Plant Cell Rep
DOI 10.1007/s00299-012-1346-5

ORIGINAL PAPER

Development of real-time PCR method for the detection and the quantification of a new endogenous reference gene in sugar beet "*Beta vulgaris* L.": GMO application

Maher Chaouachi · Akram Alaya · Imen Ben Haj Ali · Ahmed Ben Hafsa · Nesrine Nabi · Aurélie Bérard · Marcel Romaniuk · Fethia Skhiri · Khaled Saïd

Received: 12 July 2012 / Revised: 8 September 2012 / Accepted: 11 September 2012

Abstract
Key message **Here, we describe a new developed quantitative real-time PCR method for the detection and quantification of a new specific endogenous reference gene used in GMO analysis.**
Abstract The key requirement of this study was the identification of a new reference gene used for the differentiation of the four genomic sections of the sugar beet (*Beta vulgaris* L.) (*Beta*, *Corrollinae*, *Nanae* and *Procumbentes*) suitable for quantification of genetically modified sugar beet. A specific qualitative polymerase chain reaction (PCR) assay was designed to detect the sugar beet amplifying a region of the adenylate transporter (*ant*) gene only from the species of the genomic section I of the genus *Beta* (cultivated and wild relatives) and showing negative PCR results for 7 species of the 3 other sections, 8 related species and 20 non-sugar beet plants. The sensitivity of the assay was 15 haploid genome copies (HGC). A quantitative real-time polymerase chain reaction (QRT-PCR) assay was also performed, having high linearity ($R^2 > 0.994$) over sugar beet standard concentrations ranging from 20,000 to 10 HGC of the sugar beet DNA per PCR. The QRT-PCR assay described in this study was specific and more sensitive for sugar beet quantification compared to the validated test previously reported in the European Reference Laboratory. This assay is suitable for GMO quantification in routine analysis from a wide variety of matrices.

Keywords Adenylate transporter · GMO · Real-time PCR · Sugar beet · TaqMan · Quantification

Abbreviations

C_t	Cycle threshold
CTAB	Cetyl trimethylammonium bromide
dNTP	Deoxynucleotide triphosphate
EC	European Commission
EST	Expressed sequence tag
EU	European Union
EU-RL	European Reference Laboratory
GS	Glutamine synthase
HGC	Haploid genome copy

Introduction

Over the past 10 years, the occurrence of a 0.9 % mandatory labeling threshold means that analytical methods for

Communicated by J. Register.

M. Chaouachi (✉) · A. B. Hafsa · N. Nabi · F. Skhiri · K. Saïd
Laboratoire de Génétique, Biodiversité et Valorisation des Bioressources, Institut Supérieur de Biotechnologie de Monastir (ISBM), Université de Monastir, Avenue Tahar Haddad 5000, BP74 Monastir, Tunisia
e-mail: maher.chaouachi@gmail.com

A. Alaya
Laboratoire de Toxicologie. Hopital Fattouma Bourguiba, Université de Monastir, Monastir, Tunisia

I. B. H. Ali
Laboratoire de Biotechnologie Végétale. Institut National des Sciences Appliquées, Tunis Cedex, Tunisia

A. Bérard
Unité Etude du Polymorphisme des Génomes Végétaux (EPGV) UR1279, Centre National de Génotypage (CNG), 2 rue Gaston Crémieux 91057, CP5721 Evry cedex, France

M. Romaniuk
Laboratoire de Méthodologies de la détection des OGM (MDO), Institut National de la Recherche Agronomique (INRA), UR 256 PMDV/MDO, Route de Saint-Cyr, 78026 Versailles cedex, France

Published online: 28 September 2012

GMO testing must: quantify DNA; be highly sensitive, reliable and reproducible; be adaptable to various kind of material such as raw commodity or processed food product and detect the largest number of commercialized GMOs (in European Union and third countries) (Fagan et al. 2010).

To date, real-time PCR-based detection and quantitative methods for several GM events in different species have already been developed and validated by international interlaboratory collaborative study (Kodama et al. 2009), and adopted as standard analytical methods (European Commission, EU-RL). In fact, many experiments were conducted using simplex or multiplex quantitative real-time PCR (QRT-PCR) and targeted the authorized or unauthorized GMOs in EU (Dinon et al. 2011; Kluga et al. 2012; Barbau-Piednoir et al. 2012; Holst-Jensen et al. 2012). QRT-PCR allows quantification either through the use of standard curves obtained with reference material, certified or not, or through the use of the ΔC_t method between, e.g., a GMO-specific sequence and taxon reference gene such as the *Adh* gene for corn. The percentage of GMO is calculated from a combination of two absolute quantification values: one for the specific GMO target and the second for the taxon endogenous gene. A plant endogenous reference gene must be nuclear, present in low copy number, taxon specific and exhibit high homogeneity among cultivars (Chaouachi et al. 2008). A considerable number of taxa specific tests were previously reported targeting those species that have already been genetically transformed and authorized for feed or food and complying with the respective regulations. A summary of the reference systems was previously listed (Chaouachi et al. 2008; Wang et al. 2010). Moreover, taxa-specific genes were used for the construction of new standard plasmids as calibrators for GMO quantification and representing an alternative to genomic DNA (Wang et al. 2011).

In 2007 (Chaouachi et al. 2007), bottlenecks faced when designing reference gene assays were discussed. Among the cited bottlenecks was the difficulty in differentiating closely related taxa and of detecting introgression events. One of the closely related taxa that requires differentiation and additionally is subject to introgression events is the case of the Chenopodiaceae and particularly sugar beet (*Beta vulgaris* L.). This later has an estimated genome size of 758 Mbp. There was little sequence information in the public databases prior to our study. Sugar beet (*B. vulgaris* L.) is an important crop plant accounting for 30 % of the world's sugar production annually. The genus *Beta* is a distant relative of currently sequenced taxa within the core eudicotyledons, and genomic characterization of sugar beet is essential to make its genome accessible to molecular dissection. Several projects (such as BeetSeq consortium (http://www.gabi.de) are underway for the generation and annotation of the sugar beet genome sequence and then the identification of a large number of genes (Dohm et al. 2011). Because of the lack of gene sequences and the possibility of introgression events, the Chenopodiaceae presents a particular challenge when developing a new endogenous reference gene assay. Most endogenous reference gene PCR assays developed to date have not examined related wild taxa that are thought to be involved in gene introgressions (based on phylogenetic studies), except for maize, whose putative ancestor, "Teosinte" has been studied (Paternò et al. 2009) and the solanaceae (tomato and potato) (Tranzschel 1927). For example, in the case of the Chenopodiaceae family, a reference gene detection system has been reported using the glutamine synthetase reference gene. It was revealed to be not specific and could not discriminate between sugar beet (*Sugar beet*) and autumn beet (*Brassica rapa*) as mentioned in the validation report of the EU-RL for the validation of the event-specific method for the quantification of sugar beet line H7-1 using real-time PCR (Community Reference Laboratory for GM Food and Feed 2012). The genus *Beta* is divided into four genomic sections (Beta, Corrollinae, Nanae and Procumbens) (Ford-Lloyd and Williams 1975). The ploïdy number of the genus *Beta* can be diploid, tetraploid and hexaploid with a chromosome number of $x = 9$. All wild and cultivated *Beta* species are capable of hybridizing. Thus, the design of taxa reference gene particularly for species with a complex genome requires necessarily the study of the genetic characteristic such as ploïdy and eventual introgression and the determination of inter- and intra-species sequence variability. Moreover, no endogenous reference genes have been reported for sugar beet, taxa genetically transformed and authorized in feed and food such as the event H7-1, GTSB77 and T120-7 (http://cera-gmc.org).

In the present work, we first described a theoretical analysis required for designing a specific reference system, based on the selection of the gene and the fragment to be studied, here a region of the adenylate transporter gene (*ant*). A second step consisted in the sequencing of the target to examine the intra- and inter-species sequence variability using lines, cultivars and related wild species and also using species involved in the introgression of genes of the three sugar beet genomic sections. The conserved region of our gene of interest was used for the design of an optimized real-time PCR method specific for the first genomic section *Beta*. A representing collection of the genetic and the geographic diversity of the Chenopodiacea were established for the experimental study of the specificity.

Materials and methods

Description of the plant material

A large number of sugar beet varieties were used in this study to maximize the representativity of the samples and

Table 1 Sugar beet cultivars used in this study and covering the four genomic sections and the genetic diversity of the taxa

Species name	Varieties	Genomic section
Sugar beet (*Beta vulgaris* L.)	*Agile*	Section I
	Alezan	Section I
	Alliage	Section I
	Alpage	Section I
	Ariana	Section I
	Beluga	Section I
	Brazzil	Section I
	Cathy	Section I
	Clarine	Section I
	Crocodile	Section I
	Expair	Section I
	Impec	Section I
	Khazar	Section I
	Laredo	Section I
	Pedro	Section I
	Radar	Section I
	Reggaë	Section I
	Soléa	Section I
	Sonate	Section I
	Titus	Section I
	Véronica	Section I
	Virgo	Section I
Fodder beet (*Beta vulgaris* L.)	*Polyfourra*	Section I
	Brigadier	Section I
	Eckendorf Gelb	Section I
	Eckendorf Rot	Section I
	Jamon	Section I
	Monro	Section I
	Jauna	Section I
	Merveille	Section I
	Starmon	Section I
	Splendide	Section I
Garden beet (*Beta vulgaris* L.)	*Noire d'Egypte*	Section I
	Pablo F1	Section I
	Bétina	Section I
	Rétina	Section I
Leaf beet (*Beta vulgaris* L. ssp. *vulgaris)*	*Verte à couper*	Section I
	Blonde à Carde blanche	Section I
	Lucullus	Section I
Beta vulgaris L. ssp. *maritima*	–	Section I
Beta vulgaris L. ssp. *adanensis*	–	Section I
Beta patula Ait	–	Section I
Beta macrocarpa	–	Section I
Beta trygina W	–	Section II
Beta intermedia	–	Section II
Beta corolliflora	–	Section II
Beta nana	–	Section III
Beta procumbens	–	Section IV
Beta patellaris	–	Section IV
Beta webbiana	–	Section IV
Atriplex halimus	–	–
Atriplex hortensis	–	–
Arthrocnemum glaucum	–	–
Suadea vera	–	–
Beta LL (GTSB77)	*GM event*	–
Beta RR line (A5/15)	*GM event*	–
Spinach (*Spinacia oleracea*)	*Gigente America*	–
	Monstrueux de Viroflay	–
	F1 Lazio	–
Arabidopsis thaliana	*Columbia*	–
Brassica napus	*Westar*	–
Brassica oleracea	*Gemmifera*	–
Brassica nigra	*Capitata*	–
Brassica juncea	*TPM-1*	–
Brassica rapa	*Oleifera*	–
Nicotiana tabacum	*K326*	–
Triticum durum	*Cham1*	–
Triticum aestivum	*Arminda*	–
Zea mays	*Mo17*	–
Medicago truncatula	*F11-005*	–
Oryza sativa	*Arélate*	–
Phaseolus aureus	*Aureus*	–
Pisum sativum	*Copernic*	–
Secale cereale	*Petkus*	–
Glycine max	*A1439*	–
Gossypium barbadense	*Giza69*	–
Gossypium hirsutum	*5189*	–
Hordeum vulgare	*Estérel*	–
Linum usitatissimum	*Ariane*	–

The table contains also the taxa used for the experimental specificity (close relatives and non-related species)

to avoid false positives in the case of specificity tests. The collection included cultivars and wild species representative of the four genomic sections and composed of: section I: *Beta*: 22 varieties of sugar beet, 10 varieties of fodder beet, 4 varieties of garden beet and 3 varieties of leaf beet; section II: *Corollinae*: 3 species (*Beta patula, Beta adanensis, Beta maritime, Beta macrocarpa*); section III: *Nanae*: 1 specie (*Beta nana*); section IV: *Procumbentes*: 3 species (*Beta procumbens*, *Beta patellaris and Beta webbiana*). The study of specificity included also 20 non-sugar beet plants and 8 closely related species in the

Chenopodium genus of the Chenopodioideae subfamily and namely, *Atriplex halimus*, *Atriplex hortensis*, *Arthrocnemum glaucum* and *Suadea vera* and 3 varieties of Spinach (*Spinacia oleracea*). The transgenic material used was composed of two GM events, GTSB77 and RR line (A5/15). All the names of the species used for the specificity assessment are listed in Table 1. All of the provided seeds were germinated in the greenhouse and stored at −20 °C to provide enough fresh leaves for genomic DNA extraction. All the samples were provided as leaves, roots or seeds from different INRA groups (Laboratoire de Génétique et d'Amélioration des Fruits et Légumes, INRA; Domaine St Maurice, Avignon; Amélioration des Plantes et Biotechnologies Végétales, INRA; Agrocampus Rheu, Laboratoire d'Amélioration et de Santé des Plantes, INRA; Clermont Ferrand and Laboratoire de Méthodologies de détection des OGM, INRA, Versailles, France).

Isolation and quantification of plant genomic DNA

The isolation of sugar beet plant DNA was carried out by the cetyltrimethylammonium bromide (CTAB) protocol described in EN ISO 21571 (2002). All the DNA samples were quantified using the Nanodrop® (ND-1000 spectrophotometer, Chatsworth, CA) quantification method (Chaouachi et al. 2007). PCR reactions were performed using universal primers (Taberlet et al. 1991) and electrophoresis to check the quality of the DNA.

Primers and probes

The previous reported oligonucleotide primers and TaqMan® fluorescent probes for the sugar beet reference system using the glutamine synthetase gene (*GS*) and reported by the EU-RL (http://gmo-crl.jrc.ec.europa.eu/) were used in the specificity study. For the detection and the quantification of the new reference gene (*ant*), a 135-bp fragment is amplified using two newly designed specific primers Bet1F and Bet1R. PCR products are measured during each cycle by means of a target-specific oligonucleotide probe labeled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end. Degenerate primers used for sequencing the region of the *ant* gene in sugar beet were previously reported (http://gmo-crl.jrc.ec.europa.eu/). Sequences of primers and probe are listed in Table 2.

Primer design and DNA sequencing

All of the primers for reference genes were designed for conserved regions on the basis of comparison of the results of sequencing using sugar beet *ant* partial sequence. Conserved regions suitable for designing PCR primers were identified from the alignment, and the primers were designed to fit the sugar beet *ant* region. 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. G50 gel filtration [Sephadex TM G-50 superfine (Amersham Biosciences AB)] was used for the purification of reaction products. ABI3730XL 96 capillary sequencer was used for DNA sequencing. The web multialign program (http://multalin.toulouse.inra.fr/) was used for sequence alignments. Then, consensus regions were targeted for the design of specific TaqMan® probes. The detection of polymorphism was performed using the software GENALYS (available at http://software.cng.fr).

Table 2 Primers and probes used in this study

Primers	Name	Sequence 5′–3′	Amplicon size (bp)	Reference
TR03	Sens	TCT GCC CTA TCA ACT TTC GAT GGT A	137	(Taberlet et al. 1991)
TR04	Antisens	AAT TTG CGC GCC TGC TGC CTT CCT T		
DegBetF	Sens	TGGAGAGGAAACACTGCYTAATGT	516	(Chaouachi et al. 2007)
DegBetR	Antisens	ATGTTRGCACCAGCWCCCTTGA		
Betantpr	Probe	FAM-TCTCAATTACTCATAGTGAGAT-MGB	135	This study
BetantF	Sens	CAGTATATTTTAGTCAATTCCAAG		
BetantR	Antisens	ACATTTTCTGTCTGGTCTACTACC		
GluAF	Sens	GACCTCCATATTACTGAAAGGAAG	121	(European Commission 2012)
GluAR	Antisens	GAGTAATTGCTCCATCCTGTTCA		
Glu D1	Probe	FAM-CTACGAAGTTTAAAGTATGTGCCGCTC-TAMRA		

Qualitative PCR conditions

Qualitative PCR was run on MJ Research Thermocyclers (MJ research, Waltham, MA, USA). PCR amplifications were performed in a final volume of 25 µl. Each reaction mixture had 1× PCR buffer, 0.2 mM dNTP, 0.4 µM each primer, 25 ng of each DNA sample, 1 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). The amplicons were resolved by electrophoresis on 2 % agarose gels (Invitrogen, Carlsbad, CA, USA). Gels were scanned with Image Master VDS (Amersham Bioscience, Amersham, UK).

Real-time PCR conditions

Several real-time PCR chemistries generally perform as well as the currently and the most broadly was applied TaqMan® chemistry (Gašparič et al. 2010). PCRs were run on the apparatus the ABI Prism 7900HT sequence detection system with a final volume of 25 µL. The real-time PCR for the *ant* gene detection contained the following reagents: 1× PCR buffer, 200 µM each of dNTPs, 400 nM primers, 200 nM TaqMan probes, 1.5 U of Taq DNA polymerase, and 3 mM $MgCl_2$ and 5 µL of template DNA samples. The real-time PCR for exogenous gene detection contained the following reagents: 1× PCR buffer, 200 µM each of dATP, dGTP, dCTP, and dTTP, 400 nM primers, 400 nM TaqMan probes, 1.25 U of Taq DNA polymerase, and 6 mM $MgCl_2$ and 5 µL of template DNA samples. The real-time PCR profile was 10 min at 94 °C followed by 40 cycles of 15 s at 94 °C and 1 min at 60 °C. The fluorescent signal was monitored during every PCR cycle at the annealing step. Data were analyzed with Rotor gene 3000 software version 6.0 (Corbett Research). All of the PCR reagents were purchased from Biocolor Company (Shanghai, China) except for primers and probes.

Construction of standard curves

Standard curves for ant assays were established with five dilutions of DNA from sugar beet plant leaves. Five serial diluted concentrations (15.2–0.0114 ng per reaction, respectively) of sugar beet genomic DNA were used for the preparation of standard curves, containing approximately 20,000, 2000, 200, 20, 15 and 10 copies of haploid genome per reaction according to the haploid sugar beet genomic DNA quantity (0.76 pg per haploid genome) (Arumuganathan and Earle 1991).

Results and discussion

Selection of the specific reference gene

The design of reference specific DNA has three requirements: that it be species specific, have a single or a stable low copy number, and have low intra-species variability (Chaouachi et al. 2007). In this part of the work, the different steps previously proposed for the design of a reference gene system were applied. The cited strategy is divided into three parts, namely the in silico study, the determination of the gene sequences in the taxa targeted and finally the experimental study (Chaouachi et al. 2007). Here, the main problem encountered for the selection of sugar beet reference gene was the lack of gene sequences in the web databases. For this, a strong bibliography was established to isolate studies working on genetic maps and then the identification, cloning of the Expression Sequence Tags (EST) in sugar beet. The pre-selection demonstrated the presence of 42 functional genes that have been assigned to the nine linkage groups of sugar beet (Schneider et al. 1999). These genes encoded products that act in different pathways such as Calvin Cycle, Glycolysis, Sucrose degradation and transport processes. Using EST sequences identified with the accession number through the DNA databases such as NCBI, we searched for reference gene sequences that belong to low copy number gene families. The *ant* (Acc. Number AF173648) sugar beet gene was selected. The gene product is involved in the biochemical pathway and particularly in the transport processes.

According to the literature, the genus *Beta* is comprised of four sections: (1) *Beta*, to which sugar beet belongs; (2) Corollinae; (3) Nanae; and (4) Procumbentes. All species within the *Beta* section (*B. vulgaris* ssp. *maritime* and *B. macrocarpa*) are sexually cross-compatible and produce fertile progeny. Hybrids between sugar beet and members of the other sections do not occur naturally, and cross-breeding with other members of the Chenopodiaceae is unlikely. Assuming proximity, synchronicity of flowering, and suitable conditions sugar beet may freely hybridize with the botanical varieties within the *vulgaris* subspecies. Wild relatives that are sexually compatible with sugar beet exist in parts of Europe, Asia, and Central and South America. Hybrids resulting from crosses between cultivated sugar beets and *B. macrocarpa*, and those with *B. vulgaris* ssp. *maritima* have been reported in Europe. The potential therefore exists for introgression of the glyphosate tolerance trait from H7-1 into these wild relatives.

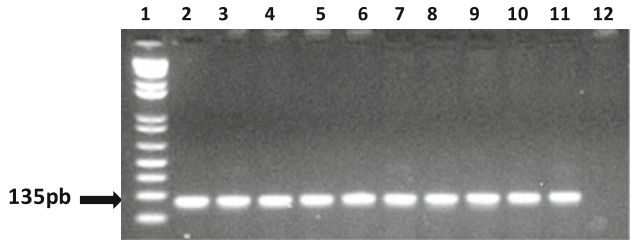

Fig. 1 *Lane 1* size ladder 50 bp, *lanes 2 and 3* sugar beet var *Alezan* and var *Alliage*, *lanes 6 and* 7 fodder beet var *Eckendord gelb* and var *jamon*, *lanes 8 and 9* leaf beet var *Verte à couper*, Garden beet var *Noire d'Egypte* and *lanes 10 and 11* GM sugar beet event GTSB77 and LL liberty RR, *lane 12* (no template control *NTC*)

Study of the theoretical specificity

Bioinformatics analysis of sequence information was performed applying the EMBOSS software package (Sarachu and Colet 2005). Relevant DNA sequence data were retrieved from public databases, patents, and scientific literature. The result of the "blast" used for homology search of the targeted sequence of the *ant* gene demonstrates that there were no sequences producing significant alignments and no homologies observed with any genomic plant DNA. Then, in silico study confirmed the specificity of the region used for the design of the sugar beet reference gene. Primer pairs preferentially comprised in a consensus region of the gene *ant* were designed using the ''Primer Express'' program from Applied Biosystems (version 3.0). A bioinformatics specificity analysis for each primer is performed by "blasting" each primer against several public and GMO DNA sequence databases such as NCBI. 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.

Validation of the performance criteria

To evaluate the performance of the testing methods, some performance criteria were proposed and extensively described in the Budapest document of the codex alimentarius (Codex Committee on Methods of Analysis and Sampling 2012). The most common criteria for detection methods are precision, accuracy, sensitivity, specificity. These parameters are strongly related to the usual terms used to describe the performance of a given analytical method such as applicability, calibration, trueness, precision, recovery, operating range, limit of quantification (LOQ), limit of detection (LOD), and ruggedness (Bertheau et al. 2002). Many of these criteria are today accepted by various international organisms. However, standardisation and validation of GMO testing and quantification

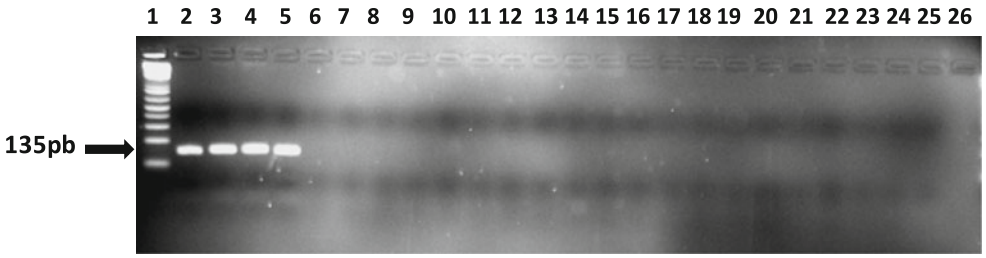

Fig. 2 PCR products (*ant* 135 bp) were electrophoresed in triplicate on a 4 % agarose gel. The primers (Betant F and R) for sugar beet detection were used. *Lane 1* (50 bp size ladder), *lanes 2–5* corresponds to positive samples (species *B. vulgaris maritima*, *B. vulgaris adanensis*, *B. vulgaris* var *belluga* and *B. macrocarpa*), *lanes 6–25* (*B. nana*, *B. procumbens*, *B. patellaris*, *B. webbiana*, *Atriplex halimus*, *Suadea vera*, *Zea mays*, *Oryza sativa*, *Glycine max*, *Brassica napus*, *Brassica rapa*, *Solanum tuberosum*, *Gossypium hirsutum*, *Hordeum vulagare*, *Triticum aestivum*, *Triticum durum*, *Pisum sativum* and *Medicago truncatula*), respectively, *lane 26* (no template control *NTC*). No amplification was obtained with closed relative or non-sugar beet species

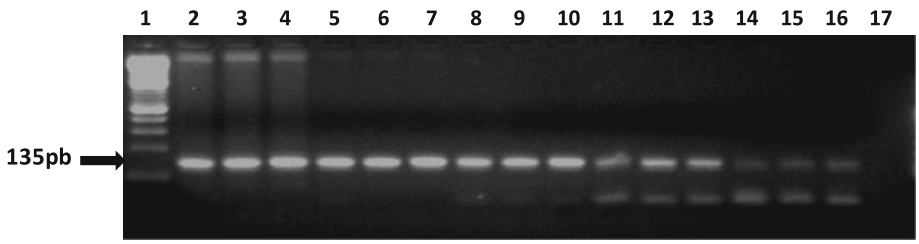

Fig. 3 PCR products (*ant* 135 bp) were electrophoresed in triplicate on a 4 % agarose gel. The primers (Betant F and R) for sugar beet detection were used. *Arrowhead* indicates the expected PCR amplification. Template DNA for each lane was as follows: *lane 1* 50 bp size ladder, *lanes 2–4* 20,000 HGC, *lanes 5–7* 2000 HGC), *lanes 8–10* (200 HGC), *lanes 11–13* 20 HGC and *lanes 14–17* 15 HGC

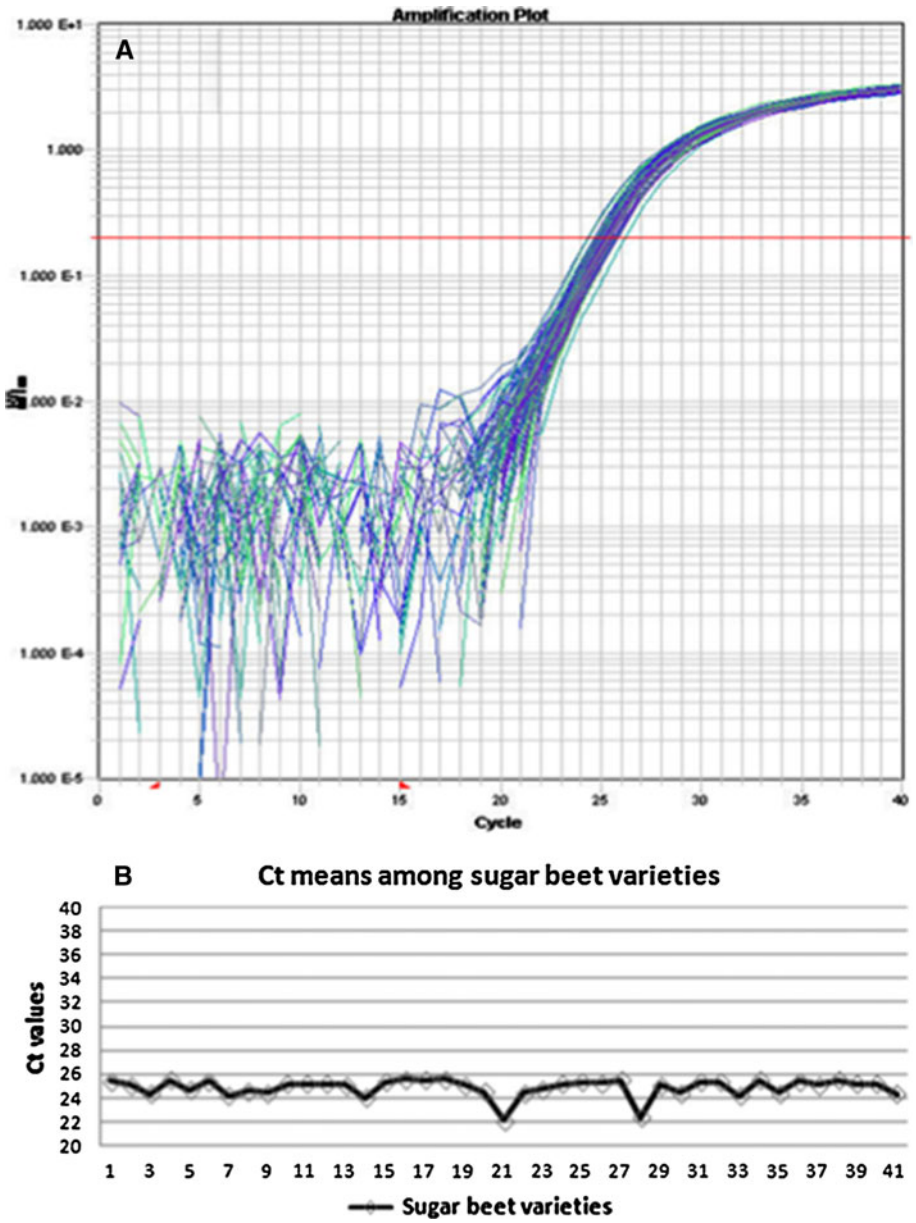

Fig. 4 **a** Amplification plot of the QRT-PCR with a DNA template of 20,000 HGC (15.2 ng/µl) using 39 species and varieties of the section *Beta* and the two GM sugar beet GTSB77 and LL beet. All the samples were tested in triplicate. **b** The calculated C_t means ranged from 24.2 to 25.7 except for the two varieties of fodder beet, *Polyfourra* and *Brigadier* giving C_t values of 22.2 and 22.36, respectively

Table 3 Amplification data used for the determination of the LODa

DNA template (HGC sugar beet/ng)	Total repeats (signal rate)	Mean C_t	SD of the observed C_t
20,000/15.2	6/6	25.36	0.19
2000/1.52	6/6	28.58	0.25
200/0.152	6/6	32.68	0.16
20/0.0152	6/6	35.73	0.24
15/0.0114 (LODa)	6/6	36.91	0.20
10/0.00152	3/6	39.69	NA

NA not applicable

methods by harmonized and accepted protocols are still in its early phases (Anklam et al. 2002). In this study, major performance criteria were tested in both qualitative and QRT-PCR and used the sugar beet diploid variety *Alpage*.

Study of the experimental specificity

To assess the specificity of the *ant* PCR system using the primers (BetantF and BetantR) qualitative PCR was performed using 50 ng of genomic DNA extracted from different species of plant, including all the species and varieties of the four genomic sections of the genus *Beta* of sugar beet, closely related species and also non-related species as templates (Table 1). The electrophoresis results of the qualitative PCR showed that a 135-bp band expected size was observed from the sugar beet samples of the first genomic section, and there were no amplification products

observed from any of the species tested other than this section (Figs. 1, 2). The specificity results suggested that the developed qualitative PCR detection method for the sugar beet was suitable for practical use in the monitoring and identification of GMO.

Sensitivity of primer pairs designed for sugar beet detection

Serial dilutions were obtained for the determination of sensitivity [absolute limit of detection (LODa)] and ranged from 20,000 to 10 HGC (Fig. 3). Sugar beet DNA preparations are first measured spectrophotometrically and diluted to obtain solutions with a precisely known, fixed amount of target copies. The sensitivity test was performed to determine the LODa of the new qualitative PCR method by three times repeat. The LODa for sugar beet was 10 HGC (Fig. 3). Considering the lowest threshold value, which is 0.9 % of EU, the results of the sensitivity test demonstrated that the developed qualitative PCR method can meet the requirements for monitoring GMO labeling systems worldwide.

Experimental performance criteria with QRT-PCR

For performance evaluation LOD, quantification (LOQ), dynamic range, PCR efficiency, and repeatability were compared. All the evaluations were based on the "acceptance criteria" compiled by the European Network of GMO Laboratories (ENGL) according to the recent key documents entitled "Definition of minimum performance requirements for analytical methods of GMO testing (2008)" and "Verification of analytical methods for GMO testing when implementing interlaboratory validated methods (2011)" (http://gmo-rl.jrc.ec.europa.eu/).

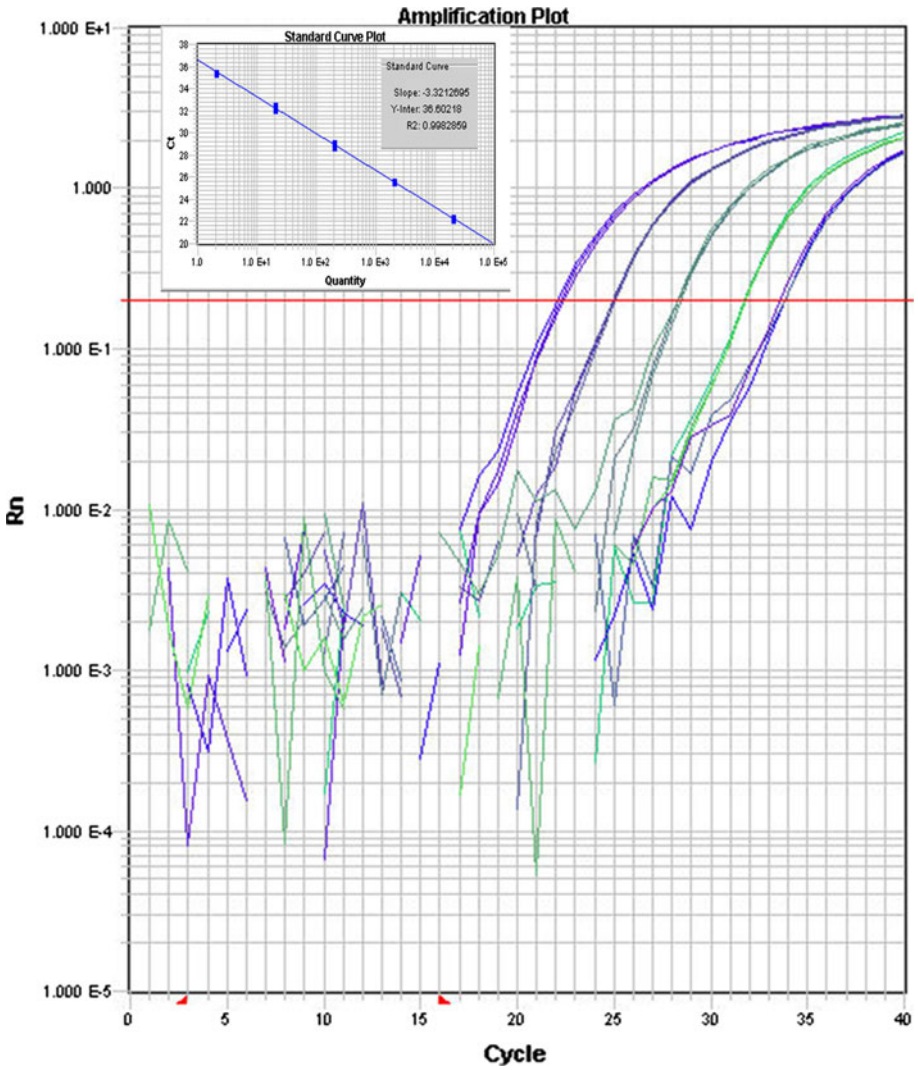

Fig. 5 **a** Amplification profile of the sugar beet DNA using six serial dilutions (20,000, 2,000, 200, 20, 15 and 10 HGC). **b** Standard curve obtained from the cited dilutions. No amplification curve was observed with 10 HGC. The LODa was confirmed as 15 HGC and the slope of the standard was -3.32 and R^2 was 0.99

Table 4 Values of standard curve slope, PCR efficiency and linearity (R^2) for the sugar beet reference gene (*ant*)

Number of runs	Sugar beet reference gene "*ant*"		
	Slope	PCR efficiency (%)	Linearity (R^2)
Run1	−3.45	95	0.98
Run2	−3.39	97	0.95
Run3	−3.48	94	0.94
Run4	−3.43	95	0.98
Run5	−3.35	99	0.99
Mean	−3.42	96	0.97

Conservation of C_t values among the genomic section I of the genus *Beta* and copy number of *ant* gene

The stability of the target sequences poorly studied for most genes is a critical point in species-specific QRT-PCR test development. The intra-species conservation of gene copy number is necessary for the reliability of the method (Fig. 4). For this purpose, real-time PCR reactions were performed in triplicate using 15.2 ng DNA templates from the entire collection of sugar beet composed of 39 species and varieties of the section *Beta* and the two GM sugar beet GTSB77 and LL beet. All the samples were tested in triplicate. The mean cycle threshold (C_t) was calculated. Using the same primer pair for amplification of *ant*, stable similar average C_t values were obtained for sugar beet (25.7 ± 0.29) wild and cultivated species and varieties from the genomic section I of the genus *Beta*. A significant C_t variability was observed between for 24 triploid varieties (24.2 ± 0.12) and for the polyploid fodder beet ($2n + 4n$) (22.28 ± 0.19). This reveals the possible influence of ploidy level in the absolute quantification and this parameter may be taken into consideration when using a reference system for GMO analysis. In fact, this problem was widely discussed by Trifa and Zhang (2004) and particularly in the case of seed kernels which are composed of different level of ploidy (triploid endosperm and diploid tegument and embryo) (Trifa and Zhang 2004).

Standard curves, repeatability, and sensitivity

Six serially diluted DNA samples using the diploid variety *Agile* of sugar beet were prepared for the construction of a standard curve. The standard curve was constructed by plotting the C_t values against the logarithm of the DNA copy number. According to the AFNOR Standard XP-V-03-020-2 (2003), the LOD corresponds to the smallest target DNA concentration for which six replicates give a positive result. The sensitivity of the method was also assessed by determining the LODa and the LOQ with the lower dilution of 15 HGC of sugar beet DNA (Table 3).

PCR efficiencies and linearity

For the generation of standard curves, five runs were conducted with the same template used in the sensitivity test. Figure 5 shows two typical standard curves in one of the five real-time PCR runs. The regression correlation coefficient (R^2) values of the standard curves were from 0.996 to 0.999 in five PCR runs, indicating excellent linearity between the DNA copy numbers in the template and the fluorescence values (C_t). The PCR reaction efficiencies were from 99.4 to 100.2 % for the standard curves of the sugar beet ant reference system, indicating highly efficient reactions. The values of the slopes from which the PCR efficiency is calculated using the formula $(10^{(-1/\text{slope})} - 1) \times 100$ (1) of the standard curves and the R^2 (expressing the linearity of the regression) reported for the developed reference system is summarized in Table 4. Two out of the five conducted runs (run 2 and run 3) showed R^2 values of 0.95 and 0.94, respectively. These later results do not comply with the EU-RL requirements ($R^2 \geq 0.98$) and needed to be taken into consideration in further studies for

Table 5 QRT-PCR amplification data used for the determination of the repeatability and reproducibility of the developed method based on the C_t values obtained

Template (HGC)	Mean C_t values (triplicate) (different periods)								Mean C_t values (triplicate) (same day)							
	R1	R2	R3	R4	R5	Mean	SD^r	$\%RSD_r$	R1	R2	R3	R4	R5	Mean	SD^R	$\%RSD_R$
"*ant*" sugar beet reference system									"*ant*" sugar beet reference system							
15	36.84	36.21	36.44	36.80	36.39	36.53	0.216	0.591	35.91	36.37	36.70	36.49	36.91	36.47	0.377	1.035
20	35.79	35.88	35.29	35.19	35.69	35.56	0.308	0.866	35.27	35.71	35.93	35.85	35.53	35.65	0.264	0.742
200	32.12	32.19	32.58	32.39	32.43	32.34	0.186	0.575	32.26	32.22	32.19	32.58	32.74	32.39	0.247	0.762
2000	28.55	28.22	28.13	28.71	28.63	28.44	0.257	0.903	28.29	28.36	28.73	28.96	28.49	28.56	0.276	0.969
20,000	25.44	25.29	25.49	25.37	25.88	25.49	0.228	0.894	25.80	25.76	25.73	25.36	25.43	25.61	0.204	0.799

SD^r repeatability standard deviation, SD^R reproducibility standard deviation, RSD_r relative standard deviation of repeatability, RSD_R relative standard deviation of the reproducibility, *R1, R2, R3, R4 and R5* runs conducted for the repeatability and reproducibility assay

the interlaboratory validation procedures which is not the scope of this study.

Repeatability and reproducibility of the method

The relative reproducibility standard deviation (RSD_R) should be below 33 % at the target concentration and over the majority of the dynamic range, while it should be below 50 % at the lower end of the dynamic range (http://gmo-rl.jrc.ec.europa.eu/). As it can be observed in Table 5, the method fully satisfies the EU requirement. In fact, the highest value of RSD_R (%) is 1.032 calculated with the C_t values and 8.00 obtained with the copy numbers (Table 6) complying well with the acceptance criterion. In the same table the relative repeatability standard deviation (RSD_r) values are also reported. According to the EU-RL requirements, it accepts that RSD_r is below 25 % (0.59 and 10.69 % calculated with the C_t values and copy number, respectively) as indicated by the ENGL (http://gmo-crl.jrc.ec.europa.eu/). As it can be observed from the values calculated, the method developed in this study complies with the requirements throughout the whole dynamic range tested. To test the repeatability and reproducibility, five independent real-time PCR runs completed at different times over a period of 30 days with six replicates per run were performed for each sample dilution. The repeatability and reproducibility of the reactions for the real-time PCR system were calculated using data for triplicate reactions obtained from the five concentration levels of sugar beet DNA. The ant gene of the sugar beet was repeatedly amplified three times in the same day with the same operator. Table 5 shows the results of the reproducibility study of the sugar beet DNA for a different period and different operator using the established method. The values of SD and RSD were all in the acceptable range according to ENGL (http://gmo-crl.jrc.ec.europa.eu/) and were from 0.204 to 0.377 and from 0.742 to 1.035 % for 1 day, respectively. The SD and RSD values obtained were in the range of 0.186–0.308 and 0.591–0.903 % for different days, respectively. All the later values were calculated according to the C_t values. However, the Table 6 shows the repeatability and the reproducibility studies according to the copy number as required in the EU-RL key documents find in the (http://gmo-crl.jrc.ec.europa.eu/). The values of SD and RSD ranged from 1.19 to 83.63 HGC and 0.41 to 8.32 % for the same day. The SD and RSD values for different period ranged from 1.05 to 81.41 HGC and from 0.4 to 10.69 %, respectively (Table 6). In conclusion, in this paper, specific primer pair (BetantF and BetantR) were designed in qualitative and quantitative PCR assays for common sugar beet. A PCR product with a 135-bp length and its related qualitative and quantitative PCR cycling conditions were found to be suitable for the detection and

Table 6 QRT-PCR amplification data used for the determination of the repeatability and reproducibility of the developed method based on the copy numbers obtained from the standard curves

Template (HGC)	Mean copy number (triplicate) (different periods)								Mean copy number (triplicate) (same day)							
	R1	R2	R3	R4	R5	Mean	SD^r	$\%RSD_r$	R1	R2	R3	R4	R5	Mean	SD^R	$\%RSD_R$
"*ant*" sugar beet reference system									"*ant*" sugar beet reference system							
15	15.9	14.3	16.1	13.9	14.1	14.86	1.05	7.06	14.9	13.3	14.1	15.9	16.1	14.8	1.19	8.00
20	21.1	18.9	19.6	17.3	18.2	18.86	1.99	10.69	22.1	19.2	18.6	19.6	17.8	19.4	1.62	8.32
200	223.9	246.1	234.3	224.1	253.6	236.4	13.24	5.6	214.2	235.1	214.9	244.6	225.9	226.9	13.1	5.77
2000	2,066.9	2,085.2	2,068.1	2,082.3	2,079.1	2,076.3	8.34	0.41	2,065.2	2,058.8	2,074.7	2,046.2	2,052.1	2,059.4	11.13	1.04
20,000	20,006.6	20,169.3	20,188.6	20,183.6	20,204.4	20,150.5	81.41	0.40	20,288.5	20,302.2	20,106.3	20,207.1	20,159.1	20,212.6	83.63	0.41

SD^r repeatability standard deviation, *SD^R* reproducibility standard deviation, *RSD*, relative standard deviation of repeatability, *RSD_R* relative standard deviation of the reproducibility, *R1, R2, R3, R4 and R5* runs conducted for the repeatability and reproducibility assay

the cycling conditions were similar to those of other widely used endogenous genes, such as maize and soybeans, in GMO detection (Shindo et al. 2002; Matsuoka et al. 2000). In addition, the sensitivities of 15 HGC of sugar beet genomic DNA for qualitative and QRT-PCR were acceptable for monitoring the GM crops. Whilst there were minor deviations in relation to C_t values with polyploidy fodder beet, the primers and probe of the designed assay were able to correctly detect and quantify the presence of the *ant* amplicons and generated the correct characteristic DNA profiles used in this study for a range of test samples and the positive controls. The results in this paper demonstrate that this *ant* fragment met the requirements for a specific reference system DNA sequence for PCR analyses without false results and may be subjected to large scale collaborative trial validation in the near future and this approach may provide an alternative route for quantifying GM sugar beet ingredients for those laboratories that have access to real-time PCR.

Acknowledgments We thank all the INRA groups who provided us samples and all the members of the EPGV-INRA unit in Evry/France and MDO laboratory in INRA Versailles/France for their precious help for providing samples and for the paper redaction.

References

AFNOR Standard XP-V-03-020-2 (2003) Détection et quantification des organismes végétaux génétiquement modifiés et produits dérivés. Partie 2: Méthodes basées sur la réaction de polymérisation en chaîne, pp 1–20. AFNOR (FR)

Anklam E, Gadani F, Heinze P, Pijnenburg H, Van den Eede G (2002) Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products. Eur Food Res Technol 214:3–26

Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9:208–218

Barbau-Piednoir E, Lievens A, Vandermassen E, Mbongolo-Mbella EG, Leunda-Casi Nancy Roosens, Sneyers A, M Van Bulcke den M (2012) Four new SYBR_Green qPCR screening methods for the detection of Roundup Ready, LibertyLink and CryIAb traits in genetically modified products. Eur Food Res Technol 234:13–23

Bertheau Y, Diolez A, Kobilinsky A, Magin K (2002) Detection methods and performance criteria for genetically modified organisms. J AOAC Inter 85:801–808

Chaouachi M, Giancola S, Romaniuk M, Laval V, Bertheau Y, Brunel D (2007) A strategy for designing multi-taxa specific reference gene systems. Example of application—ppi phosphofructokinase (ppi-PPF) used for the detection and quantification of three taxa: maize (*Zea mays*), cotton (*Gossypium hirsutum*) and rice (*Oryza sativa*). J Agric Food Chem 55:8003–8010

Chaouachi M, El Malki R, Berard A, Romaniuk M, Laval V, Brunel D, Bertheau Y (2008) Development of a real-time PCR method for the differential detection and quantification of four solanaceae in GMO analysis: potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and pepper (*Capsicum annuum*). J Agric Food Chem 56:1818–1828

Codex Committee on Methods of Analysis and Sampling (2012) In-house method validation, Codex Alimentarius Commission, CX/MAS 98/8, Budapest, Hungary, 1998, November 23–27. http://www.fao.org/. Accessed April 2012

Community Reference Laboratory for GM Food and Feed (2012) Home. http://gmo-crl.jrc.ec.europa.eu/. Accessed April 2012

Dinon AZ, Prins TW, Van Dijk JP, Arisi AC, Scholtens MJ, Kok EJ (2011) Development and validation of real-time PCR screening methods for detection of cry1A.105 and cry2Ab2 genes in genetically modified organisms. Anal Bioanal Chem 400:1433–1442

Dohm JC, Lange C, Holtgräwe D, Rosleff Sörensen T, Borchardt D, Schulz B, Lehrach H, Weisshaar B, Himmelbauer H (2011) Palaeohexaploid ancestry for caryophyllales inferred from extensive gene-based physical and genetic mapping of the sugar beet genome (*Beta vulgaris*). Plant J. doi:10.1111/j.1365-313X.2011.04898.x

EN ISO 21571 (2002) International Organization for Standardization, Geneva, Switzerland

European Commission (2012) Community reference laboratory for GM food and feed. Event specific method for the quantification of sugar beet line H7-1 using real-time PCR (protocol, accessed April 2012)

Fagan J, Schoeler B, Haegert A, Moore J, Beeby J (2010) Performance assessment under field conditions of a rapid immunological test for transgenic. J. Int of Food Science and Technology 36:357–367

Ford-Lloyd BV, Williams JT (1975) A revision of Beta section Vulgares (Chenopodiacea), with a new light on the origin of cultivated beets. Bot J Lin Soc 71:89–1402

Gašparič MB, Tengs T, La Paz JL, Holst-Jensen A (2010) Comparison of nine different real-time PCR chemistries for qualitative and quantitative applications in GMO detection. Anal Bioanal Chem 396:2023–2029

Holst-Jensen A, Bertheau Y, De Loose M, Grohmann L, Hamels S, Hougs L, Morisset D, Pecoraro S, Pla M, den Bulcke MV, Wulff D (2012) Detecting un-authorized genetically modified organisms (GMOs) and derived materials. Biotechnol Adv. doi:10.1016/j.biotechadv.2012.01.024

Kluga L, Folloni S, Van den Bulcke M, Van den Eede G, Querci M (2012) Applicability of the ''Real-Time PCR-Based Ready-to-Use Multi-Target Analytical System for GMO Detection'' in processed maize matrices. Eur Food Res Technol 234:109–118

Kodama T, Kuribara H, Minegishi Y, Futo S, Watai M, Sawada C, Watanabe T, Akiyama H, Maitani T, Teshima R, Furui S, Hino A (2009) Evaluation of modified PCR quantitation of genetically modified maize and soybean using reference molecules: inter-laboratory study. J AOAC Int 92:223–233

Matsuoka T, Kawashima Y, Akiyama H, Miura HA (2000) Method of detecting recombinant DNAs from four lines of genetically modified maize. J Food Hyg Soc Jpn 41:137–143

Paternò A, Marchesi U, Gatto F, Verginelli D, Quarchioni C, Fusco C, Zepparoni A, Amaddeo D, Ciabatti I (2009) Finding the joker among the maize endogenous reference genes for genetically modified organism (GMO) detection. J Agric Food Chem 57:11086–11091

Sarachu M, Colet M (2005) wEMBOSS: a web interface for EMBOSS. Bioinformatics 21:540–541

Schneider K, Borchardt DC, Schäfer-Pregl R, Nagl N, Glass C, Jeppsson A, Gebhardt C, Salamini F (1999) PCR-Based cloning and segregation analysis of functional gene homologues in *Beta vulgaris*. Mol Gen Genet 262:515–524

Shindo Y, Kuribara H, Matsuoka T, Futo S, Sawada CA (2002) Validation of real-time PCR analysis for line-specific quantitation of genetically modified maize and soybean using new reference molecules. J AOAC Int 85:1119–1126

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

Tranzschel W (1927) Die Arten der Gattung Beta. Bull Appl Bot Plant Breed 17:203–223

Trifa Y, Zhang D (2004) DNA content in embryo and endosperm of maize kernal (*Zea mays* L.): impact on GMO quantification. J Agric Food Chem 52:1044–1048

Wang C, Jiang L, Rao J, Liu Y, Yang LK, Zhang D (2010) Evaluation of four genes in rice for their suitability as endogenous reference standards in quantitative PCR. J Agric Food Chem 58:11543–11547

Wang X, Teng D, Yang Y, Tian F, Guan Q, Wang J (2011) Construction of a reference plasmid molecule containing eight targets for the detection of genetically modified crops. Appl Microbiol Biotechnol 90:721–731