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Event-specific qualitative and quantitative PCR detection of roundup ready event GT73 based on the 3′-integration junction

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Abstract With the development of genetically modified organisms, labeling regulations have been introduced, which require appropriate detection methods. Event-specific qualitative and quantitative polymerase chain reaction (PCR) detection methods have become the internationally agreed state-of-art. This paper describes an event-specific PCR method for qualitative and quantitative of Roundup Ready canola event GT73. The 3′-integration junction was characterized by two methods: inverse-PCR and thermal asymmetric interlaced-PCR. In the conventional qualitative PCR assay, the event-specific primers designed were confirmed to be specific and the limit of detection (LOD) was 0.05% (approximates to ten haploid genome copies). In the quantitative TaqMan real-time PCR assay, the LOD and the limit of quantification were five and ten haploid genome copies, respectively. In addition, for further quantitative detection, a reference molecule which contained the canola endogenous gene and event-specific sequence was constructed and standard curves were set up. The goodness of the linearity and high efficiency of the PCR reaction indicated the usability of the plasmid and the established PCR system. Moreover, mixed samples with different GT73 content (6, 3, 1 and 0.5%) were quantified using the established real-time PCR system to evaluate the trueness and precision of the system. The trueness expressed as bias varied from 2.00 to 18.00%. The precision expressed as variation coefficient were different from 6.40 to 32.95%. From above results, we believed that the established event-specific qualitative and quantitative PCR systems for GT73 in this study were acceptable and suitable for genetic modified canola detection.

Keywords Event-specific · Roundup ready canola event GT73 (*Brassica napus*) · Integration junction · Inverse PCR · Thermal asymmetric interlaced · TaqMan real-time PCR

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Introduction

Genetic modification can render crops resistant to herbicides, insects, microorganisms or viruses, increase their yields and improve their qualities, making crops satisfy human beings' needs better. With such advantage, genetic modified (GM) crops have been extensively cultivated and commercialized in 1990s. Up to 2005, it is estimated the global area of GM crops has reached to 90 million hectares (James 2005). However, recognition of genetically modified organism (GMO)-derived products by the public has been controversial, and concerns about their safety exist among consumers.

To sustain the commercialization of GMO, informing the consumer for GMO presence through labeling of products, has been considered a milestone. In many countries, the threshold levels for labeling products with GMO presence have been established. For example, in Europe, the threshold is defined as 0.9% (European Commission Regulation 2003).

While in Korea and Japan, the threshold is 3 and 5%, respectively (Ministry of Agriculture and Forestry of Korea 2000; Food and Marketing Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan 2000).

Accordingly, the method for detection, identification and quantification of the GMO presence in crops and food/feed products is essential to fulfill the labeling requirement (Anklam et al. 2002). Nowadays, the key technologies applied are the ''polymerase chain reaction (PCR)'' based on the DNA fragment (Holst-Jensen et al. 2003; Lipp et al. 2001) and the ''enzyme-linked immunosorbent analysis'' based on the expressed proteins (Van Duijn et al. 2002; Ermolli et al. 2006; Xu et al. 2005). The PCR technology has been the first choice of most analytical laboratories interested in detection of GMO and derived materials due to its potentially high degree of sensitivity and specificity. Concerning PCR-based GMO test, four different detection strategies: screening, gene-, construct- and event-specific PCR have been currently used to discriminate between GM- and non-GM-derived DNA (Holst-Jensen et al. 2003). The first three methods are available at present and specific when detecting the different inserted elements. However, these methods cannot differentiate between GM organisms containing the same insert (Rønning et al. 2003). The event-specific method, which is based on the principle of the current transformation techniques, can identify the different transformation events by amplifying a fragment of the unique junction between the inserted DNA and the plant DNA. Nowadays, event-specific detection methods are available for many transformation events, for example, GTS 40-3-2 soybean (Berdal and Holst-Jensen 2001; Terry and Harris 2001; Taverniers et al. 2001), MON1445, MON531 cotton (Yang et al. 2005), MON810 (Holck et al. 2002; Hernández et al. 2003), Bt11 (Rønning et al. 2003), GA21 (Hernández et al. 2004), NK603 (Nielsen et al. 2004), T25 maize (Collonnier et al. 2005) and so on. As far as we know, only one publication reporting on the event GT73 canola (Taverniers et al. 2005) has been published, but there is no report on an independent characterization and method development for this event.

In this paper, we will characterize the 3′-integration junction of GT73 canola by inverse-PCR (I-PCR) and thermal asymmetric interlaced (TAIL)-PCR and develop an event-specific qualitative and quantitative real-time PCR methods for this event.

GT73 canola, which is an herbicide-resistance event, was transformed with a plasmid vector named PV-BNGT04. The vector includes two genes encoding proteins conferring glyphosate tolerance, *cp4-epsps* gene and *gox v247* gene. The *cp4-epsps* gene is fused to the following regulatory sequences: the 35S promoter from a modified figwort mosaic virus (P-CmoVb), the 3′ end of the pea *rbcS* E9 gene (E9 3′) and a chloroplast transit peptide sequence AEPSPS/CTP2 delivering bacterial EPSPs to the chloroplasts of higher plants. And the regulatory sequences fused to the *gox* gene as follows: the 35S promoter from a modified figwort mosaic virus (P-CmoVb), the 3′ end of the pea *rbcS* E9 gene (E9 3′). By the action of the N-terminal of the small submit 1A of the ribulose 1, 5-biosphosphate carboxylase chloroplast transit peptide sequence Arab-SSU1A/CTP1, *gox v247* gene was targeted to the plasmid (Fig. 1a). The exogenous DNA was transferred into the canola genome as a single and stable insert by an *Agrobacterium* mediated transformation system (Australia New Zealand Food Authority 2001).

Materials and methods

Materials

GT73 canola (heterozygous) and its non-GM parent seeds were kindly provided by Monsanto Company. The seeds were sowed, after growing 3 months the leaves were picked and subsequently preserved in –80°C until further use. Other GM materials, T45, MS1, RF3, MS8 and Bt11 were developed and supplied by Bayer BioScience. GTS40-3-2 soybean developed by Monsanto Company was also used.

DNA extraction

GT73 and its non-GM parent genomic DNAs were extracted from their leaves, while genomic DNAs of other crops were extracted from their seeds. Leaves and seeds were lyophilized for 24 h and then ground using a Retch ball-mill in liquid nitrogen. The obtained powders were incubated for 30 min at 65°C in the CTAB extraction buffer containing α-amylase (Sigma, USA) (10 µl of a 10 mg/ml solution) and RNase A (Sigma) (10 µl of a 20 mg/ml solution). During incubation the tubes were mixed every 3 min by inversion. Twenty microliters of a 40 mg/ml solution of proteinase K (Sigma) was added and the mixture was further incubated for 45 min at 50°C. Peptides were extracted with chloroform, and the DNA was precipitated with a 0.25 vol. of 10 M of ammonium acetate and a 2/3 vol. of isopropyl alcohol on ice for 1 h. The pellet was recovered by centrifugation at 15,000×*g* and 4°C for 30 min. The pellet was washed with 500 µl of 70% ethanol, recovered by centrifugation, and dried under vacuum at room temperature. The DNA was dissolved for 1 h in 0.1× TE buffer (1 mM Tris, 0.1 mM EDTA, pH 8) (Sambrook and Russell 2001). Plasmid DNAs were extracted and purified using QIAGEN Plasmid Midi kit (Qiagen, Germany).

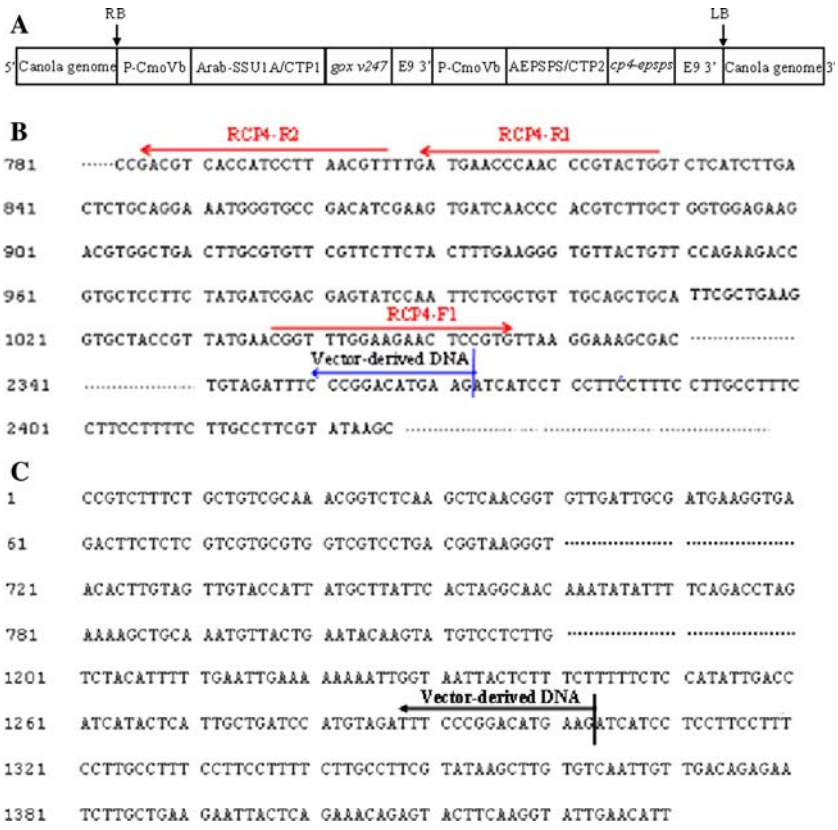

Fig. 1 Genome organization of event GT73 canola. **a** Schematic diagrams of the genomic arrangement of event GT73 canola. **b** 3′-integration junction sequences obtained by inverse-PCR. Primers for PCR amplifications detailed in Table 1 are *underlined*. **c** 3′-integration junction sequences obtained by TAIL-PCR

The quantity of DNAs was evaluated with the picogreen ds DNA quantification kit (Molecular Probes, Leiden, Netherlands). Fluorescence was detected using the FLx800 microplate fluorescence reader (Bio-Tek, USA) and analyzed by KC4 software (2000). The copy numbers were measured according to the DNA quantity and DNA average size.

Characterization of the GT73 canola 3′-integration junction

Identifying the 3′-integration junction sequences by I-PCR

The 3′-integration junction sequences of GT73 canola were identified by the I-PCR method described by Rønning et al. (2003). The experiment was performed with slight modifications.

GT73 canola genomic DNA (1 μg) was digested using 16 units of *Hind*III (Promega, USA) for 3 h at 37°C in a total volume of 60 μl, and successively was purified with the phenol/chloroform extraction protocol (Zimmermann et al. 2000). The digested DNA was self-ligated in order to get small circularized genomic DNA as follows: 6 units of T4 DNA ligase (Promega) was used to ligate 40 ng the digested DNA in a total volume of 50 μl for 24 h at 4°C. The ligase was subsequently heat inactivated at 72°C for 15 min and purified by phenol–chloroform extraction protocol used above. The pellet was resuspended in 20 μl ddH_2O.

All PCR reactions were carried out on a ABI2720 thermal cycler (Applied Biosystems, USA), in final volumes of 30 μl, containing 1× reaction buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.3 μM of each primer and 2.5 units of Pyrobest DNA polymerase (TaKaRa Biotechnology Co., Ltd, China) in a nested approach. The first reaction contained 2 μl of previously purified ligated DNA and the primers RCP4-R1 and RCP4-F1 (Table 1), using a PCR program consisting of a first 94°C denaturation step for 5 min followed by 30 cycles of 30 s denaturation at 94°C, 40 s annealing at 58°C and 1 min 40 s extension at 72°C. The second reaction contained 2 μl of the amplification product from the first PCR reaction and the primers RCP4-R2 and RCP4-F1 (Table 1), using a PCR program consisting of a first 94°C denaturation step for 5 min followed by 35 cycles of 30 s denaturation at 94°C, 40 s annealing at 59°C and 2 min extension at 72°C. All the PCR products were separated by electrophoresis on 1.2% agarose gel at constant voltage 120 V in TAE buffer. The fragments were successively isolated from the gel by cutting the gel under UV-light and purifying using Biospin gel extraction kit (Bioflux, China) and ligated into pGEM T-Easy Vector (Promega).

Table 1 Primers and probes used for identifying the 3′-integration junction and for the qualitative and quantitative PCR

PCR assay	Name	Sequence of oligonucleotide 5′–3′	Target	Size (bp)	Reference
Inverse-PCR	RCP4-F1	CGGTTTGGAAGAACTCCGTG	*cp4-epsps*		This work
	RCP4-R1	ACCAGTACGGGTTGGGTTCA			
	RCP4-R2	ACGTTAAGGATGGTGACGTCG			
TAIL-PCR	LP1-F	TGTTCCAGAAGACCGTGCTCC	*cp4-epsps*		This work
	LP2-F	AAAGCGACCGTCTTTCTGCTG			
	LP3-F	CCGTCTTTCTGCTGTCGCA			
	AD3	AG(A/T)GNAG(A/T)ANCA (A/T)AG A			
Qualitative	acc1F	GAGAATGAGGAGGACCAAGCTC	Canola endogenous	196	Hernández et al. (2001)
Qualitative, quantitative	acc1R	GGCGCAGCATCGGCT			
Quantitative	acc2F	GGTGAGCTGTATAATCGAGCGA		104	
Quantitative	accP	AACACCTCTTCGACATTCGTTCCATTGGTCGA			
Qualitative	GT1F	TGAACTTTCCTTTATGTAATTTTCCAGAA	GT73 3′-integration junction	522	This work
Qualitative, quantitative	GT1R	GCTTATACGAAGGCAAGAAAAGGA			
Quantitative	GT2F	CCATATTGACCATCATACTCATTGCT		108	
Quantitative	GT-P	TCCATGTAGATTTCCCGGACATGAAGATCA			
Construction of plasmid standard	pGTF	CGCGGATCCTGAACTTTCCTTTATGTAATTTTCCAGAA	GT73 3′-integration junction	540	This work
	pGTR	GCTTATACGAAGGCAAGAAAAGGAGGATCCGCG			
	paccF	CGGAATTCGAGAATGAGGAGGACCAAGCTC	Canola endogenous	212	
	paccRR	GGCGCAGCATCGGCTGAATTCCG			

Sequencing of the cloned DNA fragment was performed by TaKaRa Biotechnology Co., Ltd.

Identifying the 3′-integration junction sequences by TAIL-PCR

The 3′-integration junction sequences of GT73 canola were also identified using the TAIL-PCR method described by Liu and Whittier (1995). Slight modifications for isolation of the 3′-integration junction of GT73 canola were as follows. The first TAIL-PCR amplification was performed in a total volume of 30 µl containing: 1×reaction buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.5 µM primer LP1-F, 5 µM primer AD3, 2.5 units of *TaKaRa Ex Taq* HS DNA polymerase (TaKaRa Biotechnology Co.), and 50 ng of GT73 canola template DNA. The PCR program for the primary TAIL-PCR consisted of a denaturation step of 10 min at 95°C followed by 6 min at 68°C, and the following amplification cycles: 5 cycles of 30 s at 94°C and 6 min at 68°C, 1 cycle of (15 s at 94°C, 3 min at 44°C and 6 min at 68°C), 5 cycles of (15 s at 94°C, 30 s at 44°C and 6 min at 68°C), and a final step of 12 cycles of (15 s at 94°C, 7 min at 68°C, 15 s at 94°C, 7 min at 68°C, 15 s at 94°C,30 s at 44°C and 7 min at 68°C). Secondary TAIL-PCR amplification was carried out in a total volume of 30 µl containing: 1×reaction buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.2 µM primer LP2-F, 2 µM primer AD3, 5 units of *TaKaRa Ex Taq* HS DNA polymerase (TaKaRa Biotechnology Co.) and 1 µl of a 25-fold dilution of the primary PCR products. The secondary TAIL-PCR program was 10 min at 95°C, followed by 15 cycles of (15 s at 94°C, 7 min at 68°C, 15 s at 94°C, 7 min at 68°C, 15 s at 94°C, 30 s at 44°C and 7 min at 68°C). Tertiary TAIL-PCR was the same as the secondary amplification described above, except that the primer LP2-F was replaced by LP-3F and the template was 1 µl of a 25-fold dilution of the secondary PCR products. The tertiary TAIL-PCR program was 20 cycles of (30 s at 94°C, 44 s at 68°C and 6 min at 72°C), and the last step of 5 min at 72°C. All PCR reactions were carried out on an ABI2720 thermal cycler (Applied Biosystems). The three rounds PCR amplification products were analyzed by agarose gel electrophoresis with ethidium bromide staining, and the

possible amplified fragments were purified with Biospin gel extraction kit (Bioflux) and ligated into pGEM T-Easy Vector (Promega). After restriction endonuclease analysis, sequencing of the cloned DNA fragment was performed by TaKaRa Biotechnology Co., Ltd.

Preparing GM canola DNA samples used in qualitative and quantitative detection of GT73

DNA solutions extracted from GT73 and its non-GM parent were quantified using the pico-green method with a BioRad VersaFluor fluorimeter and the concentrations were adjusted to 100 ng/μl with TE buffer and homogenized by gentle agitation at 4°C overnight. Once homogenized, the concentration of the dilution was checked once again. GM canola DNA samples with different concentrations were produced by blending DNA from GM and non-GM canola. The detailed procedure was as follows [taking 10 ng/μl of GM DNA (=10% GM) for example]: 100 μl of the 100 ng/μl GM DNA were added to 900 μl of the 100 ng/μl non-GM DNA, then homogenized by gentle agitation and stored at –20°C.

Cloning and preparing the plasmid standards

The primer pairs of pGTF/R and paccF/R (Table 1) were used to amplify the 3′-integration junction sequences (540 bp) and endogenous reference gene (212 bp), respectively. The PCR amplified fragments, were co-cloned 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 linearized with *Xha*I 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 10^6 copies per 1 μl, from which a tenfold dilution series was made to serve as standard curves for real-time PCR.

Qualitative PCR

In qualitative PCR assay, all the PCR reactions were performed in final volumes of 30 μl, containing 1× reaction buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.3 μM of each primer 2.5 units of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd.) and 1 μl DNA template. All PCR reactions were carried out on a ABI2720 thermal cycler (Applied Biosystems) with the program as follows: one step of 5 min at 94°C, 35 cycles of 10 s at 94°C, 20 s at 58°C and 30 s at 72°C, one step of 5 min at 72°C. PCR products were separated by electrophoresis on 2% agarose gel at constant voltage 120 V in TAE buffer. Each reaction of one test was repeated three times and each time with triple replication.

Quantitative PCR

The oligonucleotide primers and corresponding TaqMan fluorescent dye-labeled probes between the primers were designed using ABI Prism Primer Express Version 2.0 software (Applied Biosystems). All PCR primers and fluorogenic probes were obtained from Shanghai Sangon Biotechnology Corporation (China). The probes were labeled with the fluorescent FAM reporter dye on the 5′-end and the fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) was located on the 3′ end of the probes.

Real-time PCR reactions were run on an ABI PRISM 7000 sequence detection system (Applied Biosystems) using the following program: 2 min at 50°C, 10 min at 95°C, 40 cycles of (15 s at 95°C and 1 min at 60°C). The PCR reaction was performed with the TaqMan PCR core reagents (Applied Biosystems) in a 20 μl reaction mixture containing 1×TaqMan buffer A (include ROX as a passive reference dye for real-time PCR), 400 μM each of dATP, dCTP, dGTP, 800 μM dUTP, 1 unit of AmpliTaq Gold DNA polymerse, 0.2 units of AmpErase uracil *N*-glycosylase (UNG), 3 or 4 mM $MgCl_2$ and 5 μl of the DNA solution. The concentration of primers and probes of each system were optimized according to the manufacturer's protocol.

Results and discussion

Characterization of the 3′-integration junction of GT73 canola

The aim of this work was to design an event-specific PCR system for qualitative and quantitative analysis of GT73 canola according to the 3′-integration junction. The crude genetic map of the genetic construct was obtained from disclosed information of GT73 canola (Fig. 1a) (http://www.monsanto.com/Monsanto/content/sci_tech/prod_safety/roundup_canola/es.pdf), and the primers were designed based on the *cp4-epsps* gene to amplify the 3′-integration junction by I-PCR (Fig. 1b) and TAIL-PCR (Fig. 1c).

A modified I-PCR was successfully used to obtain the 3′-integration junction of the GT73 canola. During the first amplification reaction, no fragments were detectable, subsequently, a fragment of over 2 kb was observed in a

nested PCR. The result of sequence analysis indicated that 2,576 bp from the obtained 2.63 kb fragment were identical to the sequences of *cp4-epsps* and E9 3′, and about 54 bp showed 100% homology with the sequence (NCBI accession number AX685147). AX685147 is a chimeric genomic and insert sequence and not from T-DNA, so the 54 bp may be from the unknown canola genome (Fig. 1b).

However, one the difficulties of I-PCR resides in finding suitable restriction sites in the target region to produce circularized DNA with a suitable size. We selected *Hind*III to digest the genomic DNA according to the available information of the crude genetic map. The BLAST result in NCBI showed that the target region contained the *Hind*III site, so *Hind*III might not be an ideal restriction endonuclease when expecting longer unknown sequences.

By using TAIL-PCR, the aim was also achieved. Amplified products in the three rounds were detected with agarose gel electrophoresis. The non-GM canola was used as the control, and no amplification products were observed. Although the target products in the PCR were verified by the size shift of the products (Liu et al. 1995), in our substantial experiments we found the products in the three rounds PCR always produce several bands. It is difficult to ascertain the target products by the size shift. So we designed several sets of specific primer pairs located within the known sequence to identify the amplified products. After each round of the hemi-nested PCR, we identified the PCR products by these specific primer pairs. Then all the possible products were sequenced. We analyzed some of the sequence results. The results showed that a fragment of 1,430 bp from a tertiary PCR was our target product, because the sequence alignments showed 1,303 bp, showed 100% homology with a sequence (NCBI accession number DD417675, which is a synthetic construct having Glyphosate tolerant), and the rest sequences gave a 100% match with a sequence (NCBI accession number AX685147) and part of it are identical with the 54 bp obtained by I-PCR, so the 127 bp may be from the unknown canola genome (Fig. 1c).

Each strategy has its own characteristic. I-PCR, described by Ochman et al. (1988), is a variant of PCR when only one internal sequence is known. It involves a series of digestions and self-ligation before cutting by an endonuclease, resulting in known sequences at either end of the unknown sequence. It is extensively used and is a useful, relative fast, reliable tool in identifying flanking sequences. However, the procedure is complex. TAIL-PCR, which is an efficient tool for the recovery of DNA fragments adjacent to known sequences, has been successfully used to isolate sequences (Terauchi and Kahl 2000; Liu and Whittier 1995; Liu et al. 1995; Rolland et al. 2003). This method has advantage over other methods with respect to simplicity, specificity, efficiency, speed and sensitivity. However, the designation of the primers and the cycle order are important and difficult. In addition, main significant limitations of this technique are its 20–40% failure rate and the size of the obtained DNA products, usually in the ranges from 300 bp to 1 kb (Antal et al. 2004). Moreover, in our experiment we found the complexity of the PCR amplification, the lowly complete repetitiveness and the phenomenon of bands migration. It may be caused by the complexity of the amplification program and the randomicity of arbitrary primers. Taking the above into account, we employed the two methods to achieve our aim. From the results of sequence analysis, we concluded that both methods were useful and the results obtained with the two alternative approaches yielded corresponding results, putting more strength to the reliability of the resulting data.

Qualitative detection of GT73 canola by conventional PCR

After characterizing the 3′-integration junction, an event-specific PCR system for GT73 canola was established with event-specific primer pair GT1F/1R, located within the pea *rbcS* E9 gene and the canola genomic sequence flanking the insert, respectively. This yielded a PCR product of 522 bp. The canola species-specific gene *acc* was used as an endogenous reference control (Hernández et al. 2001). A single fragment of the expected size was obtained using the GT73 canola as template, while no fragment was observed using other GM canola lines (T45, MS1, RF3 and MS8), GM maize (Bt11), GM soybean (GTS40-3-2) and no template control (Fig. 2a). The observation of the expected 196 bp fragment of the *acc* gene in all canola samples indicated that the PCR reactions were not inhibited (Fig. 2b).

The sensitivity of detection was also determined. Samples with six different levels of GT73 canola's content: 10, 5, 2, 1, 0.1 and 0.05% were prepared from GT73 canola and its non-GM parent DNA samples. The results showed that the target fragment could be detected from all of the levels tested, which meant that the lowest tested level was 0.05%, which approximates to ten haploid genome copies on the basis of canola genomic DNA of 5.6 pg per haploid genome. (Arumuganathan and Earle 1991) (Fig. 2c).

Quantitative detection of GT73 canola by TaqMan real-rime PCR

Determination the LOD and LOQ of the event-specific quantitative assay

To determine the limit of detection (LOD) and the limit of quantification (LOQ) of event-specific quantitative assay, a series of PCR reactions were carried out using known amounts of GT73 genomic DNA ranging from 50 to

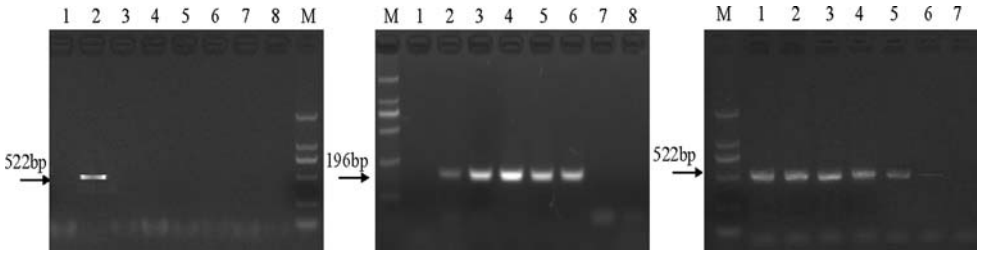

Fig. 2 Specificity and sensitivity qualitative detection of GT73 canola. **a** Specificity of event-specific qualitative results. *Lane 1* no template control (NTC); *lanes 2–8*: GT73, T45, MS1, RF3 and MS8 canola, Bt11 maize, GTS40-3-2 soybean; *lane M*: DL2000 DNA marker. **b** Canola endogenous reference control, *acc* gene, species-specific result. *Lanes* are the same as in **a**. **c** Sensitivity test of GT 73 canola qualitative detection. *Lane 1–6*: 10, 5 2, 1, 0.1 and 0.05% GT73 canola contents, respectively; *lane 7*: NTC; *lane M*: DL2000 DNA marker

0.005 ng, which were a tenfold dilution series, and the event-specific primers and probe were used. Each reaction was tested in three parallel reactions and repeated three times. The fluorescence signals could be detected in eight PCR reactions among all the nine PCR reactions, when the amount of the DNA template was lowered to 0.005 ng. In statistical, the LOD given by this does not meet the definition of LOD [The LOD usually refers to the concentration at which detection can be achieved reliably with an acceptable probability (usually 95% confidence)]. And from Poisson distributions a theoretical LOD below five copies does not make sense. So we did the experiment with the sample containing 0.025 ng GT genomic DNA (which approximates to five haploid genome copies), tested the sample nine times and this time the fluorescence signals can be detected in all nine times, which indicated that the LOD of the quantitative PCR approximate to five haploid genome copies. However, DNA content at this level still could not be quantified. We obtained standard curves when samples with DNA amounts ranging from 50 to 0.05 ng, the positive fluorescence signals can be detected in all reactions and the repeatability of experiments are all acceptable. One of the standard curves generated showed an R^2 of 0.9949 and a slope of –3.29 (the theoretically optimal value is –3.32) (Fig. 3b). This indicated that an acceptable correlation between the original amount of DNA and the Ct values and a high efficiency of the PCR reaction. According to above, we concluded that the LOQ of the event-specific real-time PCR assay was about ten haploid genome copies.

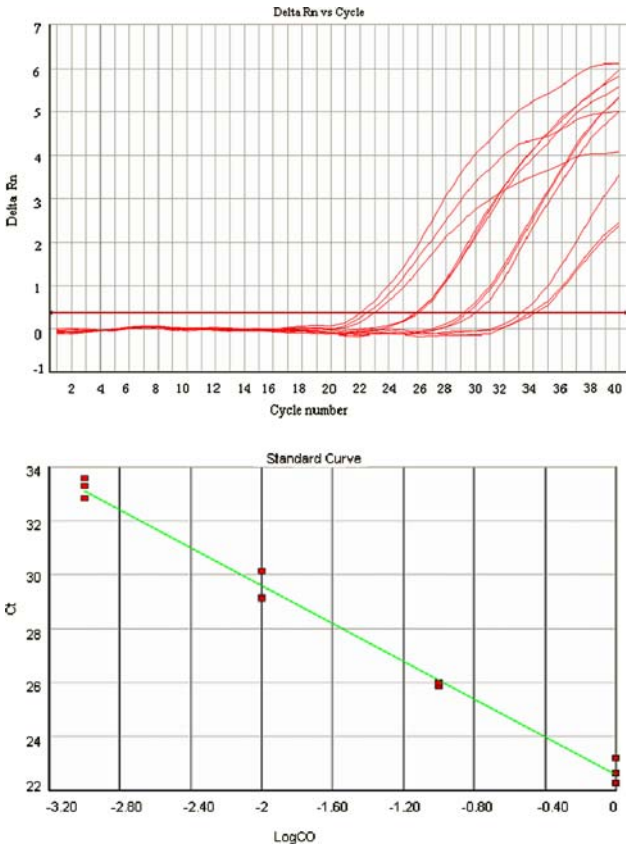

Fig. 3 Quantitative amplification with event-specific primers-probe system. **a** Amplification plots generated by a tenfold serial dilution of GT73 canola genomic DNA ranging from 50 to 0.05 ng with the GT2F/1R primers and the GTP probe. **b** Standard curve generated from the amplification data given in Fig. 3a

Construction of reference molecule

The certified reference materials (CRMs) are generally used as reference materials in real-time quantification of GMOs. 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 labor- and cost-intensive tasks (Trapmann et al. 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

Table 2 Data of plasmid standard constructing of the standard curves

RM copies	Replicate	Species-specific gene							Event-specific sequence						
		Ct			Mean	Measured copy number	SD	CV (%)	Ct			Mean	Measured copy number	SD	CV (%)
		Run 1	Run 2	Run 3					Run 1	Run 2	Run 3				
	1	36.93	37.91	38.06					37.97	38.29	38.43				
10	2	37.21	37.68	37.74	37.59	10.32	0.43	1.11	38.21	37.96	38.09	38.16	9.82	0.63	1.67
	1	33.86	34.19	34.67					33.82	34.91	34.37				
100	2	33.61	34.01	34.39	34.12	99.25	0.38	1.11	34.06	34.81	34.59	34.43	109.09	0.43	1.24
	1	30.16	30.69	30.48					30.85	31.13	30.79				
1,000	2	30.42	31.03	30.78	30.59	992.27	0.31	1	31.18	31.07	31.04	31.01	992.41	0.38	1.22
	1	27.54	26.93	27.46					27.28	27.49	27.91				
10,000	2	26.91	27.04	27.04	27.16	9294.33	0.27	1	27.59	27.78	27.67	27.62	8854.76	0.27	0.96
	1	23.09	23.39	23.41					23.62	23.54	23.94				
100,000	2	23.42	23.74	23.73	23.46	103,822	0.24	1.04	23.83	23.93	24.23	23.85	100,973	0.25	1.04
	1	19.67	20.11	20.16					19.97	20.04	20.52				
1,000,000	2	19.94	20.01	19.87	19.96	1,017,889	0.18	0.89	20.21	20.38	20.21	20.22	1,051,918	0.21	1.01
Slope		–3.45	–3.55	–3.59	–3.53				–3.55	–3.61	–3.54	–3.57			
Y-intercept		40.65	41.31	41.55	41.169				41.47	41.92	41.69	41.695			
R^2		0.999	1	1	0.9995				0.999	1	0.999	0.9992			
E (efficiency) (%)		95	91	90	92				91	89	92	91			

DNA copy number may be doubted (Taverniers et al. 2004). To overcome these drawbacks, linearized plasmid DNA has been used and was recognized as a cheaper and more feasible alternative to CRMs. Plasmid first used by Taverniers et al. (2001) was a single-insert one. Due to some shortcomings of this kind of plasmid, a double-insert plasmid standard was used as a replacement as described by Mattarucchi et al. (2005). The experimental difference between two single-insert plasmids will not occur using a double-insert plasmid. Therefore, it is a more powerful quantitative tool. We constructed a double-insert plasmid, named pGT (12.059 kb), as the reference molecule (RM). Sequence analysis showed part of the cloned plasmid sequence matched with 3′-integration junction sequences and another part of it gave a 100% match to the *acc* gene.

Construction of standard curves

To construct standard curves, the plasmid DNA was firstly linearized at the restriction sites located outside the integrated fragments, and serially diluted from 10^6 to 10 copies per 1 μl. According to the sizes of the RM and the canola genome, we considered that the range of copy numbers from 10^1 to 10^6 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 labeling regulations in EU (0.9%), Korea (3%) and Japan (5.0%).

The species-specific gene and event-specific sequence were separately amplified to set up standard curves. Each concentration of RM was analyzed in two replicates and three independent runs were performed. Data are shown in Table 2. From the high square regression coefficients (R^2) and high efficiency of the PCR reaction (E) we could conclude that good standard curves were obtained.

The reproducibility and repeatability of real-time PCR assays can be analyzed by the data shown in Table 2. For the real-time PCR assay for species-specific gene detection, the mean Ct values varied from 19.96 to 37.59 with a standard deviation (SD) value from 0.18 to 0.43 and coefficient of variation (CV) value from 0.89 to 1.11%. The event-specific PCR gave mean Ct values from 20.22 to 38.16 with a SD value from 0.21 to 0.63 and CV value from 0.96 to 1.67%. These results also indicated that we obtained a fine standard curve with plasmid DNA.

Quantitative detection of the content of GT73 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 GT73 with four different GM contents (6, 3.0, 1.0 and 0.5%). 100 ng DNAs extracted from mixed samples were used as template in one PCR reaction, and each reaction was analyzed in three parallels. The Ct values of mixed samples were measured in the real-time 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 species-specific sequence and multiplying this number by 100. Samples with GT73 canola concentrations of 6, 3.0, 1.0 and 0.5%, gave calculated results of 6.13, 3.07, 1.11 and 0.59%, respectively

Table 3 Analysis of the trueness and precision of the real-time quantitative system

Samples (%)	PCR run	Measured copy number		GM content (%)	Trueness		Precision	
		Species-specific gene	Event-specific sequence		Mean content (%)	Bias (%)	SD	CV (%)
	1	19,267	958	5.83				
	2	21,725	1,365	5.97				
6	3	20,683	1,458	6.57	6.12	2.00	0.39	6.40
	1	17,758	571	3.22				
	2	19,231	509	2.65				
3	3	20,129	673	3.34	3.07	2.33	0.37	12.09
	1	16,591	207	1.25				
	2	19,113	171	0.89				
1.00	3	17,695	216	1.22	1.12	12.00	0.20	17.53
	1	15,634	57	0.36				
	2	16,381	116	0.71				
0.50	3	14,887	103	0.69	0.59	18.00	0.19	32.95

(Table 3). The CV value and the error in Table 3 for 0.5% is 32.95 and 18%, respectively. These high values may be caused by the low GMO concentration.

In conclusion, we reported the sequence of the 3′-integration junction of GMO canola GT73. Based on this sequence, we established the event-specific qualitative and quantitative PCR systems for the reliable and accurate detection of GT73 canola. Due to the specificity of the established systems, we believe that this method is a new contribution to the labeling system for GMOs, and also suitable for event-specific detection and quantification of food and feed products.

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References

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

Antal Z, Rascle C, Fèvre M, Bruel C (2004) Single oligonucleotide nested PCR: a rapid method for the isolation of genes and their flanking regions from expressed sequence tags. Curr Genet 46:240–246

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

Australia New Zealand Food Authority (2001) Food derived from glyphosate-tolerant canola line GT73 a safety assessment. Technical Report Series No.8, pp 5–8

Berdal KG, Holst-Jensen A (2001) Roundup ready soybean event specific real-time quantitative PCR assay and estimation of the practical detection and quantification limits in GMO analyses. Eur Food Res Technol 213:432–438

Collonnier C, Schattner A, Berthier G, Boyer F, Coué-Philippe G, Diolez A, Duplan MN, Fernandez S, Kebdani N, Kobilinsky A, Romaniuk M, De Beuckeleer M, De Loose M, Windels P, Bertheau Y (2005) Characterization and event specific-detection by quantitative real-time PCR of T25 maize insert. J AOAC Int 88:536–546

Ermolli M, Fantozzi A, Marini M, Scotti D, Balla B, Hoffmann S, Querci M, Paoletti C, Van den Eede G (2006) Food safety: screening tests used to detect and quantify GMO proteins. Accredit Qual Assur 11:55–57

European Commission Regulation (EC) No.1829/2003 and 1830/2003 (2003) Off. J. Eur Commun L 268:1–28

Food and Marketing Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan, Tokyo, Japan (2000) Notification No.1775

Hernández M, Río A, Esteve T, Prat S, Pla M (2001) A rapeseed-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. J Agric Food Chem 49:3622–3627

Hernández M, Pla M, Esteve T, Prat S, Puigdomènech P, Ferrando A (2003) A specific real-time quantitative PCR detection system for event MON810 in maize YieldGard® based on the 3′-transgene integration sequence. Transgenic Res 12:179–189

Hernández M, Esteve T, Prat S, Pla M (2004) Development of real-time PCR system based on SYBR Green I, Amplifluor and TaqMan technologies for specific quantitative detection of the transgenic maize event GA21. J Cereal Sci 39:99–107

Holck A, Vaïtilingom M, Didierjean L, Rudi K (2002) 5′ nuclease PCR for quantitative event specific detection of the genetically modified MON810 MaisGard maize. Eur Food Res Technol 214:449–454

Holst-Jensen A, Rønning SB, Løvseth A, Berdal KG (2003) PCR technology for screening and quantification of genetically modified organisms (GMOs). Anal Bioanal Chem 375:985–993

http://www.monsanto.com/Monsanto/content/sci_tech/prod_safety/roundup_canola/es.pdf

James C (2005) Global status of commercialized biotech/GM crops. ISAAA Briefs 34-2005

Lipp M, Bluth A, Eyquem F, Kruse L, Schimmel H, Van den Eede G, Anklam E (2001) Validation of a method based on polymerase chain reaction for the detection of genetically modified organisms in various processed foodstuffs. Eur Food Res Technol 212:497–504

Liu YG, Whittier RF (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics 25:674–681

Liu YG, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J 8:457–463

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. Eur Food Res Technol 221:511–519

Ministry of Agriculture and Forestry of Korea (2000) Notification No.2000-31, Seoul

Nielsen CR, Berdal KG, Holst-Jensen A (2004) Characterisation of the 5′-integration site and development of an event-specific real-time PCR assay for NK603 maize from a low starting copy number. Eur Food Res Technol 219:421–427

Ochman H, Gerber AS, Hartl DL (1988) Genetic applications of an inverse polymerase chain reaction. Genetics 120:621–623

Rolland S, Jobic C, Fe'vre M, Bruel C (2003) *Agrobacterium*-mediated transformation of *Botrytis cinerea*, simple purification of monokaryotic transformants and rapid conidia-based identification of the transfer-DNA host genomic DNA flanking sequences. Curr Genet 44:164–171

Rønning SB, Vaïtilingom M, Berdal KG, Holst-Jensen A (2003) Event specific real-time quantitative PCR for genetically modified Bt11 maize (*Zea mays*). Eur Food Res Technol 216:347–354

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor

Taverniers I, Windels P, Van Bockstaele E, De Loose M (2001) Use of cloned DNA fragments for event-specific quantification of genetically modified organisms in pure and mixed food products. Eur Food Res Technol 213:417–424

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. Anal Bioanalytical Chem 378:1198–1207

Taverniers I, Windels P, Vaitilingom M, Milcamps A, Van Bockstaele E, Van Den Eede G, De Loose M (2005) Event-specific plasmid standards and real-time PCR methods for transgenic Bt11, Bt176, and GA21 Maize and transgenic GT73 canola. J Agric Food Chem 53:3041–3052

Terauchi R, Kahl G (2000) Rapid isolation of promoter sequences by TAIL-PCR: the 5′-flanking regions of *Pal* and *Pgi* genes from yams *(Dioscorea)*. Mol Gen Genet 263:554–560

Terry CF, Harris N (2001) Event specific detection of roundup ready soybean using two different real time PCR detection chemistries. Eur Food Res Technol 213:425–431

Trapmann S, Schimmel H, Kramer GN, Van Den Eede G, Pauwels J (2002) Production of certified reference materials for the detection of genetically modified organisms. J AOAC Int 85:775–779

van Duijn G, Van Biert R, Bleeker-Marcelis H, Van Boeijen I, Adan AJ, Jhakrie S, Hessing M (2002) Detection of genetically modified organisms in foods by protein- and DNA-based techniques: bridging the methods. J AOAC Int 85:787–791

Xu W, Huang K, Zhao H, Luo Y (2005) Development and application of immunoaffinity column as cleanup tool for an enzyme linked immunosorbent assay of phosphinothricin-N-acetyltransferase detection in genetically modified maize and rape. J Agric Food Chem 53:4315–4321

Yang L, Pan A, Zhang K, Yin C, Qian B, Chen J, Huang C, Zhang D (2005) Qualitative and quantitative PCR methods for event specific detection of genetically modified cotton MON1445 and MON531. Transgenic Res 14:817–831

Zimmermann A, Lüthy J, Pauli U (2000) Event specific transgene detection in Bt11 corn by quantitative PCR at the integration site. Lebenson Wiss Technol 33:210–216