Food Chemistry 145 (2014) 49–56

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

A new dual plasmid calibrator for the quantification of the construct specific GM canola Oxy-235 with duplex real-time PCR

Maher Chaouachi *, Ahmed Ben Hafsa, Nesrine Nabi, Mohamed Salem Zellama, Khaled Said

Laboratoire de Génétique, Biodiversité et Valorisation des Bioressources, Institut Supérieur de Biotechnologie de Monastir, Université de Monastir, Avenue Tahar Haddad, 5000, BP74, Monastir, Tunisia

ARTICLE INFO

Article history:
Received 6 January 2013
Received in revised form 5 June 2013
Accepted 7 August 2013
Available online 13 August 2013

Keywords:
GMO
Plasmid
Calibration
Quantification
Oxy235
Canola
Construct specific

ABSTRACT

To overcome the difficulties of obtaining the Certified Reference Material (CRM) and according to the key documents of the European Union Reference Laboratory (EU-RL), a new standard reference molecule containing the construct specific of the canola event Oxy-235 (3′-junction Nitrilase/Tnos) and the canola endogenous reference gene (acety-CoA-carboxylase) was constructed and used for duplex real-time quantitative analysis. The limits of detection (LOD) were less than 5 Haploid Genome Copy (HGC) and the limits of quantification (LOQ) were about 10 HGC. Furthermore, mixed GM and non-GM canola samples were analysed with duplex QRT-PCR to evaluate the performance criteria as required for validation procedures in the EU-RL, namely, the precision and the accuracy. The accuracy expressed as bias ranged from 2% to 10% and the precision (repeatability and reproducibility) expressed as the RSD_r and RSD_R was from 2.2 to 5.12 and 2.15 to 5.46 respectively. All these indicated that the developed construct specific method and the reference molecule are suitable for the identification and the quantification of the canola event Oxy-235.

1. Introduction

The Certified Reference Materials (CRMs) are generally used as reference materials in real-time quantification of GMOs, since the lowest threshold level for labelling of products has been established and defined as 0.9% (European Commission Regulation (EC) No 1830/2003 of the European Parliament & of the Council of 22, 2003). However, the usage of CRMs has many limitations. Firstly, specific CRMs must be produced for each approved GM line, but just a few categories have been produced and certified until now. In addition, the production and maintenance of CRMs are very labour- and cost-intensive tasks (Trapmann, Schimmel, Kramer, Van den Eede, & Pauwels, 2002). Furthermore, PCR quantification for GMO content can only be based on genome equivalents (relative ratios of DNA molecules), while CRMs are produced on weight equivalents. Since there is no exact relationship between weight and number of DNA molecules, the suitability of CRMs for the determination of DNA copy number has been doubted (Taverniers, Van Bockstaele, & De Loose, 2004). To overcome these drawbacks, linearised plasmid DNA has been used and was recognised as a cheaper and more feasible alternative to CRMs. In the light of our knowledge, more than 20 plasmid calibrators have been successfully constructed and used for the detection and quantification of several GM maize, soybean, and cotton events. To date the quantitative real-time PCR (QRT-PCR) is considered as the reference technology for GMO quantification and actually used for all the validated methods with the Joint Research Centre (JRC) of the European Commission in Ispra, Italy, being the European Union Reference Laboratory (EU-RL) for GMO detection methods. The literature results indicated that quantification procedure is easily and reliably applied to various food products even for samples with low DNA quantity. The use of multiplex PCR in the quantification is made possible by the use of different reporter dyes, which can be detected separately in one reaction tube (Randhawa, Chhabra, & Singh, 2009; Randhawa, Sharma, & Singh, 2009). However, the number of targets for multiplexing is mainly restricted to the real-time PCR platform constraints (Dörries, Remus, Grönewald, Grönewald, & Berghof-Jäger, 2010). Multiplex reactions are not only an economical way of doing PCR, but they also allow accurate relative quantification without previous estimation of DNA quantity or copy numbers. With a multiplex reaction, a direct relation between % GMO and the results of the real-time PCR can be established. This reduces the variation and permits accurate data interpretation by simple statistical evaluation of the quantification result. It is thus considered the most appropriate to exploit further QRT-PCR-based screening approaches. For this, the construction of standard plasmids as calibrators using QRT-PCR for GMO analysis have been extensively studied as a substitute of dried powder CRMs. Two kinds of plasmids were constructed, the single target

* Corresponding author. Tel.: +216 22 20 27 73; fax: +216 73 465 404.
E-mail address: maher.chaouachi@gmail.com (M. Chaouachi).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.08.026

plasmid (STP) and the multiple target plasmid (MTP), containing one or many GMO markers respectively. In addition the European network of GMO laboratories (ENGL) has constructed a plasmid database which is not yet publicly accessed in the web. In fact, all the constructed pENGL are deposited in the official plasmid and cDNA collection (LMBP) from the Belgian Coordinated Collection of Micro-organisms (BCCMTM) of Gent (Belgium) as central master depository under the statute of full private deposit. According to the literature, many STP or MTP plasmid calibrators were also constructed and validated. For example Lievens et al. (2010), have constructed and assessed a dual-target plasmid, designated as pJANUS™-02-001, comprising part of a junction region of genetically modified soybean event GTS-40-3-2 and the endogenous soybean specific lectin gene was constructed. Taverniers et al. (2004) have described two QRT-PCR methods for Roundup Ready soybean used for relative quantification, in which two types of plasmid DNA fragments were used as calibrators. Single-target plasmids (STPs) diluted in a background of genomic DNA were used in the first method and multiple-target plasmids (MTPs) containing both sequences in one molecule were used as calibrators for the second method. Both methods simultaneously detect a promoter 35S sequence as GMO-specific target and a*lectin* gene sequence as endogenous reference target in a duplex PCR. In the other hand, Taverniers et al. (2005), have also constructed event-specific plasmid standards for the events Bt11, Bt176, and GA21 maize and the event GT73 canola. In 2007, Dalla Costa and Martinelli (2007), have developed new MTP pGEM-T Plasmids containing *adh1-lect1* and *zein-lect1* endogenous genes and found to be the most reliable calibration systems for this analysis, providing precise and accurate quantification results. In 2011, Li et al. (2011) have developed a flexible plasmid RM pNK containing three DNA fragments, namely the 5′ and 3′ event-specific sequences of maize NK603 and endogenous gene *zSSIIb* and proved the suitability of this calibrator compared with that of genuine genomic DNA. Another recent study conducted by Khoo, Cheah, and Son (2011) has developed a new recombinant plasmid DNA used as calibrator for the quantification of the unapproved Starlink corn and the approved Bt176 maize present in the maize containing foods, such as raw maize and processed food, as well as, animal feed in Malaysian market. Finally, Meng, Liu, Wang, Zhang, and Yang (2012), have reported the construction of plasmid pTC1507 for a quantification assay of the maize event TC1507 and the collaborative ring trial in international validation of its applicability as a plasmid calibrator. pTC1507 contained one event-specific sequence of TC1507 maize and one unique sequence of maize endogenous gene zSSIIb. Mattarucchi, Weighardt, Barbati, Querci, and Van den Eede (2005), have constructed and used a tandem marker plasmid as a competitor for the detection and quantification of genetically modified cotton MON-531. This plasmid contained event specific sequence of GM Bt cotton MON-531 and taxon specific sequence of cotton that is fsACP (fibre specific acyl carrier protein). More recently, Wang, Wang, Teng, Xi, and Guan (2013), have generated a new plasmid pTLH10 containing ten target genes from GM soybean, maize and cotton namely, Bt176 event-specific 30-junction (Bt176G30), MON810 event-specific 30-junction (MON810G30), CP4EPSPS gene and the endogenous maize Hmg genes. Moreover comparison studies were conducted to search the suitability of two types of DNA calibrators, i.e. plasmid DNA and genomic DNA extracted from plant leaves, for the certification of the GMO content in reference materials as copy number ratio between two targeted DNA sequences was investigated. In fact, Caprioara-Buda et al. (2007), have observed that both plasmid and leaf genomic DNA calibrators would be technically suitable as anchor points for the calibration of the real-time PCR methods applied in GMO analysis. The case study reported here illustrates the use of QRT-PCR for the duplex detection and quantification of the canola line Oxy-235 using a new dual standard plasmid pCS-Ox235 to be used for GMO analysis. Canola (*Brassica napus*) line Oxy-235 (ACS-BNØ11-5) was developed to allow the use of oxynil herbicides as weed control options in canola. Tolerance to oxynil herbicides in Oxy-235 was achieved by introduction of the *bxn* gene isolated from the bacterium *Klebsiella pneumoniae* (sub. sp *ozaenae*), which encodes a nitrilase enzyme that hydrolyses oxynil herbicides to non-phytotoxic compounds. Up to now, only simplex QRT-PCR methods were developed for the quantification of the canola line Oxy-235 (Yang, Guo, Zhang, Liu, & Zhang, 2008) targeting the 3′ and the 5′ event specific junctions and neither construct specific nor was duplex method conducted before. Moreover, up to now the construction of plasmid calibrator has not yet been reported for this line. Thus, the need for adequate reference standards is therefore twofold: firstly qualitative PCR standards are required to act as positive controls for the identification of authorised and unauthorised GM events; whilst secondly quantitative standards are required to construct standard curves for GM DNA and total plant DNA to enable the absolute quantification of GM, thereby ensuring compliance with labelling requirements for authorised GM events.

To this end, the present study shows the design of a duplex QRT-PCR assay for specific detection of the construct specific Nitrilase/Tnos and Acc reference gene, based on their DNA sequence present in GM canola Oxy-235. These assays were evaluated by QRT-PCR in terms of specificity, limit of detection efficiency precision and accuracy. Moreover due to an absence of CRMs for the majority of GM events released worldwide many research and GM testing laboratories have taken up their use and have recently become much more widely used as reference materials. Further aim of this work is the construction of a new dual standard plasmid containing both cloned junction specific and endogenous targets of the canola GM event Oxy-235.

2. Materials and methods

2.1. Plant samples

The species used for the specificity test were Arabidopsis thaliana, sugar beet (*Beta vulgaris* subsp. *vulgaris*), cotton (*Gossypium barbadense* and *Gossypium hirsutum*), Barley (*Hordeum vulgare*), Soybean (*Glycine max*), rice (*Oryza sativa*), maize and teosinte (*Zea mays* and *Zea diploperennis*). *Brassicaceae* species of the Triangle of "U" were also tested (Nagaharu, 1935), Cauliflower (*Brassica oleraceae*), Chinese cabbage (*Brassica rapa*), mustard (*Brassica juncea*), black mustard (*Brassica nigra*) and *B. napus* were provided by the Laboratoire d'Amélioration des Plantes et Biotechnologies Végétales (INRA; Rennes, France).Transgenic material used was maize T25, MON810, Bt176, Bt11, Bt10, GA21 and CBH351, soybean RR, sugarbeet GTSB77, canola (OXY-235, GT73, RF1, RF2, RF3, MS1, MS8) and rice (LL62, provided for research only from JRC; Ispra, Italy) was provided from the Laboratory of GMO detection (INRA, Versailles, France). Seeds were grown in a greenhouse and leaves were collected. Each single plant's leaves were labelled and stored at −20 °C until DNA extraction.

2.2. DNA isolation

DNA was isolated from 1 g ground leaves under liquid nitrogen, using the cetyltrimethylammonium bromide (CTAB) protocol from Rogers and Bendich (1985), modified as follows and described in ISO (2005)21571. Briefly, 15 mL 65 °C CTAB extraction buffer was added to 1 g ground leaves with thorough shaking. Then, 100 mL of α-amylase and RNase A (10 mg/mL each) was added to the tube, followed by gentle shaking and 65 °C incubation for 30 min;

200 mL of proteinase K (200 mg/mL) was then added to the tube. After gentle shaking, the mix was incubated at 65 °C for 30 min. After 10 min centrifuge at 10,000*g*, 1 equivalent volume of chloroform was added to the supernatant. The mix was centrifuged 15 min at 10,000*g* and the supernatant was precipitated using the CTAB precipitation buffer. The supernatant was discarded and the pellet was dissolved in NaCl (1.2 mM) and extracted with chloroform (1 V). After centrifugation (10 min at 12,000*g*), the supernatant was treated with cold isopropanol (0.6 V) and centrifuged for 15 min at 12,000*g*. The supernatant was discarded and the pellet was washed with ethanol 70%, vacuum-dried, and resuspended in 30 mL Tris–EDTA (TE) buffer. DNA concentration was determined on a 0.8% agarose gel, using 1X Tris–borate EDTA (TBE) buffer stained with ethidium bromide (Eurobio, Angers, France) and visualised under UV light.

2.3. Cloning and preparation of the dual standard plasmid pCS-Ox235

The primer pairs of CR-OXF/R and paccF/R (Table 1) were used to amplify the 3′-integration junction sequences (450 bp) and endogenous reference gene (212 bp), respectively. The PCR amplified fragments, were cocloned into the binary vector p3301. All the protocols were based on the Molecular Cloning written by Sambrook and Russell (2001). The plasmid was extracted and identified by restriction digests and DNA sequencing. The confirmed plasmid was named as pGT and subsequently linearised with XhaI restriction endonuclease (Promega). After purification with the Biospin gel extraction kit (Bioflux), the plasmid was quantified using the pico-green dsDNA quantification kit as above and calculated in copy numbers. The solution was diluted in TE buffer to a concentration of 106 copies per 1 μl, from which a tenfold dilution series was made to serve as standard curves for real-time PCR.

2.4. Primer design and DNA Sequencing

Primers and probes were designed based on the sequences of the Nitril/tnos and Acc that are present in GM canola line Oxy-235, GenBank accession: EU099577.1 and AF179850.1, respectively. Sequence analysis was performed at the GenBank database by using the Blastn tool (Basic Local Alignment Search Tools Nucleotide) of the National Centre for Biotechnology Information (NCBI). Primers for the extension reaction were designed using OLIGO v. 6.0 and were purchased from MWG-Biotech AG (Ebensburg, Germany). P100 (BIORAD Bio-Gel R P-100 Gel Fine 45–90 μm) was used for the purification of PCR products. The sequencing reaction was performed in a 10 μl reaction containing 10 nmol/L one primer (forward or reverse), 1 μl of buffer Big Dye and 5 μl of H_2O, 1 μl of BigDye™ Terminator Cycle Sequencing reaction mixture (Applied Biosystems) and 2 μL of the purified PCR product. Reaction products were purified using G50 gel filtration (Sephadex TM G-50 superfine (Amersham Biosciences AB). ABI3730XL 96 capillary sequencer was used for DNA sequencing. The web multialign program (http://multalin.toulouse.inra.fr/) was used for sequence alignments. The detection of polymorphism were performed using the software GENALYS, available at http://software.cng.fr.

2.5. Duplex qualitative PCR conditions: experimental plan for optimisation

The duplex PCR reaction conditions were established by optimisation of some important parameters which included annealing temperatures (55–65 °C), $MgCl_2$ concentrations (1.5–4.0 mM), and each of primer concentrations (0.1–1.0 μM). Negative controls containing water and non-GM soybean were performed in replacement of DNA template. Moreover, positive control was also carried out. The duplex PCR reaction was carried out in a 25 μL reaction mixture, which contained fixed concentrations of extracted DNA (50 ng), 1X PCR buffer, 0.30 μM dNTP, and 2.5 U Taq DNA polymerase.

2.5.1. Optimisation of the annealing temperature

The annealing temperatures were investigated from 55 to 65 °C and the PCR amplified products were detected by electrophoresis. When the annealing temperature was at 60 °C, almost no unspecific amplification products were observed. Therefore, the optimum annealing temperature of 60 °C was selected for the duplex PCR. The thermal cycling conditions were as follows: pre-denaturation at 95 °C for 5 min; followed by 40 cycles of denaturing at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s; at the end of the cycling, the reaction mixture was held at 72 °C for 5 min.

2.5.2. Optimisation of primer concentration

In the multiplex PCR system, preferential amplification of one target sequence over another frequently occur, which could lead to uneven amplification products. The optimal concentration ratio of the primer pairs was 1:4 corresponding to 0.1 μM of Acc1f/Acc2r, 0.4 μM of Tn-NLf/Tn-NLr, respectively.

2.5.3. Optimisation of Mg2+ concentration

Concentration of Mg2+ in the PCR system has a significant influence on the product yield and specificity. The effect of Mg2+ concentrations ranging from 1.5 μM to 4.0 μM on the multiplex PCR system was investigated. Results showed that primer pairs

Table 1
List of primer sequences and expected amplicon sizes for the PCR products for qualitative and quantitative real time analysis and primers used for the construction of the calibration reference molecule.

Primers	Name	Sequence 5′- 3′	Amplicon size	Reference
TR03	Sens	TCTGCCCTATCAACTTTCGATGGTA	137 bp	Taberlet, Gielly, Pautou, & Bouvet (1991)
TR04	Antisens	AATTTGCGCGCCTGCTGCCTTCCTT		
Tn-NLf	Sens	GAAGCCATCGCTTTAACCCGTCC	141 bp	This study
Tn-NLr	Antisens	CTAAAATAACGTCATGCATTACATG		
Tn-NLp	Probe	FAM- CAAGCTTGGGCTGCAGGT-MGB		
Acc1f	Sens (qualitative)	GAGAATGAGGAGGACCAAGCTC	196 bp (Qualitative)	Hernandez et al. (2001)
Acc2f	Sens (quantitative)	GGTGAGCTGTATAATCGAGCGA	104 bp (Quantitatve)	
Acc2r	Antisens	GGCGCAGCATCGGCT		
Accp	Probe	FAM-AACACCTCTTCGACATTCG TTCCATTGGTCGA -TAMRA		
CR-OXf	Sens	GGTGACGAGGACCCGAG	450 bp	This study
CR-OXr	Antisens	TACTAGATCGATCGGGAATTGA		
paccF	Sens	CGGAATTCGAGAATGAGGAGGACCAAGCTC	212 bp	Rong, Wentao, Yunbo, & Feng (2007)
pGTR	Antisens	GCTTATACGAAGGCAAGAAAAGGAGGATCCGCG		
paccRR	Probe	GGCGCAGCATCGGCTGAATTCCG		

Tn-NLf/Tn-NLr and Acc1f/Acc2r had both low amplification yields when Mg2+ concentration was in 1.5 μM. Their amplification efficiencies were observably improved by increasing the Mg2+ concentrations up to 2.0 μM. Nevertheless, concentrations above this level resulted in the non-specific amplification and specificity decreased. Consequently Mg2+ concentration of 2.0 μM was the optimum for the duplex PCR reaction.

2.6. Agarose gel electrophoresis

The PCR products were also separated in ethidium bromide-stained 3% (w/v) agarose gel electrophoresis running for 1 h in 0.5 TBE buffer using a constant voltage (85 V), then exposed to UV light to visualise DNA fragments and the electropherograms were observed by gel imaging system. PCR reactions were performed using eukaryote universal primers (TR03/TR04) in order to check the quality of the DNA.

2.7. Duplex QRT-PCR conditions

PCR amplification was performed in a 96-well microtiter plate in a total volume of 25 μL. Reaction mixture was prepared in master mix (TaqMan Universal PCR Master Mix, ABI) and contained 0.01 U/μL uracil-N-glycosylase (AmpErase UNG®); 3.5 mM $MgCl_2$; 300 nM of each primer; 200 μM probe, 200 μM dATP, dCTP, dGTP, and 400 μM dUTP; 0.025 U/μL AmpliTaq Gold; and 5 μL template DNA. Quantification experiments were performed on ABI Prism 7900 thermocycler (ABI) using the Sequence Detector Software (SDS) V2.1. The baseline fluorescence was set automatically by the SDS software. PCR program consisted in a first decontamination step of 2 min at 50 °C (UNG activation) followed by 10 min at 95 °C to activate the *Taq* polymerase and denatured double-strand DNA, and then, 45 cycles of 30 s at 95 °C and 1 min at 60 °C.

3. Results and discussion

3.1. Description of the edge fragment (3′ boundary junction of canola event Oxy-235)

In order to analyse the Oxy-235 insert, the synthetic *pat* gene cassette was sequenced by using the primers designed previously in its 3′ end. Homology search confirmed that this cassette of 1109 pb was intact. It was aligned with 3′ edge fragments previously characterised (AC No. EU099579.1). The annotated sequence of the Oxy-235 insert targeted in this study is presented in Table 2. The Table 2 summarises "blast" analysis and showed that the insert contained: (1) One partial sequence matching with the *K. pneumoniae* bromoxynil-specific nitrilase gene (BXN) (240 bp) of AC No. J03196.1 registered in GenBank by Aventis (now Bayer CropScience). (2) One truncated sequence matching with the terminator nos (Tnos, 225 bp) of AC No. AF330636. (3) One sequence of about 325 bp resulting probably from the DNA rearrangements and mismatching neither with the nitrilase gene nor the Tnos sequence. The alignment results with the major public primers (data not shown) used for the detection and the quantification of Tnos in different GMO events revealed a region of mismatches demonstrating that the Tnos sequence was truncated and could lead to false negative amplifications. In fact the DNA rearrangements are frequent in GMOs. They have been reported in both direct and indirect transformation and were, for instance, described in different GMO evens namely rice, soybean, oat, and maize (Brunaud et al., 2002; Kohli et al., 1999). In fact, recombination may occur either between plasmid molecules, e.g., multimerization of the plasmid DNA, or between plasmid and genomic DNA prior to or during the integration of the transforming DNA. Various kinds of rearrangements can occur: deletion or truncation of the exogenous DNA (Mon810, GA21, Bt176 events; recombination between plasmid DNA and host genomic DNA (GTS 40-3-2, Bt176 events); tandem or inverted repeat formation due to duplication of either plant DNA or plasmid DNA sequences (GA21, GTS 40-3-2, Bt176 events). In addition to insert recombination, it has been observed that transgenic loci in transgenic plants obtained by direct gene transfer might exhibit extreme scrambling, resulting in the integration of several short transgenic fragments interspersed by short host genomic DNA fragments (Svitashev, Pawlowski, Makarevitch, Plank, & Somers 2002). It is hypothesised that, in plants, exogenous DNA transfer elicits a wound response which activates nucleases and DNA repair enzymes. The transferred DNA is, thus, either degraded or used as a substrate for DNA repair, resulting in its potential rearrangement and incorporation in the genomic DNA (Makarevitch, Svitashev, & Somers, 2003). In higher plants, most rearrangements involve illegitimate recombination during DNA double-strand break repair (Svitashev et al., 2002). Plasmid junctions are predominantly formed by microhomology-dependent illegitimate recombination mainly based on single-strand annealing of complementary tails, followed by repair synthesis over the remaining gaps (Kohli et al., 1999). Several other mechanisms can also be involved in DNA rearrangement, such as nonhomologous end joining, synthesis-dependent strand annealing, or polymerase slipping and template switching, sometimes leading to deletion. Furthermore, Kohli et al. (1999) report that certain plasmid and construct structures seem to be frequently involved in recombination events. Among them are the 3′ end of the CaMV 35S promoter, when it is in conjunction with specific flanking sequences derived from transforming plasmid, the right border of the Ti plasmid of *Agrobacterium tumefaciens,* and the 3′ end of the nos terminator. The real implication of these elements in the frequency of DNA recombination is, however, still being discussed.

3.2. Specificity of the canola reference gene (Acc) with qualitative PCR

B. napus plants are known to outcross up to 30% with other plants of the same species, and potentially with plants of related species *B. rapa*, *B. juncea*, *Brassica carinata*, *B. nigra*, *Diplotaxis muralis*, *Raphanus raphanistrum*, and *Erucastrum gallicum*. Previous studies have demonstrated that gene flow is most likely to occur with *B. rapa*. According to the triangle of U (Nagaharu, 1935), *B. napus* is the hybrid result of crossing between *B.rapa* and *B.oleracea* but the results showed a lack of primer specificity between the *Brassica* crops (data not shown). In fact, the same PCR product using both reference genes previously reported (ACC (Hernandez, Rıo, Esteve, Prat, & Pla, 2001) and HMG (Wu, Zhang, Wu, Cao, & Lu., 2010) was found not only with *B. napus* (AACC, 2*n*) but also with *B.oleracea* (cauliflower; CC, 2*n* = 18), and *B. rapa* (Chinese cabbage; AA, 2*n* = 20) (data not shown). These results showed that a specific reference gene needs to be validated by the EU-RL in order to avoid false positives.

3.3. Specificity and sensitivity of the construct specific Nitr/Tnos with qualitative PCR

To set up the construct-specific real-time PCR systems of the GM canola line Oxy-235, the specificity of the designed TaqMan® primers and probes was tested by means of qualitative PCR analysis. A qualitative PCR system, specific to the flanking sequence of Oxy-235 canola, was checked using the genomic DNAs from GM and non-GM lines, as well as a set of other transgenic plants. In qualitative PCR system employing primer pair Tn-NLf/Tn-NLr, the target fragment 141 bp in size was obtained from only Oxy-235 canola, and no amplified fragments were obtained from the other GM, non-GM lines, and other GM crops (Fig. 1A). Similarly, the

Table 2
Description of the fragments present in the 3′ boundary junction of the canola event Oxy-235.

Position No. of nucleotides on 1109 bp sequence	Size, bp	Description of genetic elements	Aligned with (accession no. and position of corresponding bp on sequence of the accession):
1–240	240	99% homology with Comamonas sp. 7D-2 plasmid pBHB, partial sequence	JX473584.1 (bp 2187–2425)
		99% homology with the *K. pneumoniae* bromoxynil-specific nitrilase (bxn) gene, complete	J03196.1 (bp 971–1209)
241–466	225	97% homology with the cloning vector pBEO210, complete sequence	EF192606.1 (bp 23,501–23,722)
		99% homology with Plant DNA excision vector pX6-GFP, complete sequence	AF330636 (bp 3813–4022)
467–479	12	Unknown sequence	Unknown sequence in NCBI databases
480–506	26	100% homology with the *Streptomyces rimofaciens* strain ZJU5119 mildiomycin biosynthetic gene	JN999998.1 (bp 2–28)
		100% homology with the cloning vector pOJ260, complete sequence	GU270843.1 (bp 443–468)
		100% homology with the mariner transposase delivery vector pFA545, complete sequence	EU099579.1 (bp 4641–4667)
507–792	285	100% homology with the cloning vector pBEO210, complete sequence	EF192606.1 (bp 23,723–23,993)
		100% homology with the *Agrobacterium tumefaciens* octopine-type Ti plasmid, complete sequence	AF242881.1 (bp 12,888–13,158)
		100% homology with the *Agrobacterium tumefaciens* Ti plasmid pTi15955 T-DNA region	X00493.1 (bp 13,793–14,063)
		99% homology with unidentified cloning vector 793neomycin phosphotransferase and streptomycin/spectinomycin adenyltransferase genes, complete cds	M35007.1 (bp 6140–6409)
793–1109	316	97% homology with the *Brassica rapa* subsp. pekinensis clone KBrB071P24, complete sequence	AC189451.2 (bp 61,638–61,954)
		96% homology with the *Brassica rapa* subsp. pekinensis clone KBrH013D23, complete sequence	AC189591.2 (bp 120,058–120,374)

qualitative PCR primer pair, which is Acc1f/Acc2r, used in the qualitative PCR gave rise to expected specific DNA fragments as shown in Fig. 1A. In fact, the canola 196 bp endogenous fragment, *acc*, was obtained in all canola DNA samples and no fragment was obtained in NTC (No Template Control) and other GM crops. All of these indicated that designed primer pairs have high specificity and are suitable for further quantitative analysis of GM canola samples. More over all the PCR products obtained in this study was verified with the protocol of sequencing mentioned in the methodology description. According to the EU-RL Method Performance Requirements (European Union-Reference Laboratory (EU-RL)., 2008), analytical methods should detect the presence of an analyte at 1/20th of the threshold relevant for legislative requirements at least 95% of trials. The sensitivity of detection was also determined. Samples

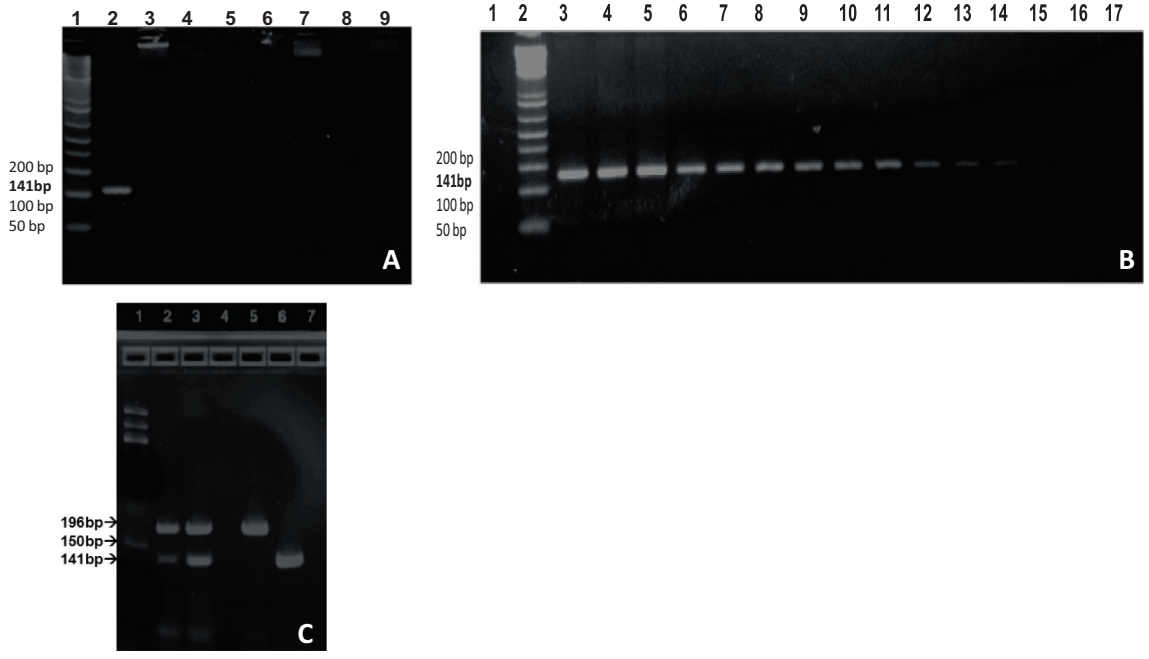

Fig. 1. (A) Example of specificity test of the construct-specific primers (Tn-NLf/Tn-NLr) with simplex qualitative PCR. The amplification product is 141 bp. Lane 1: DNA ladder (50 bp), Lane 2: PCR product from GM canola Oxy-235, Lane 3–8: GM canola GT73 and RF1, non-GM samples namely: *Brassica napus,* barley (*Hordeum vulgare*), soybean (*Glycine max*), maize (*Zea mays*) and Lane 9: NTC. (B) Sensitivity of the construct specific using qualitative PCR: determination of the absolute limit of detection (LODa). Lane 1: NTC, Lane 2: DNA ladder (50 bp), Lane 3 and 4: 10%, Lane 5 and 6: 5%, Lane 7 and 8: 1%, Lane 9 and 10: 0.5%, Lane 11 and 12: 0.1%, Lane 13 and 14: 0.05%, Lane 15–17: 0.01%. C: amplification of the canola Oxy-235 using duplex qualitative PCR (primers Acc1f/Acc2r for lectine (196 bp) and Tn-NLf/Tn-NLr for the construct specific Oxy-235 (141 bp). Lane 1: DNA ladder, Lane 2: DNA from Oxy-235 before optimisation, Lane 3: DNA from Oxy-235 after optimisation, Lane 5: simplex PCR product using DNA from lectine, Lane 6: simplex PCR product using DNA from the construct specific Oxy-235 and Lane 7: (No Template Control (NTC)).

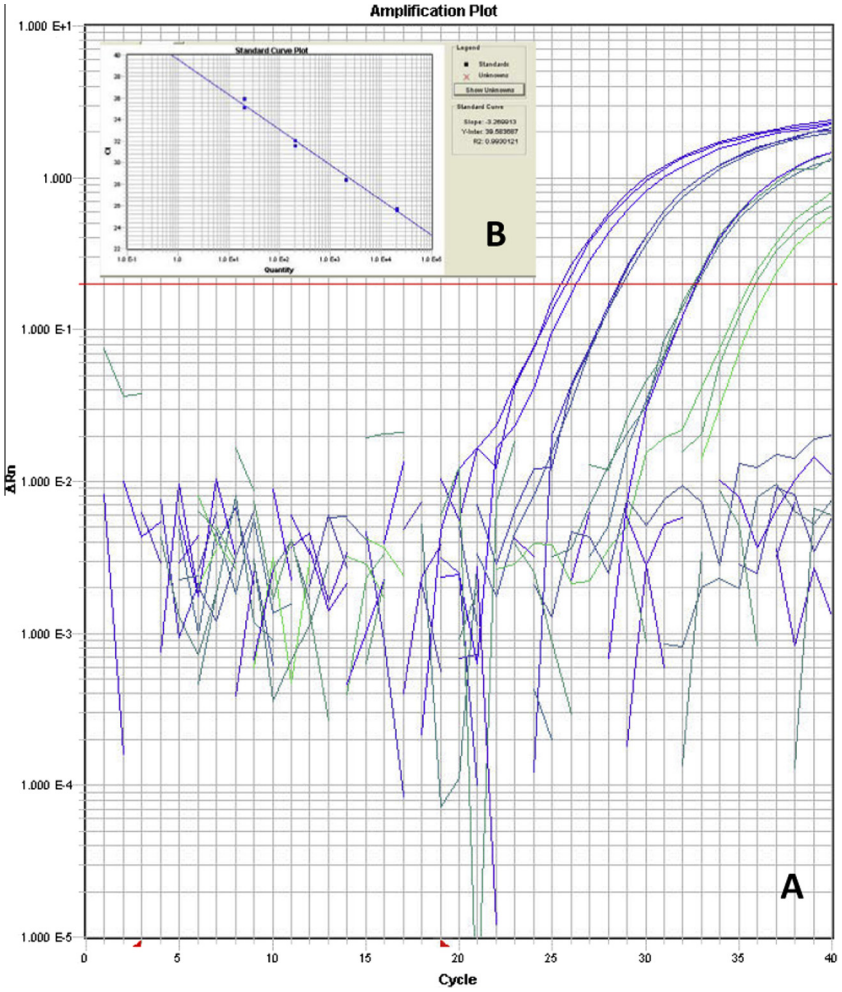

Fig. 2. Amplification plot and standard curve for canola pCS-Ox235 using duplex real time PCR assay targeting the construct specific Tnos/Nitrilase. (A) Amplification plots generated by tenfold serial dilution of canola pCS-Ox235 DNA ranging from 2000 HGC to 2 copies with the Tn-NLf and Tn-NLr primer pair and Tn-NLp probe. Assays were performed in triplicate. (B) calibration curves generated from the amplification data given in A. No amplification observed (Ct = 40) with the concentration 2 copies confirming that, the absolute LOD is less than 5 copies.

with seven different levels of Oxy-235 canola's content: 10, 5, 1, 0.1, 0.05% and 0.01% were prepared from Oxy-235 canola and its non GM parent DNA samples. The results are shown in the Fig. 1B. They demonstrated that the target fragment 141 bp could be detected from all of the levels tested, which meant that the lowest tested level was 0.05%, corresponding to 10 HGC (Haploid Genome Copies) on the basis of the canola genomic DNA of 5.6 pg per Haploid Genome (Arumuganathan & Earle, 1991). Considering the lowest threshold value permitted is 0.9% by the EU, this result indicated that the qualitative PCR method developed from this study could be used in monitoring work for the GMO labelling systems worldwide (Fig. 1B).

3.4. Qualitative duplex PCR results

Agarose gel electrophoresis (Fig. 1C) and melting curve analysis revealed that all primer pairs amplified a single PCR product with the expected size. Furthermore, sequence analysis of cloned amplicons revealed that all sequenced amplified fragments were identical to the sequences used for primer design from GenBank. Duplex PCR refers to combining two primer sets in a single reaction for detection of multiple transgene specific amplicons at once. If developed correctly, the duplexing of PCR primers for the detection of GM events can be advantageous since it can accelerate testing protocols, reduce sample loads, and conserve time and resources. One primer pairs for Oxy-235 (Tn-NLf/Tn-NLr), one primer pairs for Acc (Acc1f/Acc2r), were combined in a single PCR for simultaneous amplification of the two targets. The Fig. 1C shows the duplex PCR results before and after optimisation experimental plan.

3.5. Determination of the QRT-PCR efficiency and linearity

To check whether the different GMO detection methods comply with the ENGL criteria mean PCR efficiencies, squared coefficient of correlations R^2 and slopes of the calibration curves were calculated for all GMO detection methods. All values are the mean of a minimum of eight PCR runs, except for the last four GMO-detection methods where the values listed are the mean values of four PCR runs. The average PCR efficiencies were mostly over 96% for all endogenous and construct-specific GMO detection methods complying with the ENGL performance criteria requirements (European Union- Reference Laboratory (EU-RL)., 2008). The average squared coefficient of correlation R^2 of the calibration curves was over 0.99 for all methods tested. From the presented results it can be concluded that PCR linearity of the test indicated that quantitative PCR assays using the construct specific were stable and reliable. From the high square regression coefficients (R^2) and high

Table 3
Repeatability and reproducibility of the pCS-Ox235 using the construct specific test.

True copy number	Calculated Ct values (mean three replicates)					Mean Ct	Mean copy number	SD^{r}[a]	SD^{R}[b]	CV (%)
	Run1	Run2	Run3	Run4	Run5					
2,000,000	16.35	16.55	16.44	16.39	16.49	16.44	2100677.0	0.02	0.08	0.48
200,000	19.67	19.87	19.76	19.60	19.71	19.72	187098.30	0.03	0.1	0.5
20,000	22.54	22.71	22.81	22.65	22.59	22.66	21898.12	0.05	0.1	0.44
2000	25.13	25.25	25.21	25.27	25.22	25.21	2035.08	0.07	0.05	0.19
200	29.39	29.47	29.41	29.49	29.45	29.44	231.12	0.02	0.04	0.13
100	30.54	30.67	30.61	30.71	30.59	30.62	123.32	0.06	0.06	0.19
50	31.33	31.71	31.65	31.55	31.61	31.57	67.13	0.05	0.1	0.31
25	32.87	32.97	32.79	32.76	32.90	32.85	32.12	0.02	0.08	0.24
10	35.78	35.67	35.87	35.80	35.91	35.80	13.05	0.03	0.09	0.25
5	37.12	37.27	37.25	37.16	37.30	37.22	7.31	0.04	0.07	0.18

[a] SD^{r}: repeatability standard deviation.
[b] SD^{R}: reproducibility standard deviation.

Table 4
Study of the precision (RSDr and RSDR) and the accuracy (bias). The GMO percentages (%) described were calculated using the report (canola Oxy-235 copies/Lec copies).

True value (%)	Canola Oxy-235 copies/Lec copies			Accuracy		Precision				
	Mean 1	Mean 2	Mean 2	Mean GMO (%)	Bias (%)	SD^{R}	SD^{r}	RSD^{R}	RSD^{r}	CV (%)
5	21,567/1078	22,015/1151	22,178/1136	5.11	2.2	0.11	0.10	2.15	2.21	2.15
3	18,322/665	18,523/601	18,698/635	3.4	13.3	0.09	0.12	2.67	2.83	2.67
2	17,685/414	17,635/408	17,796/395	2.28	14	0.06	0.05	2.98	3.02	3
1	15,435/170	15,238/188	15,356/182	1.17	17	0.06	0.03	5.55	4.87	5
0.5	13,352/70	13,562/77	13,485/79	0.55	10	0.03	0.02	5.46	5.12	5

efficiency of the PCR reaction (E) we could conclude that good standard curves were obtained.

3.6. Construction and validation of the pCS-Ox235 standard for GMO quantification

To construct standard curves, the plasmid DNA was firstly linearised at the restriction sites located outside the integrated fragments, and serially diluted from 2×10^3 to 2 copies. According to the sizes of the reference molecule (RM) and the canola genome, we considered that the range of copy numbers from 2 to 2×10^3 was sufficient to quantify GMOs from 0.01% to 100% in the 100 ng of the template for one reaction. The range was supposed to be sufficient to quantify GMOs because of the quantified threshold values of labelling regulations in EU (0.9%). The species-specific gene and construct-specific sequence were separately amplified to set up standard curves. Each concentration of RM was analysed in three replicates and five independent runs were performed. After the construction of pCS-Ox235, real-time PCR assays of both Oxy-235 construct -specific sequence and endogenous acc gene using pCS-Ox235 as a calibrator were evaluated through in-house validation. For determination of the limit of detection (LOD) and limit of quantification (LOQ) of these two real-time PCR assays, five diluted pCS-Ox235 DNA solutions with concentrations from 2 to 2×10^3 copies were prepared and amplified. Each reaction was performed with five replicates and repeated by five operators. The test results showed that all of the reactions with less than 5 copies of pCS-Ox235 DNA could be detected in all three parallels and repeats in both Oxy-235 construct-specific (Fig. 2) and acc assays (data not shown). Therefore, we concluded that the LOD and LOQ of these two assays were 10 and 2 initial copies of pCS-Ox235, respectively. These results suggested that the duplex quantitative system employing pCS-Ox235 as calibrator have acceptable and high sensitivity.

3.7. Repeatability and reproducibility: determination of the RSD^{r} and RSD^{R}

Repeatability and reproducibility of both event-specific and endogenous gene real-time PCR assays using pCS-Ox235 as calibrators were determined. The SD^{r} and RSD^{r} were calculated from three tests; each test had three parallel PCR reactions. Data are described in Table 3. For the real-time PCR assay for the construct specific detection using the pCs-ox235, the mean Ct values varied from 16.44 to 37.22 with a standard deviation (SD^{r} and SD^{R}) values from 0.02 to 0.07 and 0.05 to 0.1 respectively. These results also indicated that we obtained a fine standard curve with dual plasmid calibrator.

3.8. Quantitative detection of the content of Oxy-235 canola in mixed samples

Mixed samples were prepared to evaluate the accuracy and precision of the established real-time PCR methods in this study. GM canola mixtures contained Oxy-235 with four different GM contents (5, 3, 2, 1 and 0.5%). 100 ng DNAs extracted from mixed samples were used as template in one PCR reaction, and each reaction was analysed in three parallels. The Ct values of mixed samples were measured in the QRT-PCR, and from the standard curves of plasmid, the copy numbers of mixed samples could be derived. The content of GM canola, expressed as a percentage of weight, was then calculated by dividing the measured copy number of the event-specific sequence by the copy number of the endogenous sequence and multiplying this number by 100. Samples with Oxy-235 canola concentrations of 5, 3, 2, 1 and 0.5%, gave calculated results of 5.11, 3.4, 2.28, 1.17 and 0.55%, respectively (Table 4). The CV value as reported in Table 4 for 0.5% was 5%. The RSD and RSD parameters calculated to determine the test precision ranged from 2.2 to 5.12 and 2.15 to 5.46 respectively. The quantified biases from true values were lower than the acceptance criterion (<25%) of the GMO detection method (maximum Bias of 17% for

the 1% true value), suggesting that the developed quantitative PCR assay using the pCS-Ox235 was creditable and accurate for the quantification of GM canola event Oxy-235. In conclusion, the reference molecule plasmid pCS-Ox235 obtained in this work could be used to substitute for CRMs in the quantification of Oxy-235 canola content.

4. Conclusion

With the dramatic expansion of global area under cultivation of GM crops, there is an urgent need to step up the development of robust, efficient, and reliable methods for GM quantification. To the best of our knowledge, this article is the first attempt to validate a new duplex method for the detection and the quantification of the canola line Oxy-235 and the construction of a reliable dual plasmid calibrator for its absolute quantification. In fact, in this study the 3′ boundary junction of the canola event Oxy-235 was analysed and new primers and probes specific for the construct specific detection were developed. They showed a high specificity for target detection without cross-reactivity with other non target GMOs tested. Also the limit of detection and quantitative response make both real time PCR methods adequate for the screening and detection of GMOs and GMO-derived products according to the EU-RL instructions. These methods can be implemented in further interlaboratory studies to contribute to the development of strategies for GMO detection. The parameters should comply with the ENGL Method Performance Requirements (EU-RL). Further the developed plasmid overcome the issue of the variability of plant ploidy and parental contribution problems faced with the use of CRMs and could be used as potential calibrators for the real-time PCR analysis that is used to determine the amount of potential GMO food contaminants. Because of the lack of plasmid calibrator availability for most of authorised GMOs, similar studies should be conducted in order to enrich the European official database and presented in the near future as a substation solution of the CRMs.

References

Arumuganathan, K., & Earle, E. D. (1991). Nuclear DNA content of some important plant species. *Plant Molecular Biology Reports, 9*, 208–218.

Brunaud, V., Balzergue, S., Dubreucq, B., Aubourg, S., Samson, F., Chauvin, S., et al. (2002). T-DNA integration into the Arabidopsis genome depends on sequences of pre-insertion sites. *EMBO Reports, 3*, 1152–1157.

Caprioara-Buda, M., Meyer, W., Jeynov, B., Corbisier, P., Trapmann, S., & Emons, H. (2007). Evaluation of plasmid and genomic DNA calibrators used for the quantification of genetically modified organisms. *Analytical and Bioanalytical Chemistry*. http://dx.doi.org/10.1007/s00216-012-6104-6.

Dalla Costa, L., & Martinelli, L. (2007). Development of a real-time PCR method based on duplo target plasmids for determining an unexpected genetically modified soybean intermix with feed components. *Journal of Agriculture and Food Chemistry, 55*, 1264–1273.

Dörries, H. H., 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). *Analytical and Bioanalytical Chemistry, 396*, 2043–2054.

European Commission Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 (2003). Concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from GMOs and amending Directive 2001/18/EC. *Official Journal European Union, L268*, 24–28.

European Union- Reference Laboratory (EU-RL). (2008) Definition of Minimum Performance Requirements for Analytical Methodfs of GMO Testing, 13 Oct. <http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf>.

Hernandez, M., Rıo, A., Esteve, T., Prat, S., & Pla, M. (2001). A canola specific gene, Acetyl-CoA Carboxylase, can be used as a reference for qualitative and real-time quantitative PCR detection of transgenes from mixed food samples. *Journal of Agriculture and Food Chemistry, 49*, 3622–3627.

ISO 21571 (2005). Foodstuffs—Methods of analysis for the detection of genetically modified organisms and derived products—Nucleic acid extraction. *International Standard ISO, 21571*.

Khoo, S., Cheah, Y. K., & Son, R. (2011). The used of recombinant plasmid DNA in GMO quantitative analysis of insect resistance maize targeted unapproved Starlink corn and approved Bt176 corn in food and feed sold commercially sold Malaysia. *International Food Research Journal, 18*, 167–178.

Kohli, A., Griffiths, S., Palacios, N., Twyman, R., Vain, P., Laurie, D., et al. (1999). Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology mediated recombination. *Plant Journal, 17*, 591–601.

Lievens, A., Bellocchi, G., De Bernardi, D., Moens, W., Savini, C., Mazzara, M., et al. (2010). Use of pJANUS™-02-001 as a calibrator plasmid for Roundup Ready soybean event GTS-40-3-2 detection: An interlaboratory trial assessment. *Analytical and Bioanalytical Chemistry, 396*, 2165–2173.

Li, X., Pan, L., Li, J., Zhang, Q., Zhang, S., Lv, R., et al. (2011). Establishment and application of event-specific polymerase chain reaction methods for two genetically modified soybean events, A2704-12 and A5547-127. *Journal of Agriculture and Food Chemistry, 59*, 13188–13194.

Makarevitch, I., Svitashev, S. K., & Somers, D. A. (2003). Complete sequence analysis of transgene loci from plants transformed via microprojectile bombardment. *Plant Molecular Biology, 52*, 421–432.

Mattarucchi, E., Weighardt, F., Barbati, C., Querci, M., & Van den Eede, G. (2005). Development and applications of real-time PCR standards for GMO quantification based on tandem-marker plasmids. *European Food Research and Technology, 221*, 511–519.

Meng, Y., Liu, X., Wang, S., Zhang, D., & Yang, L. (2012). Applicability of plasmid calibrator pTC1507 in quantification of TC1507 maize: An interlaboratory study. *Journal of Agriculture and Food Chemistry, 60*, 23–28.

Nagaharu, U. (1935). Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Japanese Journal of Botany, 7*, 389–452.

Randhawa, G. J., Chhabra, R., & Singh, M. (2009). Multiplex PCR-based simultaneous amplification of selectable marker and reporter genes for the screening of genetically modified crops. *Journal of Agriculture and Food Chemistry, 57*, 5167–5172.

Randhawa, G. J., Sharma, R., & Singh, M. (2009). Multiplex polymerase chain reaction for detection of genetically modified potato (*Solanum tuberosum* L.) with cry1Ab gene. *Indian Journal Agriculture Science, 79*, 368–371.

Rong, Y., Wentao, X., Yunbo, L., & Feng, G. (2007). Event-specific qualitative and quantitative PCR detection of Roundup Ready event GT73 based on the 3′-integration junction. *Plant Cell Reports, 26*, 1821–1831.

Rogers, S. O., & Bendich., A. J. (1985). Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology, 5*, 69–76.

Sambrook, J., & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory. ISBN 0879695773.

Svitashev, S. K., Pawlowski, W., Makarevitch, I., Plank, D. W., & Somers, D. A. (2002). Complex transgene locus structures implicate multiple mechanisms for plant transgene rearrangement. *Plant Journal, 32*, 433–445.

Taberlet, P., Gielly, L., Pautou, G., & Bouvet, J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology, 17*, 1105–1109.

Trapmann, S., Schimmel, H., Kramer, G. N., Van den Eede, G., & Pauwels, J. (2002). Production of certified reference materials for the detection of genetically modified organisms. *Journal AOAC International, 85*, 775–779.

Taverniers, I., Van Bockstaele, E., & De Loose, M. (2004). Cloned plasmid DNA fragments as calibrators for controlling GMOs, different real-time duplex quantitative PCR methods. *Analytical and Bioanalytical Chemistry, 378*, 1198–1207.

Taverniers, I., Windels, P., Vaitilingom, M., Milcamps, A., Van Bockstaele, E., Van den Eede, G., et al. (2005). Event-specific plasmid standards and real-time PCR methods for transgenic Bt11, Bt176, and GA21 maize and transgenic GT73 canola. *Journal of Agricultural and Food Chemistry, 53*, 3041–3052.

Yang, L., Guo, J., Zhang, H., Liu, J., & Zhang, D. (2008). Qualitative and quantitative event-specific PCR detection methods for oxy-235 canola based on the 3′ integration flanking sequence. *Journal of Agriculture and Food Chemistry, 56*, 1804–1809.

Wu, G., Zhang, L., Wu, Y., Cao, Y., & Lu., C. (2010). Comparison of five endogenous reference genes for specific PCR detection and quantification of *Brassica napus*. *Journal of Agriculture and Food Chemistry, 58*, 2812–2817.

Wang X., Wang X., Teng D., Xi D., Guan Q., Construction of a reference plasmid containing ten targets for the detection of genetically modified crops, Plasmid, 2013 Jan, 69(1), 108-13, DOI: 10.1016/j.plasmid.2012.09.005.