Elodie Barbau-Piednoir · Antoon Lievens · Els Vandermassen · Etondoh-Guillaume Mbongolo-Mbella · Amaya Leunda-Casi · Nancy Roosens · Myriam Sneyers · Marc Van den Bulcke

Received: 31 May 2011 / Revised: 28 September 2011 / Accepted: 8 October 2011 / Published online: 29 October 2011

Abstract SYBR®Green qPCR methods for the detection of the Roundup Ready® "CP4-EPSPS", LibertyLink® "PAT" and "BAR," and the *Bacillus thuringiensis* "CryIAb" traits as present in genetically modified organisms (GMO) were developed. Their specificity, sensitivity, and PCR method efficiency were determined. All methods are specific and generate amplicons of 108, 73, 109, and 69 bp, respectively, for "CP4-EPSPS," "CryIAb," "PAT," and "BAR" targets. They perform well at low target levels and can detect down to 5 copies of their respective targets extracted from a sample. The PCR efficiency of the methods ranges between 91 and 109%. Due to their trait-specific nature, these methods allow an efficient screening between the different GMO. In this way, the number of possible GMO candidates present in a sample can be reduced what results in lower global costs due to limiting of further the number of analytical identification steps. The application of these methods in CoSYPS GMO analysis is illustrated using two GEMMA proficiency test samples and a reference material from the GM rapeseed event RF3. This set of SYBR®Green qPCR trait-specific methods represents a very interesting novel set of GMO analysis methods allowing cost-effective identification of GM materials in products.

Keywords Quantitative real-time PCR · Feed/food analysis · GMO detection · Herbicide resistance · Insect resistance

Elodie Barbau-Piednoir and Antoon Lievens have equally contributed to this manuscript and should be considered both as first author.

E. Barbau-Piednoir · A. Lievens · E. Vandermassen · E.-G. Mbongolo-Mbella · A. Leunda-Casi · N. Roosens (✉) · M. Sneyers
Scientific Institute of Public Health,
Rue J. Wytsmanstraat 14, 1050 Brussels, Belgium
e-mail: nancy.roosens@wiv-isp.be

M. Van den Bulcke
Institute of Health and Consumer Protection,
Via Enrico Fermi 2749, 21027 Ispra, VA, Italy

Introduction

In 2010, 148 million hectares GM crops were cultivated globally with a total of more than 1 billion hectares of transgenic crops cultivated over the last 15 years [1]. Herein, transgenic herbicide tolerant (HT) and insect-resistant crops are the most important commercial applications of genetic modification in plants. These so-called first-generation HT glyphosate and glufosinate traits and the CryIAb insect-resistance *B. thuringiensis* (Bt) toxins are today still the most frequently engineered biotechnology traits (for a comprehensive overview, see http://www.gmo-compass.org).

The major commercial transgenic HT crops to date incorporate two different classes of genes: the bacterial phosphinotricin-*N*-acetyltransferases from *Streptomyces viridochromogenes* (*pat*) and from *Streptomyces hygroscopicus* (*bar*) [2] and the 5-enolpyruvylshikimate-3-phosphate synthase (*epsps*) from *Agrobacterium tumefasciens* strain CP4 or from plant origin (in casu petunia) [3, 4]. Both classes of HT genes have been engineered into commercially grown GM crops [5]. The CryIAb protein of *B. thuringiensis* represents still the key Bt toxin in GM-based insect control strategies [6]. Different variants of the *cryIAb* gene (e.g., the *cryIAb/Ac*) have been introduced into maize, cotton, and rice aiming at controlling different insect pests [5]. Various new HT and Bt-based insect control systems

are being developed: new herbicide tolerances such as acetolactate synthase inhibiting herbicides and novel glyphosate tolerances [5] and new insect-resistance traits such as novel Bt toxins like CryF in TC 1507 maize or Cry3 toxins in MON 863 maize [6]. The first-generation traits will however remain prominent in commercial crops, and all their derived food and feed products for a very long period.

As the total amount and number of commercialized GM crops increases, the development of sensitive, reliable but also cost-effective, and flexible screening strategies for GMO presence in products will become more and more important. A number of novel approaches have been developed applying the "Polymerase Chain Reaction" (PCR) technology [7]. Several multiplex qPCR formats are available reducing the number of analyses and facilitating high throughput but requiring multi-channel detection devices and often including costly detection probes for at least some of the targets [8–10]. In other cases, the applied chemistries are quite complex or are to be combined with other technologies that are less appropriate for routine applications [11–16]. Several of these approaches involve the use of PCR with multiple targets and consecutive detection and identification of the amplification products using microarrays [11–13]. Apart from requiring additional costly array analysis equipment, these approaches are often prone to variable quality of the array chips making them less suitable for routine or enforcement purposes [17]. Raymond et al. [14] and Nadal et al. [15] combined PCR with capillary electrophoresis in a very effective way, but requiring costly investment in additional equipment. Finally, an effective novel approach based on so-called padlock-probes [18] was developed, which could in the future represent an elegant solution to cover the broad diversity of GMO [16]. However, this technology is less sensitive and per se requires time-consuming optimization [19]. All these approaches require still broader testing by other laboratories to support their common applicability and robustness in GMO detection and may in se impose some difficulties in establishing appropriate validation schemes of these methods according to ISO or Codex Alimentarius standards.

Contrary to the above "high-tech" strategies, a number of PCR-based approaches have also been developed essentially applying the so-called combinatory or matrix-approach [20]. In such GMO screening approach, a limited set of simplex or multiplex PCR methods targeting various types of elements (endogene markers, construct-specific markers, GM traits…) is selected in such a way that multiple GMO can be detected within a single analytical run. Careful selection of the markers allows to develop an approach wherein not only most GMO are detected but also discriminated [9, 20, 21]. Combined with an informatics decision support tool, such GMO screening represents a very useful approach in managing the experimental analysis of samples for regulatory or enforcement purposes [20, 21].

CoSYPS, standing for "Combinatory SYBR®Green qPCR Screening" [20], was recently developed as a simplex qPCR GMO screening approach combining PCR methods detecting species/taxon markers, so-called generic recombinant markers as the 35S promoter of Cauliflower Mosaic Virus (CaMV) and the nopaline synthase terminator of *A. tumefasciens* [22, 23]. Here, we want to extend the system with GM trait markers such as the HT and Bt genes. All methods were developed to function under the same reaction conditions and apply SYBR®Green PCR allowing for post-PCR melting curve analysis to verify the nature of the amplification products [20]. Here, the performance and use of the SYBR®Green qPCR methods targeting the major herbicide tolerance GM traits (CP4-EPSPS for Roundup Ready® and PAT and BAR for LibertyLink®) and insect control GM trait (CryIAb) are presented. Their specificity, sensitivity, dynamic range, and PCR efficiencies are shown. The use of these GM trait PCR methods in combination with species-specific and generic recombinant SYBR®Green qPCR methods in CoSYPS is illustrated using samples from two proficiency tests and one from the Quality Control of a GM reference material. The advantage of including trait-specific methods into a GMO screening approach is discussed.

Materials and methods

Overview of test materials

Test materials were obtained from leaf tissue of in-house grown plants or were obtained from the Institute for Reference Materials and Measurements (IRMM), the American Oil Chemists' Society (AOCS), the Biotech Companies (Bayer CropScience, Monsanto, Syngenta), or from FAPAS proficiency tests (Table 2).

Standard growth conditions for all plants were at 16 h/8 h day/night regime at 25 °C with chamber humidity at 80% in a Schnijder Scientific Plant growth chamber (S1084).

All Sybricon plasmids (see below) were constructed in-house, isolated using Qiagen mini/midi plasmid preparations and verified by DNA sequence analysis [23].

Bioinformatics analysis and primer design

Bioinformatics analysis of sequence information was performed applying the wEMBOSS software package [24, 25]. Relevant DNA sequence data were retrieved from public databases, patents, and scientific literature and from

in-house DNA sequence analyses. A uniform primer design approach is applied in the development of primer pairs for the respective targets. The first step consists of identifying regions with high DNA sequence homology within the respective targets from the different GMO. Next, primer pairs (between 6 and 10 pairs) preferentially comprised in a common region are designed using the "Primer Express" program from Applied Biosystems (version 3.0). A bioinformatics specificity analysis for each primer is performed by probing each primer against several public and GMO DNA sequence databases. Any primer showing homology with non-relevant DNA sequences is discarded from further analysis. All primer pairs selected through this assessment were then evaluated further experimentally.

DNA target sequences and oligonucleotides

Primers applied in this study are listed in Table 1. Relevant physical parameters on the respective GM targets/amplicons including the primer sequences and the size of the expected PCR products are indicated.

DNA extraction, DNA quantification, and PCR analysis

DNA extraction of all the different materials (seeds, powders, leaves) was performed as described in [23]. In short, 250 mg to 1 g homogenized material was extracted by a CTAB DNA extraction protocol (except for cotton materials for which a Qiagen kit was used). The final genomic DNA (gDNA) pellet was resuspended in 200 µL of DNase- and RNase-free water (ACROS) and stored at −20 °C. The extracted gDNA is quantified using the PicoGreen dsDNA quantitation kit (Invitrogen) on a VersaFluor™ Fluorimeter (Biorad). When considered necessary (e.g., unexpected low/high DNA yield, PCR inhibition…), the concerned DNA extracts were tested for purity either spectrophotometrically (260/280 nm ratio) or by agarose gel electrophoresis. Any non-conform extracts (degraded DNA, high protein content…) were either further purified (e.g., by column separation) or discarded.

Qualitative PCR was carried out on a Biorad iCycler using Amplitaq Gold (Applied Biosystems) and supplied buffers. Real-time qPCR was carried out on ABI 7300 PCR System (Applied Biosystems) using the SYBR®Green PCR mastermix (Diagenode, Liege-Belgium).

For both types of PCR, a standard 25 µl reaction volume was applied containing 5 µl of template (10 ng/µl gDNA or 40 copies plasmid DNA/µl), 1× SYBR®Green PCR Mastermix, and 250 nM of each primer). The thermal program consisted of a single cycle of DNA polymerase activation for 10 min at 95 °C, followed by 40 amplification cycles of 15 s at 95 °C (denaturing step) and 1 min at 60 °C (annealing-extension step). After completion of the run, a melting point analysis was performed by stepwise temperature increase (±1.75 °C/min) from 60 to 95 °C.

A fluorescent reporter signal was measured against an internal reference dye (ROX) signal to normalize for non-PCR-related fluorescence fluctuations between samples. The threshold cycle (C_t) for each sample was calculated in automatic mode according to the manufacturer's specifications. If considered necessary (e.g., due to highly diverging baseline values close to the exponential phase), the threshold and baseline were adjusted manually.

Cloning of the SYBR®Green amplification products obtained with the qPCR methods for CP4-EPSPS, CryIAb, PAT, and BAR

In order to confirm the correct nature of the amplification products obtained with each of the qPCR methods, DNA sequence analysis was performed on the amplicons obtained with representative GM events. For this, all amplicons were cloned into a pUC18 vector and the insert DNA sequence was determined applying dideoxy sequence

Table 1 GM trait-specific targets, their corresponding primer sequences, and the amplicon size, the EMBL reference, and the Sybricon BCCM reference

SYBR®Green qPCR target name	Primer name	Primer sequence	Amplicon size (bp)	EMBL Reference	Sybricon (BCCM)
CP4-EPSPS	CP4 synthetic F	GCATGCTTCACGGTGCAA	108	FN610849	LMBP 5663 (CP4-EPSPS I)
	CP4 Synthetic R	TGAAGGACCGGTGGGAGAT		FN610850	LMBP 5664 (CP4-EPSPS II)
	CP4 synth Rbis	TGAAGGACCTGTGGGAGAT			
CryIAb	CryIAb_Bt.Cott_Fwd	ACCGGTTACACTCCCATCGA	73	FN650616	LMBP 5454 (Bt11)
	CryIAb_Bt.Cott_Rev	CAGCACCTGGCACGAACTC		FN610851	LMBP 5693 (Mon 810)
PAT	Pat–Pat Fwd	CCGCGGTTTGTGATATCGTT	109	FN650617	LMBP 5455
	Pat–Pat Rev	TCTTGCAACCTCTCTAGATCATCAA			
BAR	Pat–Bar Fwd	CGTCAACCACTACATCGAGACAA	69	FN650618	LMBP 5457
	Pat–Bar Rev	GTCCACTCCTGCGGTTCCT			

analysis. These plasmids were designated as "Sybricons" (= **SYBR**Green ampl**icons**). Each of the obtained sequences was verified for homology by Blast sequence comparison against public DNA sequence databases (NBCI, EMBL). Only DNA sequences matching the expected traits at stringent homology could be retrieved (data not shown). All constructed plasmids have been registered under "Safe Deposit" at the "Belgian Coordinated Collections of Micro-organisms" in the "Plasmid Collection" (BCCM™/LMBP, Gent-Belgium) (Table 1) and were certified for their authenticity. The amplicon sequences were deposited at the EMBL sequence database (Accession number listed in Table 1).

Specificity of the SYBR®Green qPCR methods

The criteria for specificity set by Barbau-Piednoir et al. [23] for SYBR®Green qPCR methods were applied in this study. A recorded signal upon SYBR®Green qPCR analysis is considered positive when the four criteria are met: (1°) an (exponential) amplification at a similar level to the endogenous control marker (ΔCt (Endogene–GM marker) < 3) is obtained with 100% GM template DNA comprising the target sequence(s), while negative controls (the so-called No Template Controls (NTC) and the gDNA from wild-type crop plants) do not yield such amplification; (2°) with all target-containing template DNA, the obtained PCR product(s) represents a single peak upon melting analysis with a unique T_m value corresponding to the nominal T_m value obtained with the respective Sybricon as template DNA (with an acceptable SD ± 1 °C), while no specific peaks are detectable in the negative controls, and (3°) a single band on agarose gel analysis with (4°) an amplicon length of the predicted size (SD ± 10 bp).

Primer specificity was assessed by testing amplifications of positive and negative controls (50 ng of DNA template). "No Template" controls (NTC) were included in the assay to assess possible interference by primer dimer formation or due to a-specific background fluorescence.

Sensitivity of the SYBR®Green qPCR methods

To test the sensitivity of the real-time PCR methods in terms of (approximate) target copy number, serial dilutions of a well-characterized positive control were analyzed [23]. The LOD was set as outlined in the former AFNOR standard XP V 03-020-2 (2003) [26]. The LOD represents the required target copy number in a PCR reaction well to obtain a reproducible (= six-times repeatable) amplification. From these analyses, also the PCR efficiency (E) for each of the methods can be calculated according to [27]:

$$E = \left(10^{-1/\text{slope}}\right) - 1$$

The PCR efficiency (E) could be expressed in percentage:

$$E = (10^{-1/\text{slope}} - 1) \times 100$$

Agarose gel analysis

Agarose gel electrophoresis was performed on 3% Ready-Agarose 96 Plus Gel (Biorad) supplied with Ethidium Bromide. Standard electrophoresis running conditions were applied (as running buffer 89 mM TRIS–borate—2 mM EDTA was used; current was set at 100 v).

DNA sequencing

DNA sequencing analysis was carried out on a CEQ8000 Genetic Analysis System (Beckman Coulter) with the Genomelab Dye Terminator Cycle Sequencing (DTCS) kit (Beckman Coulter).

CoSYPS screening analysis

In short, two subsamples (250 mg) of a homogenized powder were extracted using the formerly described CTAB protocol [23]. From each subsample, a single analysis of 50 ng of extracted DNA, as measured by Picogreen, is performed for each target. The CoSYPS analysis was performed according to [20] but documenting explicitly the use of the four methods described in this study. The analysis for the presence of all markers in a sample was performed simultaneously on the same run. The obtained T_m and C_t values obtained from each target in each run are then independently scored applying a priori established acceptance ranges for both the T_m and the C_t value of each method as described in [22, 23] and in this study. The interpretation of the results is done according to the decision criteria described in [20].

Results and discussion

Bioinformatics analysis and development of PCR primers for Roundup Ready (*CP4-EPSPS)*, LibertyLink® (*PAT and BAR*), and the Bt CryIAb traits

The LibertyLink® (LL) traits (PAT and BAR phosphinothricin herbicide tolerance), the Roundup Ready® (RR) traits (CP4-EPSPS-based glyphosate herbicide tolerance), and the CryIAb Bt traits are present in a large number of GMO [7] (Table 2). Aiming at developing primers to highly conserved regions, a bioinformatics analysis (Align

program) was performed on a number of available DNA sequences for the respective traits.

For the LL traits, no regions of homology could be defined and specific primers were developed for the PAT and the BAR gene separately (Genebank AY346130.1; DQ156557.1). For the CP4-EPSPS genes existing to date, two types of DNA sequences can be distinguished: a sequence-type I EPSPS found in soybean, maize, and cotton GM events (indicated CP4-EPSPS I) and a sequence-type II EPSPS found in GM rapeseed and sugar beet (indicated CP4-EPSPS II) [28, 29]. A duplex qPCR method was developed comprising a small highly homologous region close to the NH_2-terminus of the EPSPS mature protein (the forward primer matching both sequence types of CP4-EPSPS) and two reverse primers specific to each of the respective chloroplast transit peptide sequences of the EPSPS I and II (Genebank AY125353.1; own unpublished data). For the *cryIAb* gene (Genebank AF465640; EF094884.1), a single primer pair was developed allowing the detection of the major CryIAb events on the market (Bt11 and MON 810 GM maize, MON531 GM cotton). For both the CP4-EPSPS and the CryIAb targets, the respective different types have been specified in Table 2. The DNA sequence of the selected primers are listed in Table 1.

Development of Sybricon control plasmids for CP4-EPSPS, CryIAb, PAT, and BAR targets

To minimize bias due to the genetic background in determining the nominal value of the melting temperature for each target, so-called Sybricon plasmids containing the respective amplification products of the respective CP4-EPSPS, CryIAb, PAT, and BAR amplicons were constructed. All constructed plasmids have been registered under "Safe Deposit" at the "Belgian Coordinated Collections of Micro-organisms" in the "Plasmid Collection" (BCCM™/LMBP, Gent-Belgium) (Table 1) and were certified for their authenticity. All DNA sequences of the respective Sybricon inserts have been deposited at EMBL (for references, see Table 1). T_m values for the different amplicons are all distinct from each other: T_m values at 85 and 80.5 °C for the CP4-EPSPS I and II, respectively, at 77.2 and 78.9 °C for CryIAb from Bt11 and MON 810 maize, respectively, at 75.3 °C for PAT and at 78.4 °C for BAR. It is generally accepted that the T_m obtained with SYBR®Green qPCR can vary between 0.5 and 1 °C for the same amplicon [30, 31]. Therefore, to cover slight deviations in the T_m value between reference materials and samples due to analyte impurities, a standard deviation (SD) of ± 1 °C on the nominal T_m value will be applied, as the acceptance range, in further analysis. In the case of the CryIAb targets, the T_m values of the amplicons are overlapping, and in this case, the acceptance range of a positive signal is set at a T_m = 78.1 ± 2 °C.

Specificity assessment of the SYBR®Green qPCR Methods for CP4-EPSPS, CryIAb, PAT, and BAR presence in GM events

The specificity of the respective qPCR methods was demonstrated on both wild type and genetically modified control samples from different species (including *Zea mays, Glycine max, Gossypium hirsutum var Coker, Brassica napus, Oryza sativa var japonica, Beta vulgaris,* and *Solanum tuberosum)* (Table 2). The results of these analyses demonstrated that each method recognized the correct targets in the respective GMO and that wild-type crop material did not give any aberrant signals (summarized in Table 2). Melting curve analysis of the produced amplicons yielded clearly defined peaks for all qPCR products in the positive controls and no primer dimer formation could be detected (data not shown). The amplification products obtained by qPCR using the respective trait-specific methods were analyzed by 3% agarose gel electrophoresis. All qPCR methods produce a single DNA product of expected size in the positive controls (data not shown). Using genomic DNA, the T_m values obtained for the respective amplicons were consistently slightly higher than the $T_m$values obtained with pure plasmid DNA due to the lower initial target copies in the plasmid samples (copy number at about 200 in the case of plasmids vs. >1,000 in case of gDNA).

Identification of GMO by melting curve analysis

Melting curve analysis yielded clearly defined qPCR products in all tested GMO. In case of the BAR and PAT positive controls, a clearly distinct T_m value was observed for both traits (T_m (BAR) at 78.4 °C; T_m (PAT) at 75.3 °C) (see Table 2). These results confirm the low homology at DNA level between both targets as deduced from DNA sequence data. With the CP4-EPSPS SYBR®Green qPCR method, a distinct T_m value was observed with different GMO. In the case of GTS 40-3-2 soybean and NK 603 and MON88017 maize, melting analysis of the amplicons yielded a T_m value = 85 °C. In case of MON 89099 soybean, GT73 oilseed rape, H7 sugar beet, and MON 1445 cotton, a T_m value = 80.5 °C was observed. Again, these results are in line with reported DNA sequences and confirm the very distinct open reading frames applied in the different classes of Roundup Ready® GMOs. The plant EPSPS as present in GA21 maize was not recognized by this method.

For the CryIAb SYBR®Green qPCR method, two distinct amplicon types were generated: one type with a T_m value at 77.2 °C corresponding to the Bt11 type CryIAb and a second type with T_m value at 78.9 °C corresponding

Table 2 Specificity assessment of the four SYBR®Green GM trait qPCR methods: "CP4-EPSPS" and "CryIAb," "PAT," and "BAR." Specificity assessment of the four GM trait SYBR®Green qPCR methods using gDNA from relevant GM events and wild-type gDNA as template, using "Sybricon" plasmids as reference for the T_m value

Sample name	Species	GM % (m/m)	Origin	CP4-EPSPS			CryIAb			PAT			BAR		
				Expected presence	C_t	T_m	Expected presence	C_t	T_m	Expected presence	C_t	T_m	Expected presence	C_t	T_m
Sybricon004 (CryIab–Bt11)	NA	NA	This study	No			Yes	+	+						
Sybricon005 (PAT)	NA	NA	This study	No			No			Yes	+	+			
Sybricon007 (BAR)	NA	NA	This study	No			No			No			Yes	+	+
Sybricon018 (EPSPS I)	NA	NA	This study	Yes	+	+	No			No			No		
Sybricon019 (EPSPS II)	NA	NA	This study	Yes	+	+	No			No			No		
Sybricon020 (CryIAb–MON810)	NA	NA	This study	No			Yes	+	+	No			No		
Wt Soybean	Soybean	0	Monsanto	No	−	−	No	−	−	No	−	−	No	−	−
A 2704-12	Soybean	100	AOCS	No			No			Yes	+	+	No		
GTS40-3-2	Soybean	100	Monsanto	Yes[I]	+	+	No			No			No		
MON 89788	Soybean	100	AOCS	Yes[II]	+	+	No			No			No		
Wt Maize	Maize	0	IRMM	No	−	−	No	−	−	No	−	−	No	−	−
MON 88017	Maize	100	AOCS	Yes[I]	+	+	No			No			No		
MON 89034	Maize	100	AOCS	No			Yes[Bt11]	+	+	No			No		
Bt11	Maize	5	IRMM	No			Yes[Bt11]	+	+	Yes	+	+	No		
Bt176	Maize	5	IRMM	No			Yes[Bt11]	+	+	No			Yes	+	+
GA21	Maize	4.29	IRMM	Yes*	−	−	No			No			No		
DAS59122	Maize	9.87	IRMM	No			No			Yes	+	+	No		
MIR 604	Maize	100	AOCS	No			No			No			No		
MON 810	Maize	5	IRMM	No			Yes[Mon]	+	+	No			No		
MON 863	Maize	9.86	IRMM	No			No			No			No		
NK 603	Maize	4.91	IRMM	Yes[I]	+	+	No			No			No		
T25	Maize	100	AOCS	No			No			Yes	+	+	No		
TC 1507	Maize	9.86	IRMM	No			No			Yes	+	+	No		
Wt Oilseed Rape	Oilseed Rape	0	AOCS	No	−	−	No	−	−	No	−	−	No	−	−
GT73	Oilseed Rape	100	AOCS	Yes[II]	+	+	No			No			No		
RF1*	Oilseed Rape	100	Bayer	No			No			No			Yes	+	+
RF2*	Oilseed Rape	100	Bayer	No			No			No			Yes	+	+
RF3	Oilseed Rape	100	AOCS	No			No			No			Yes	+	+
T45*	Oilseed Rape	100	AOCS	No			No			Yes	+	+			
MS1*	Oilseed Rape	100	Bayer	No			No			No			Yes	+	+
MS8	Oilseed Rape	100	AOCS	No			No			No			Yes	+	+
Topas 19/2*	Oilseed Rape	100	Bayer	No			No			Yes	+	+	No		
Wt Rice	Rice	0	AOCS	No	−	−	No	−	−	No	−	−	No	−	−
LL601	Rice	100	JRC	No			No			No			Yes	+	+
Wt Cotton	Cotton	0	AOCS	No	−	−	No	−	−	No	−	−	No	−	−
MON 1445	Cotton	100	AOCS	Yes[II]	+	+	No			No			No		
MON 531	Cotton	100	AOCS	No			Yes[Bt11]	+	+	No			No		
MON 15985	Cotton	100	AOCS	No			Yes[Bt11]	+	+	No			No		
LL25	Cotton	100	AOCS	No			No			No			Yes	+	+
Wt Sugar beet	Sugar beet	0	IRMM	No	−	−	No	−	−	No	−	−	No	−	−
H7-1	Sugar beet	100	IRMM	Yes[II]	+	+	No			No			No		
Wt Potato	Potato	0	AOCS	No	−	−	No	−	−	No	−	−	No	−	−
EH92-527-1	Potato	100	AOCS	No			No			No			No		

Table 2 continued

Sample name	Species	GM % (m/m)	Origin	CP4-EPSPS			CryIAb			PAT			BAR		
				Expected presence	C_t	T_m	Expected presence	C_t	T_m	Expected presence	C_t	T_m	Expected presence	C_t	T_m
NTC	NA	NA	NA	No	–	–	No	–	–	No	–	–	No	–	–

Annotations: For the C_t values, a "+" means (exponential) amplification and a C_t value above the LOD, "−" means no amplification or amplification below the LOD; For the T_m values, a "+" means that the observed T_m value equals the T_m of the complementary Sybricon ±1 °C, while a "−" means that the observed T_m value differs more than ±1 °C from the T_m of the complementary Sybricon; "*" means "GM event only tolerated below 0.5% in the EU." Yes^{I}: CP4-EPSPS class I; Yes^{II}: CP4-EPSPS class II; Yes*: EPSPS not related to the CP4-EPSPS; Yes^{Mon}: CryIAb (MON 810); Yes^{Bt11}: CryIAb (Bt11); NA: not applicable; Wt: Wild type

to the MON 810 type CryIAb. The CryIAb amplicons obtained from maize GMO Bt176 and Bt10 had a similar T_m value as GMO Bt11. The cotton GMO MON 531 and MON 15,985 generated CryIAb amplicons with a similar T_m value as GM Bt11 maize.

Efficient detection of CP4-EPSPS, CryIAb, PAT, and BAR targets in GMO at low copy numbers

The LOD for the four SYBR®Green qPCR methods was estimated by serial dilution using leaf DNA from GTS 40-3-2 soybean, MS8 rapeseed, or Bt11 maize as representative materials that contained each of the traits at least as a single copy (Table 3). All methods performed adequately within a range from 1 to 400 copies [32], and the LOD was estimated lower than 5 copies of the respective targets (two, four, two, and a single theoretical copy for the PAT, BAR, CP4-EPSPS, and CryIAb targets, respectively). It should be stressed that these values represent only an estimated copy number. In the dilution series of the CP4-EPSPS analysis, at the 0.2 copy dilution, no positive signals could be observed anymore (data not shown). The PCR efficiencies of these 4 SYBR®Green qPCR methods are estimated at about 91, 92, 96, and 109% for the PAT, BAR, CP4-EPSPS, and CryIAb qPCR methods, respectively (Table 3). All methods thus meet the acceptance criteria set by the European Network of GMO Laboratories (ENGL) (a LOD below 20 target copies with a PCR efficiency between 89.6 and 110.2%) [27].

Application of the CP4-EPSPS, CryIAb, PAT, and BAR SYBR®Green qPCR methods in CoSYPS GMO analysis

This set of trait-specific SYBR®Green qPCR methods completes the GMO analysis methods as described and applied in combinatory SYBR®Green qPCR screening (CoSYPS) [20]. In short, CoSYPS for GMO detection applies 6 SYBR®Green qPCR methods detecting the four major GM traits, the CryIAb, the CP4-EPSPS, the PAT, and the BAR (this paper), two generic recombinant markers, the CaMV P-35S promoter and the *Agrobacterium* T-Nos terminator targets, as the two most frequently present targets in GMO to date [23]. These GM markers are combined with the RBCl plant marker and a lectin, an alcohol dehydrogenase, and a cruciferine species marker for the detection of materials derived from soybean, maize, and rapeseed, respectively [22]. CoSYPS allows to detect the presence of most EU-authorized GMO [7].

To illustrate the CoSYPS approach, three examples were chosen: two of them represent the GMO screening results of GEMMA proficiency tests (PT), the third one is an in-house verification of a reference material (Table 4). Both GEMMA proficiency tests were flours containing, respectively, the GM soybean event GTS 40-3-2 or the GM maize event Bt11.

In the first GEMMA PT, CoSYPS screening gave positive signals for the generic plant marker and for the following GM crop markers: lec (soy), P-35S, T-Nos, and the CP4-EPSPS trait. Then, the possible GMOs were determined by Prime Number Modulation analysis according to Van den Bulcke et al. [20]. These evaluation concluded that GTS 40-3-2 and MON 89788 soybean, Bt63 rice, MON 1445 and MON 531 and MON 15985 cotton and H7-1 sugar beet could be present in the sample (7/27 GMO; data not shown). The presence of these GMO was then verified applying the event-specific Taqman® qPCR methods validated by the EURL-GMFF (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm). This identification analysis showed that only GM soybean GTS 40-3-2 was present (data not shown).

A similar analysis was performed for a second GEMMA PT containing GM maize Bt11. For this PT, CoSYPS gave positive signals for the generic plant marker, the lec (soy), the adh (maize), the P-35S, the T-Nos, the CryIAb-Bt11, and the PAT marker. Prime Number Modulation analysis identified the following GMO as possibly present in the sample: A2704-12 soybean, T25 and GA21 and Bt11 and Bt10 and MON 810 and TC 1507 and DAS 59122 and MON 863 maize and Bt63 rice (10/27 GMO; data not

Table 3 Sensitivity assessment of the four SYBR®Green RT-qPCR methods: "PAT," BAR," "CP4-EPSPS," and "CryIAb"

Target	Slope	PCR Efficiency (%)	pg gDNA/assay*	0.5	0.125	0.063	0.031	0.016	0.008	0.004	0.002	0.001	NTC
PAT	3.5687	91	Theoretical copy number/assay	100	25	12.5	6.25	3.125	*1.56*	0.78	0.39	0.19	0
			Signal ratio (positive/total number of reaction)	6/6	6/6	6/6	6/6	6/6	*6/6*	5/6	3/6	nt	0/6
			C_t mean of positive signals	26.02	28.13	29.39	30.14	31.45	*33.13*	33.22	33.46	nt	NA
			C_t SD	0.72	0.26	0.39	0.61	0.73	*1.09*	0.88	0.44	nt	NA
BAR	3.5422	92	Theoretical copy number/assay	100	25	12.5	6.25	*3.12*	1.56	0.78	0.39	0.19	0
			Signal ratio (positive/total number of reaction)	6/6	6/6	6/6	6/6	*6/6*	4/6	2/6	4/6	nt	0/6
			C_t mean of positive signals	27.30	29.35	30.19	31.50	*32.89*	33.47	34.81	35.64	nt	NA
			C_t SD	0.16	0.15	0.49	0.63	*0.65*	1.19	0.82	0.26	nt	NA
CP4 EPSPS	3.4345	96	Theoretical copy number/assay	400	100	50	25	12.5	6.25	3.125	*1.56*	0.78	0
			Signal ratio (positive/total number of reaction)	nt	6/6	6/6	6/6	6/6	6/6	6/6	*6/6*	3/6	0/6
			C_t mean of positive signals	nt	27.48	28.67	29.61	30.58	31.63	32.90	*34.03*	34.69	NA
			C_t SD	nt	0.14	0.19	0.30	0.91	0.49	0.59	1.05	0.87	NA
CryIAb	3.1272	109	Theoretical copy number/assay	100	25	12.5	6.25	3.125	1.56	*0.78*	0.39	0.19	0
			Signal ratio (positive/total number of reaction)	6/6	6/6	6/6	6/6	6/6	6/6	*6/6*	5/6	nt	0/6
			C_t mean of positive signals	25.64	27.73	28.70	29.69	30.24	31.28	*32.71*	32.98	nt	NA
			C_t SD	0.29	0.36	0.42	0.55	0.50	0.34	*0.51*	0.81	nt	NA

Slope and PCR efficiency for the respective performed analysis, average C_t values from a six repeats sensitivity assessment on gDNA template. The LOD_6 is indicated in italics. nt: not tested; NA: not applicable; assay*: gDNA template in the respective assays were from Bt11 maize (PAT and CryIAb), MS8 rapeseed (BAR), GTS40-3-2 soybean (CP4-EPSPS)

Table 4 CoSYPS application of the trait-specific SYBR®Green qPCR methods: detection of GTS40-3-2 soybean and Bt11 maize in two different proficiency tests and a Quality Check of RF3 rapeseed reference material

Trait	RBCl		Lec		Adh		Cru		P-35S		T-Nos		EPSPS I and II		CryIAb		PAT		BAR	
Class	Plant		Soy		Maize		Rape		GM		GM		GM		GM		GM		GM	
	T_m	C_t	T_m	C_t	T_m	C_t	T_m	C_t	T_m	C_t	T_m	C_t	T_m	C_t	T_m	C_t	T_m	C_t	T_m	C_t
Proficiency test 1 (1.58% GTS40-3-2 soybean)																				
RUN 1	**75.9**	**29.5**	**79.3**	**19.8**	69.4	40.0	69.1	40.0	**75.4**	**28.4**	**71.1**	**26.1**	**84.1**	**25.0**	70.9	40.0	72.1	40.0	72.1	40.0
RUN 2	**75.4**	**27.1**	**79.5**	**19.8**	69.8	40.0	69.1	40.0	**75.0**	**29.0**	**71.4**	**26.0**	**84.4**	**24.9**	**77.6**	**33.7**	72.1	40.0	72.1	40.0
Screening evaluation	+	+	+	**+**	−	−	−	−	+	**+**	+	**+**	+	**+**	−	−	−	−	−	−
Proficiency test 2 (0.95% Bt11 maize)																				
RUN 1	**76.0**	**28.0**	**79.7**	**31.6**	**76.0**	**18.5**	69.4	40.0	**76.0**	**29.6**	**72.0**	**28.3**	73.9	40.0	**78.7**	**26.4**	**76.9**	**27.9**	79.0	40.0
RUN 2	**76.0**	**27.9**	**79.7**	**30.1**	**75.6**	**18.5**	69.4	40.0	**76.0**	**29.5**	**72.0**	**28.2**	72.4	40.0	**78.7**	**26.4**	**76.9**	**28.2**	78.4	40.0
Screening evaluation	+	+	**+**	**+**	**+**	**+**	−	−	**+**	**+**	**+**	**+**	−	−	**+**	**+**	**+**	**+**	−	−
Reference material verification (100% RF3 rapeseed)																				
RUN 1	**76.1**	**14.1**	76.4	40.0	**76.4**	40.0	**79.4**	**20.9**	**76.1**	40.0	**71.8**	**18.7**	75.8	39.0	74.9	40.0	73.7	40.0	**79.8**	**19.8**
RUN 2	**75.8**	**15.0**	77.0	40.0	**76.4**	39.0	**79.4**	**20.9**	**75.8**	**36.0**	**71.8**	**18.8**	76.4	40.0	74.6	40.0	73.7	40.0	**79.5**	**19.9**
Screening evaluation	+	+	−	−	**+**	−	**+**	**+**	**+**	−	**+**	**+**	−	−	−	−	−	−	**+**	**+**

Bold values indicate specific amplification

+: present above LOD; −: below LOD

Trait markers, lec: lectin from soybean; adh: alcohol dehydrogenase from maize; cru: cruciferin from rapeseeds; Class: the hierarchal status of the respective markers; plant: general recognition of plant material; soy, maize, rape: the respective plant; GM: present in genetically modified materials

shown). A similar verification analysis as described above correctly showed that only Bt11 maize was present in the sample.

Finally, an example of the Quality Control verification at our Institute is shown testing a 100% GM rapeseed RF3 reference material. In this case, CoSYPS gave positive signals only for the generic plant marker, the cru (rapeseed), the T-Nos and the BAR marker. Prime Number Modulation analysis identified only the so-called Seed-Link™ rapeseed family as possible candidates (MS1, RF1, RF2, MS8, RF3) (5/27 GMO; data not shown). A verification analysis as described above correctly showed that in the reference material sample only RF3 GM rapeseed was present.

In all three cases, CoSYPS indicated correctly the possible presence of the GM event present in the sample out of the GMO universe defined at June 2010. The CoSYPS (10 screening tests plus seven (PT1) or ten (PT2) or five (QC) identification tests, respectively) reduced the number of tests required compared to for example testing with all event-specific methods (27 tests in all three cases), applying a P-35S screening (one screening test plus 19 identification tests for both PTs; RF3 would not be detected in the QC testing) or even a P-35S/T-Nos screening (2 screening tests plus 19 identification tests for both PTs and 12 tests for the RF3 reference material). Considering that identification tests are costly due to the presence of the fluorescently labelled probes, CoSYPS also represents a reduction in cost. CoSYPS functioned appropriately when the expected targets were present in a complex matrix such as was the case for the Bt11 material.

Conclusion

In this study, four novel SYBR®Green qPCR methods that perform at equal efficiency were developed to allow the detection of the most common GM traits present in genetically modified crops to date. Successful use of these SYBR®Green qPCR methods together with species and generic recombinant targets (such as P-35S, T-Nos …) in a CoSYPS GMO screening approach is shown. The inclusion of such methods allows a very efficient screening for potential GMO presence and will therefore generally result in a limited number of further identification steps to be considered, as such reducing the cost of analysis. CoSYPS represents a powerful tool to determine GMO presence in food/feed products and is readily amendable into an efficient tool for enforcement purposes and GMO traceability along the food and feed chain. For this, the applicability, the robustness, and the reproducibility of the CoSYPS need, however, to be confirmed for example in an inter-laboratory trial.

Acknowledgments The authors would like to greatly thank Dirk VAN GEEL for his technical assistance and Alexandre PATAK (EC-JRC) for assistance with bioinformatics analysis. This research was financially supported by the GMODETEC project (RT-06/6) of the Belgian Federal Ministry ''Health, Food Chain Safety and Environment" and by the European Commission through the Integrated Project Co-Extra, Contract No. 007158, under the 6th Framework Program. All applied analyses were acceptable for accreditation under ISO standard 17025 by the official Belgian ISO accreditation organization "Belac."

References

1. James C (2011) Global status of commercialized biotech/GM crops: 2010. ISAAA brief
2. Wehrmann A, Van VA, Opsomer C, Botterman J, Schulz A (1996) The similarities of bar and pat gene products make them equally applicable for plant engineers. Nat Biotechnol 14(10): 1274–1278
3. Kishore G, Shah D, Padgette S, Dells-Cioppa G, Gasser C, Re D, Hironak C, Taylor M, Wibbenmeyer J, Eichholtz D, Hayford M, Hoffmann N, Delannay X, Horsch R, Klee H, Roger S, Rochester D, Brundage L, Sanders P, Fraley RT (1988) 5-Enolpyruvylshikimate 3-Phosphate Synthase, in American Chemical Society, (ed); Biochemistry to genetic engineering of glyphosate tolerance, pp 37–48
4. Padgette SR, Re DB, Barry GF, Eichholtz DE, Delannay X, Fuchs RL, Kishore GM, Fraley RT (1996) New weed control opportunities: development of soybeans with a Roundup Ready gene. Herbicide-resistant crops: agricultural, environmental, economic, regulatory, and technical aspects, pp 53–84
5. Agronomy eUpdate (2009), Nr 205 www.agronomy.ksu.edu
6. Bravo A, Gill SS, Soberon M (2007) Mode of action of Bacillus thuringiensis Cry and Cyt toxins and their potential for insect control. Toxicon 49(4):423–435
7. Querci M, Van den BM, Zel J, Van den EG, Broll H (2010) New approaches in GMO detection. Anal Bioanal Chem 396(6):1991–2002
8. Dörries HH, Remus I, Grönewald A, Grönewald C, Berghof-Jäger K (2010) Development of a qualitative, multiplex real-time PCR kit for screening of genetically modified organisms (GMOs). Anal Bioanal Chem 396:2043–2054
9. Lu IJ, Lin CH, Pan TM (2010) Establishment of a system based on universal multiplex-PCR for screening genetically modified crops. Anal Bioanal Chem 396:2055–2064
10. Bahrdt C, Krech AB, Wurz A, Wulff D (2010) Validation of a newly developed hexaplex real-time PCR assay for screening for presence of GMOs in food, feed and seed. Anal Bioanal Chem 396:2103–2112
11. Chaouachi M, Chupeau G, Berard A, Mckhann H, Romaniuk M, Giancola S, Laval V, Bertheau Y, Brunel D (2008) A high-throughput multiplex method adapted for GMO detection. J Agric Food Chem 56:11596–11606
12. Morisset D, Dobnik D, Hamels S, Zel J, Gruden K (2008) NAIMA: target amplification strategy allowing quantitative onchip detection of GMOs. Nucleic Acids Res 36:e118
13. Hamels S, Glouden T, Gillard K, Mazzara M, Debode F, Foti N, Sneyers M, Nuez TE, Pla M, Berben G, Moens W, Bertheau Y,

Audeon C, Van den Eede G, Remacle J (2009) A PCR-microarray method for the screening of genetically modified organisms. Eur Food Res Technol 228:531–541
14. Raymond P, Gendron L, Khalf M, Paul S, Dibley KL, Bhat S, Xie VRD, Partis L, Moreau ME, Dollard C, Coté MJ, Laberge S, Emslie KR (2010) Detection and identification of multiple genetically modified events using DNA insert fingerprinting. Anal Bioanal Chem 396(6):2091–2102
15. Nadal A, Esteve T, Pla M (2009) Multiplex polymerase chain reaction-capillary gel electrophoresis: a promising tool for GMO screening–assay for simultaneous detection of five genetically modified cotton events and species. J AOAC Int 92(3):765–777
16. Prins TW, van Dijk JP, Beenen HG, Van Hoef AMA, Voorhuijzen MM, Schoen CD, Aarts HJM, Kok EJ (2008) Optimised padlock probe ligation and microarray detection of multiple (non-authorised) GMOs in a single reaction. BMC Genomics 9:584
17. Luo J, Schumacher M, Scherer A, Sanoudou D, Megherbi D, Davison T, Shi T, Tong W, Shi L, Hong H, Zhao C, Elloumi F, Shil W, Thomas R, Lin S, Tillinghast G, Liu G, Zhou Y, Herman D, Li Y, Deng Y, Fang H, Bushel P, Woods M, Zhang J (2010) A comparison of batch effect removal methods for enhancement of prediction performance using MAQC-II microarray gene expression data. Pharmacogenomics J 10:278–291
18. Nilsson M, Malmgren H, Samiotaki M, Kwiatkowski M, Chowdhary BP, Landegren U (1994) Padlock probes: circularizing oligonucleotides for localized DNA detection. Science 265(5181):2085–2088
19. Krishnakumar S, Zheng J, Wilhelmy J, Faham M, Mindrinos M, Davis R (2008) A comprehensive assay for targeted multiplex amplification of human DNA sequences. Proc Natl Acad Sci USA 105(27):9296–9301
20. Van den Bulcke M, Lievens A, Barbau-Piednoir E, Mbongolo Mbella G, Roosens N, Sneyers M, Leunda-Casi A (2010) A theoretical introduction to "Combinatory SYBR®Green qPCR Screening", a matrix-based approach for the detection of materials derived from genetically modified plants. Anal Bioanal Chem 396(6):2113–2123
21. Waiblinger HU, Grohmann L, Mankertz J, Engelbert D, Pietsch K (2010) A practical approach to screen for authorised and unauthorised genetically modified plants. Anal Bioanal Chem 396(6):2065–2072
22. Mbongolo Mbella EG, Lievens A, Barbau-Piednoir E, Sneyers M, Leunda-Casi A, Roosens N, Van den Bulcke M (2011) SYBR®Green qPCR methods for detection of endogenous reference genes in commodity crops: a step ahead in combinatory screening of Genetically Modified Crops in food and feed products. Eur Food Res Technol 232(3):485–496
23. Barbau-Piednoir E, Lievens A, Mbongolo-Mbella G, Roosens N, Sneyers M, Leunda-Casi A, Van den Bulcke M (2010) SYBR®-Green qPCR screening methods for the presence of "35S promoter" and "NOS terminator"elements in food and feed products. Eur Food Res Technol 230(3):383–393
24. Rice P, Longden I, Bleasby A (2000) EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet 16(6):276–277
25. Sarachu M, Colet M (2005) wEMBOSS: a web interface for EMBOSS. Bioinformatics 21(4):540–541
26. Association Française de Normalisation (AFNOR) (2003) XP V 03-020-2 Détection et quantification des organismes végétaux génétiquement modifiés et produits dérivés- Partie 2: Méthodes basées sur la réaction de polymérisation en chaîne
27. European Network of GMO Laboratories (ENGL) (2008) Definition of minimum performance requirements for analyticals methods of GMO testing. http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf
28. Krieb R, Zeng, Q (2001) Canola event pv-bngt04 (rt73) and compositions and methods for detection thereof. Patent WO02/36831
29. Barry GF, Kishore GM, Padgette SR., Stallings WC (1997) Glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthases. US patent 5633435
30. Donohoe GG, Laaksonen M, Pulkki K, Ronnemaa T, Kairisto V (2000) Rapid single-tube screening of the C282Y hemochromatosis mutation by real-time multiplex allele-specific PCR without fluorescent probes. Clin Chem 46(10):1540–1547
31. Herrmann MG, Durtschi JD, Wittwer CT, Voelkerding KV (2007) Expanded instrument comparison of amplicon DNA melting analysis for mutation scanning and genotyping. Clin Chem 53(8):1544–1548
32. Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Reporter 9:208–218