Food Chemistry 112 (2009) 232–238

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Event-specific qualitative and quantitative PCR detection of genetically modified rapeseed Topas 19/2

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ARTICLE INFO

Article history:
Received 4 September 2007
Received in revised form 21 May 2008
Accepted 24 May 2008

Keywords:
Topas 19/2
Genetically modified organism
Herbicide-tolerant rapeseed
Event-specific method
Real-time PCR
Quantitative PCR

ABSTRACT

The herbicide-tolerant transgenic rapeseed Topas 19/2 (synonym HCN92) has been approved for environmental release in Canada, Japan, Australia and the USA, and exported to a number of other countries as raw material. The purpose of this study was to establish event-specific qualitative and quantitative detection methods for Topas 19/2. The 3′-integration junction sequence spanning the host plant DNA and the integrated transgene of the Topas 19/2 event was isolated and identified. The event-specific qualitative detection method was established to produce an amplicon of 110 basepairs (bp) with an absolute detection limit of 10 initial template copies. The event-specific quantitative detection method was developed with the limit of detection (LOD) and limit of quantification (LOQ) being approximately 5 and 50 initial template copies, respectively. The developed real-time PCR systems were assessed using two mixed rapeseed samples with known Topas 19/2 contents. Expected results were obtained.

1. Introduction

In recent years, more than 100 genetically modified (GM) plant events have been approved for commercialization in different countries, among which 15 transformation events of rapeseed (*Brassica napus*) are included (Agbios, 2007a). GM rapeseed is commercially planted in Canada, the USA and Australia. Millions of tons of GM rapeseed seeds and draft rapeseed oil are exported from producing countries as food and feed material every year (Lu, Xiao, & Wu, 2005).

The commercialization and exportation of genetically modified organisms (GMOs) has led to the introduction of labeling regulations in more than 30 countries and regions in order to protect the consumers' right to information (Matsuoka, 2001). At present, the legislation for GM food labeling varies in different countries. For example, there is a 0.9% regulatory threshold of GMO content for labeling in the European Union (EU) and in Russia (Chief Medical Officer of the Russian Federation, 2007; European Commission, 2003a, 2003b), while 3% and 5% in South Korea and Japan, respectively, (Matsuoka, 2001; Ministry of Agriculture, 2000). In China, no mandatory labeling threshold has been set so far, though label is mandatorily implemented for 17 food materials derived from 5 species of GM plants (Ministry of Agriculture of the People's Republic of China, 2002). The legislative requirements for labeling mean that establishment of an efficient and accurate detection method for every specific GMO is imperative.

The most generally accepted GMO detection methods are based on the polymerase chain reaction (PCR), because of the high stability of the DNA molecule and the capability to amplify specific DNA fragments from highly processed materials (Hübner, Waiblinger, Pietsch, & Brodmann, 2001; Miraglia et al., 2004). Four PCR strategies can be distinguished depending on the amplification target sequences: screening, gene-, construct- and event- specific detection (Miraglia et al., 2004). Each method has a different sensitivity and different capacity to discriminate a specific GM-derived DNA from others (Anklam, Gadani, Heinze, Pijnenbrug, & Van den Eede, 2002; Blook & Schwarz, 2004; Wolf et al., 2000; Yang et al., 2005). The event-specific PCR is considered the most appropriate for GMO labelling purposes according to a threshold.

The event-specific PCR method targets the junction border between the insert and the host genome. Because the integration of a transgenic construct into the genome can not be replicated at the same locus with current technology, the junction sequence is unique for both copy number and location despite possible rearrangements of the genome. Unlike the other detection methods, the event-specific method can distinguish different GM lines transformed with the same plasmid such as in the transgenic events Rf1 and Rf2 of Aventis (US Food and Drug Administration, 1999). Essential for the event-specific method is isolation of the junction sequence. Zimmermann et al. first used an inverse PCR strategy to isolate the junction fragment of Bt11 corn and established an unambiguous Bt11 detection PCR system (Zimmermann, Lüthy, &

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doi:10.1016/j.foodchem.2008.05.105

Pauli, 2000). The EU funded the QPCRGMOFOOD project in February 2000, and has published so far the junction sequences and correspondent event-specific detection methods for GTS 40-3-2 soybean, Mon810, Bt11, Bt176, T25, CBH 351, GA21 and DBT418 maize (Qpcrgmofood, 2003). Other laboratories have established the event-specific detection methods for other transgenic crops, including Roundup Ready Soybean (Berdal & Holst-Jensen, 2001; Taverniers et al., 2005; Terry & Harris, 2001), StarLink™ maize (Windels et al., 2003), maize NK603 (Huang & Pan, 2004), herbicide-tolerant rapeseed such as GT73 (Taverniers et al., 2005), T45 (Yang et al., 2006), Ms1 × Rf1 and Ms1 × Rf2 (Wu, Wu, Xiao, & Lu, 2007). In Europe the Community Reference Laboratory for GM Food and Feed (CRL–GMFF) is the major institution for the scientific assessment and validation of detection methods for GM food and feed. The validated event-specific detection protocols are published on the website of CRL–GMFF, which cover most of the major commercialized GM plants (Community Reference Laboratory, 2007). However, the event-specific detection protocol for Topas 19/2 is not yet available.

Topas 19/2 (synonym HCN92) is a herbicide-tolerant transgenic rapeseed cultivar developed by the Aventis CropScience company (Lyon, France), which has been approved for environmental release in Canada, Japan, Australia and the USA since 1995 (Agbios, 2007a; Lu et al., 2005) and for import as raw materials to such countries as China, the EU and Mexico. Molecular characterization of Topas 19/2 demonstrated that a synthetic *pat* gene coding for phosphinothricin acetyltransferase (PAT) and a *neo* gene coding for neomycin phosphotransferase II (NPT II) were inserted into the genome of rapeseed (Agbios, 2007b). Expression of the *pat* gene was regulated by the Cauliflower Mosaic Virus (CaMV) 35S promoter and terminator sequences. The *neo* gene was driven by the nopaline synthase promoter and an octopine synthase terminator sequence (Agbios, 2007b; Japanese Biosafety Clearing House, Ministry of Environment, 2007).

This research was designated to isolate the 3′ end junction sequence of the Topas 19/2 event, and to establish event-specific methods for qualitative and quantitative PCR detection of Topas 19/2.

2. Materials and methods

2.1. Plant materials

Genuine seeds of GM rapeseed Ms8 × Rf3, Ms1 × Rf1, Ms1 × Rf2, Oxy-235, T45 and Topas 19/2 were provided by Bayer CropScience Co. (Monheim, Germany). Genuine seeds of GM rapeseed RT73 were provided by Monsanto Co. (St. Louis, MO, USA).

Non-transgenic seeds of *Brassica napus* cv. 821, *Arabidopsis thaliana*, *B. rapa*, *B. oleracea*, *B. juncea*, *Glycine max*, *Oryza sativa*, *Zea mays* and *Gossypium hirsutum* were collected by our laboratory (Wuhan, China).

Seeds of Topas 19/2, non-transgenic *B. napus* and *Zea mays* were sown and cultivated in a greenhouse at 22 °C. One month after sowing, the fresh leaves were collected for DNA extraction.

2.2. DNA extraction

For the genome walking, the primers/probes screening, the real-time PCR optimization and the standard curve development, genomic DNA was extracted and purified from young leaves of *B. napus* and *Z. mays* following a cetyltrimethylammonium bromide (CTAB) based protocol as described by Saghai-Maroof, Soliman, Jorgensen, and Allard (1984).

For the sample detection experiments, genomic DNA was extracted and purified from seeds with the DNA Extraction Kit for GMO Detection Ver. 2.0 (Takara, Shiga, Japan).

DNA concentrations were estimated with a spectrophotometer Lambda 25 (Perkin Elmer, Ames, IA, USA) and further checked by agarose gel electrophoresis, ethidium bromide staining and calculated with Quality One software (Bio-Rad, Hercules, CA, USA).

2.3. Junction isolation

The 3′ end junction sequence of the Topas 19/2 event was isolated using a GenomeWalker Universal Kit (Clontech, Mountain View, CA, USA).

The Topas 19/2 genomic DNA libraries were constructed according to the manufacturer's manual. Based on the released preliminary schematic map of the transgenic vector pOCA18/Ac (Japanese Biosafety Clearing House, Ministry of Environment, 2007), the primers were designed to isolate the genome-Topas 19/2 event junction according to the sequence of the NPTII gene (GenBank accession no. AF485783). Details of the primers are shown in Table 1.

The primary PCR was carried out with the KOD-Plus-kit (Toyobo, Osaka, Japan) in a volume of 50 μL containing 5 ng Topas 19/2 genomic DNA library, 1 × KOD-Plus-buffer, 1 mM $MgSO_4$, 200 μM each of dNTPs, 100 nM each of kit-provided adapter primer AP1 and gene-specific primer TOPLB-1, and 1 unit KOD-Plus-DNA polymerase. The PCR reactions were performed on a GeneAmp® 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using a program consisting of a 94 °C initial denaturation step for 2 min; followed by 45 cycles of 94 °C denaturation for 15 s, 68 °C annealing and extension for 3 min; and a 68 °C final extension for 7 min. Each of the primary PCR products was diluted with water (1–49 μL), and then 1 μL each of the diluted products was used as the template of the secondary PCR.

Using the adapter primer AP2 provided in the kit and the gene specific primers TOPLB-2, the secondary PCRs were carried out in the same buffer as the primary PCRs. The program for the secondary PCRs consisted of a 94 °C initial denaturation step for 2 min; followed by 25 cycles of 94 °C denaturation for 15 s, 68 °C

Table 1
Primers and fluorogenic probes used in this research

Purpose	Name	Sequence (5′–3′)	Amplicon size (bp)	Specificity
Genome walker kit adapter primers	AP1	GTAATACGACTCACTATAGGGC		Genome walker kit adapter
	AP2	ACTATAGGGCACGCGTGGT		
Isolation of junction fragments in Topas 19/2	TOPLB-1	TCGCCCAATAGCAGCCAGTCCCTTCC		NPTII gene
	TOPLB-2	AGTCCCTTCCCGCTTCAGTGACAACG		
Conventional and qualitative PCR analysis of *HMG-I/Y*	*hmg*-F	GGTCGTCCTCCTAAGGCGAAAG	99	Genome
	hmg-R	CTTCTTCGGCGGTCGTCCAC		
	hmg-P[a]	CGGAGCCACTCGGTGCCGCAACTT		
Conventional and qualitative PCR analysis of 3' junction fragments	TOPLG	CGGCCTTAATCCCACCCCAG	110	Genome
	TOPLV	AGTTCCAAACGTAAAACGGCTT		Construct
	TOPLP[a]	TCCCGGTCATATATCAGCGCCGGTC		Junction site

[a] The probes were labeled with 5′-FAM and 3′-TAMRA.

annealing and extension for 3 min; and a 68 °C final extension for 7 min.

The PCR products were checked by electrophoresis on 1% agarose gels (TAE buffer) and subcloned into the pZErO-2 vector (Invitrogen, Carlsbad, CA, USA) through the *Eco*RV restriction enzyme site. All the constructs containing the junctions were sequenced using the M13 forward primer and M13 reverse primer (Sunbiotech, Beijing, China).

Each sequence was aligned with those in the GenBank database using Blastn analysis to confirm that the isolated junctions truly span the integration border between the genome and the integrated construct.

2.4. Primers and probes

Oligonucleotide primers and TaqMan® fluorescent probes were designed with the aid of the software Primer Premier Version 5.00 (PREMIER Biosoft International, Palo Alto, CA, USA) specifying an optimal amplicon size of no more than 150 bp and a melting temperature (tm) of 60 °C for primers and 70 °C for probes, respectively. The 5′ ends of the TaqMan® fluorescent probes were labelled with the fluorescent reporter 6-carboxy-fluorescein (FAM), and the 3′ end with the fluorescent quencher 6-carboxytetramethylrhodamine (TAMRA). All the primers and probes (see Table 1) were synthesized by Sangon (Shanghai, China).

The primer TOPLV was designed to anneal with the inserted transgene sequence of the Topas 19/2 event, and the TOPLG primer was concordant with the rapeseed genome. Combined with the TaqMan® probe TOPLP, the primer pair TOPLG/TOPLV was employed in the event-specific qualitative and quantitative PCR detections of the 3′-junction of the Topas 19/2 event and yielded a product of 110 bp.

The primers and probe for the quantitation of the *High-mobility-group protein I/Y* (*HMG-I/Y*) gene were synthesized according to the protocol published by Weng et al. (2005).

2.5. Qualitative PCR conditions

In the qualitative PCR assay 100 ng of genomic DNA was used as template in a volume of 25 μL. The reaction mixture contained 1 × PCR buffer (10 mM Tris·HCl, pH 8.3, 50 mM KCl), 200 μM of each dNTPs, 2.5 mM $MgCl_2$, 250 nM each primers, and 1 unit Hot Start Taq (Takara, Shiga, Japan). The PCR amplifications were carried out on a GeneAmp® 9700 thermal cycler using the following program: a 94 °C initial denaturation step for 2 min; followed by 40 cycles of 94 °C denaturation for 15 s, 60 °C annealing for 30 s, and 72 °C extension for 30 s; and a 72 °C final extension for 2 min. The PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide for visualization.

2.6. Quantitative PCR conditions

The real-time quantitative PCR reactions were performed for the *HMG-I/Y* gene and the 3′-junction of the Topas 19/2 event separately on a fluorometric thermal cycler (DNA Engine Opticon® 2 Continuous Fluorescence Detector, MJ Research, Waltham, MA, USA). Fluorescence signals were monitored and analyzed by the software Opticon Monitor® 2 Version 2.02 (MJ Research, Waltham, MA, USA).

The real-time PCR assay of the *HMG-I/Y* gene was performed according to the protocol of Weng et al., 2005. The reaction mixture contained 100 ng genomic DNA as template, 1 × TaqMan® buffer (50 mM KCl, 10 mM Tris–HCl, 10 mM EDTA, pH 8.3), 5 mM $MgCl_2$, 500 nM each primer *hmg*-F and *hmg*-R, 300 nM probe *hmg*-P, 200 μM each dATP, dCTP and dGTP (Fermentas, Vilnius, Lithuania), 400 μM dUTP (Fermentas, Vilnius, Lithuania), 0.2 unit UNG Amperase Uracil *N*-glycosylase (UNG, Applied Biosystems, Foster City, CA, USA) and 1.25 units AmpliTaq™ Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μL.

The concentrations of $MgCl_2$, dNTPs/dUTP and primers/probe were optimized sequentially through the PCR amplifications on the real time PCR machine. Firstly, the PCR reactions were performed in a volume of 20 μL containing 0.1 ng Topas 19/2 genomic DNA in a total of 100 ng rapeseed DNA as template, 1 × TaqMan® buffer, 400 nM each primer TOPLG and TOPLV, 100 nM probe TOPLP, 300 μM each of dATP, dCTP and dGTP, 600 μM dUTP, 0.2 unit UNG, 1.25 units AmpliTaq™ Gold DNA polymerase and 2–6 mM of $MgCl_2$. For each reaction 3 replicates were performed. The best concentration of $MgCl_2$ was selected based on the replicability, efficiency and fluorescence intensity of the amplicons. With the optimized concentration of $MgCl_2$ as default, varied amounts of dNTPs/dUTP was tested to determine the best result, and then followed by the concentration optimization of the Primers/Probe. The event-specific real-time PCR assay of Topas 19/2 was performed according to the optimized conditions.

All real-time PCR reactions were carried out with the same program as follows: a pre-digest step of 50 °C for 2 min, and a 95 °C DNA initial denaturation and UNG deactivation step for 10 min; followed by 50 cycles of 95 °C denaturation for 15 s, 60 °C annealing and extension for 1 min, and fluorescence measured after annealing and extension.

2.7. Standard curves

The standard curve method was used to establish the quantification assays, since it is superior to the ΔCt method when the two targets are not amplified with the same PCR efficiency (Taverniers, Van Bockstaele, & De Loose, 2004).

The haploid genome size of rapeseed was estimated to be 1187 Mbp (Arumuganathan & Earle, 1991), corresponding to a weight of 1.3 pg. Therefore, the copy number of the rapeseed genome was calculated by the weight of the rapeseed DNA divided by 1.3 pg.

For the quantitation of the total rapeseed DNA in a sample, a standard curve for the *HMG-I/Y* gene was used, which was developed by using 100 ng for each concentration of a 100% to 0.0013% dilution series of rapeseed DNA diluted by maize genomic DNA.

For the quantitation of the Topas 19/2 event, the standard curve of the 3′-junction sequence was used, which was developed by using 100 ng for each concentration of the genomic DNA from Topas 19/2 diluted by DNA from the non-transgenic rapeseed cultivar 821.

2.8. Quantitative analysis of Topas 19/2

The genomic DNA of mixed samples containing known concentrations of Topas 19/2 in the non-transgenic 821 was used as the PCR template. Three replicate reactions of 100 ng of these mixed DNA samples were used as the template concurrently with the serially diluted standards as described above. The quantitative estimated results were computed by the software Opticon Monitor® 2 version 2.02. All reactions were repeated three times to verify the repeatability of the PCR.

To evaluate the accuracy and precision of the quantitative PCR systems for practical detection, two GM rapeseed mixtures were used as unknown samples. One mixed GM sample (S1) contained 1.5% Topas 19/2 seeds in the non-transgenic 821, and the other (S2) contained 0.5% Topas 19/2 seeds. Genomic DNA was extracted from S1 and S2 as described earlier. A quantity of about 100 ng template DNA was employed in three replicates and the assays

were repeated three times. The relative content of Topas 19/2 to total rapeseed DNA (%) was computed as: (mean weight of GM-rapeseed of three replicates)/(mean weight of total rapeseed DNA of three replicates) × 100.

The amount of total rapeseed DNA and genome DNA of Topas 19/2 were calculated with the standard curves as described earlier.

3. Results and discussion

3.1. Characterization of the 3′-integration junction

A PCR product of 823 bp was amplified from the Topas 19/2 genomic DNA libraries. Sequencing and Blastn analysis indicated that 550 bp of the isolated fragment came from the transgenic construct and the rest had high homology to the genome sequence of *A. thaliana* (GenBank accession no. AB007645). The junction site between the construct-source sequence and host genome was thought to be the 3′-integration junction of Topas 19/2 event.

Part of the consensus alignments including the transgene 3'-integration junction is shown in Fig. 1. The complete isolated sequence was submitted to GenBank under the accession number EU124676.

3.2. Qualitative analysis of Topas 19/2

To ensure the specificity of the event-specific primer pairs used in this research, the genomic DNA from different transgenic and non-transgenic rapeseed cultivars was used as template for PCR assays. The electrophoresis results showed that the expected amplicons of the endogenous reference gene *HMG-I/Y* could be detected in all rapeseed samples and could not be found in reactions with non-rapeseed genome DNA of *A. thaliana*, *B. rapa*, *B. oleracea*, *B. juncea*, *G. max, O. sativa*, *Z. mays* and *G. hirsutum* as templates. The primer pair TOPLG/TOPLV amplified specific fragments from Topas 19/2, but there was no amplification observed from other GM or non-transgenic lines including Ms8 × Rf3, Ms1 × Rf1, Ms1 × Rf2, Oxy-235, T45, RT73 and cv. 821 (Fig. 2). In further tests with non-rapeseed genome DNA of *A. thaliana*, *B. rapa*, *B. oleracea*, *B. juncea*, *G. max, O. sativa*, *Z. mays* and *G. hirsutum* as templates, no PCR product was observed with the event-specific primer pair.

In practice DNA extracted from GM food or feed tends to be highly degraded or very low in quantity. For the evaluation of the detection limit of the established qualitative detection system, the samples containing 100 ng to 0.0013 ng Topas 19/2 genomic DNA were used as templates for PCR analysis. Specific products of 110 bp could be observed in the reactions with no less than 0.013 ng or 10 copies of Topas 19/2 genomic DNA as templates (Fig. 2). These results indicated that the absolute detection limit of the qualitative method established in this research was as low as 10 copies, which was similar to the detection limit of the *HMG-I/Y* (Weng et al., 2005). By combining use of the endogenous gene and the event-specific protocols, the detection limit may reach as low as 0.013 ng of Topas 19/2 genomic DNA. The size of event-specific PCR product is small enough to meet the requirements of ISO, CRL–GMFF and the labelling policy of most countries (ISO, 2005; Weighardt, 2007).

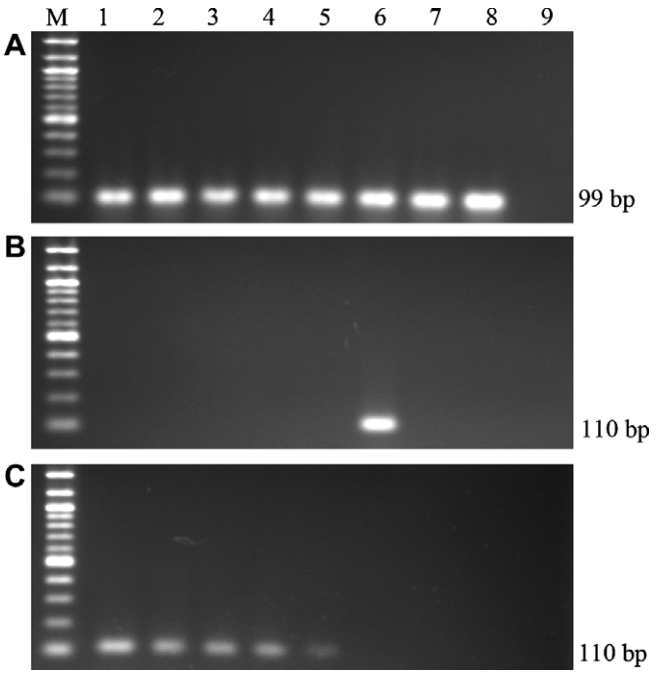

Fig. 2. Qualitative PCR assays of the Topas 19/2 genomic DNA. A: Detection of the *HMG-I/Y* gene. Lane M: 100 bp DNA Ladder (New England Biolabs, Beverly, MA, USA). Lanes 1-9: Amplifications with DNA from Ms8 × Rf3, Ms1 × Rf1, Ms1 × Rf2, Oxy-235, T45, Topas 19/2, GT73, non-transgenic 821, and no template control. B: Specificity assessment of the event-specific detection system of Topas 19/2. Lanes 1–9: Amplifications with DNA from Ms8 × Rf3, Ms1 × Rf1, Ms1 × Rf2, Oxy-235, T45, Topas 19/2, GT73, non-transgenic 821, and no template control. C: Sensitivity of the event-specific detection systems of Topas 19/2. Lane 1–7: Amplifications with 100, 13, 1.3, 0.13, 0.013 and 0.0013 ng of Topas 19/2 genomic DNA, respectively.

3.3. Quantitative analysis of Topas 19/2

After optimization of the $MgCl_2$, dNTPs/dUTP and primers/probe concentrations, the best real-time PCR assay system for the Topas 19/2 was set as follows: in a volume of 20 μL there are 100 ng genomic DNA as template, 1 × TaqMan® buffer, 4 mM $MgCl_2$, 100 nM each primer TOPLG and TOPLV, 50 nM probe TOPLP, 200 μM each of dATP, dCTP and dGTP, 400 μM dUTP, 0.2 unit UNG and 1.25 units AmpliTaq™ Gold DNA polymerase.

Fig. 3 shows the standard curves for the gene *HMG-I/Y* and for the event Topas 19/2. Based on the slope of the standard curve derived from the Topas 19/2 genomic DNA dilution series, the PCR efficiency of this event-specific quantitative system was calculated to be 92%. There was a good agreement between the amount of template and the threshold of cycle (*Ct*) values with the determination coefficients (R^2) of 0.995 for the three replicates, which was

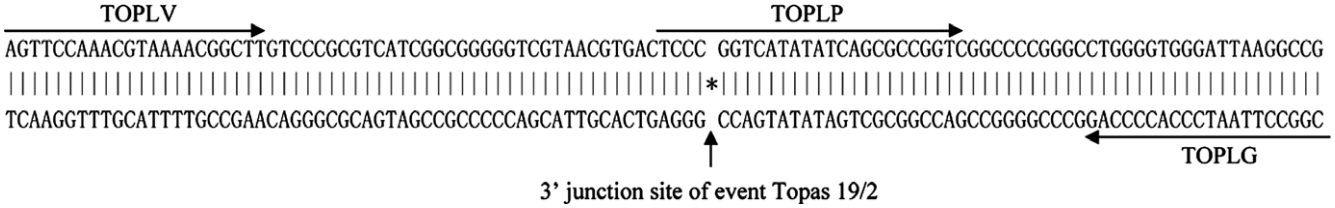

Fig. 1. Sequence of the 3′-integration junction regions and the location of the primers and the TaqMan® probes for the event-specific detection of the Topas 19/2 event. The junction sites between host DNA and the insert are indicated by an asterisk. The arrows indicate the primers' and probe's sequences and directions.

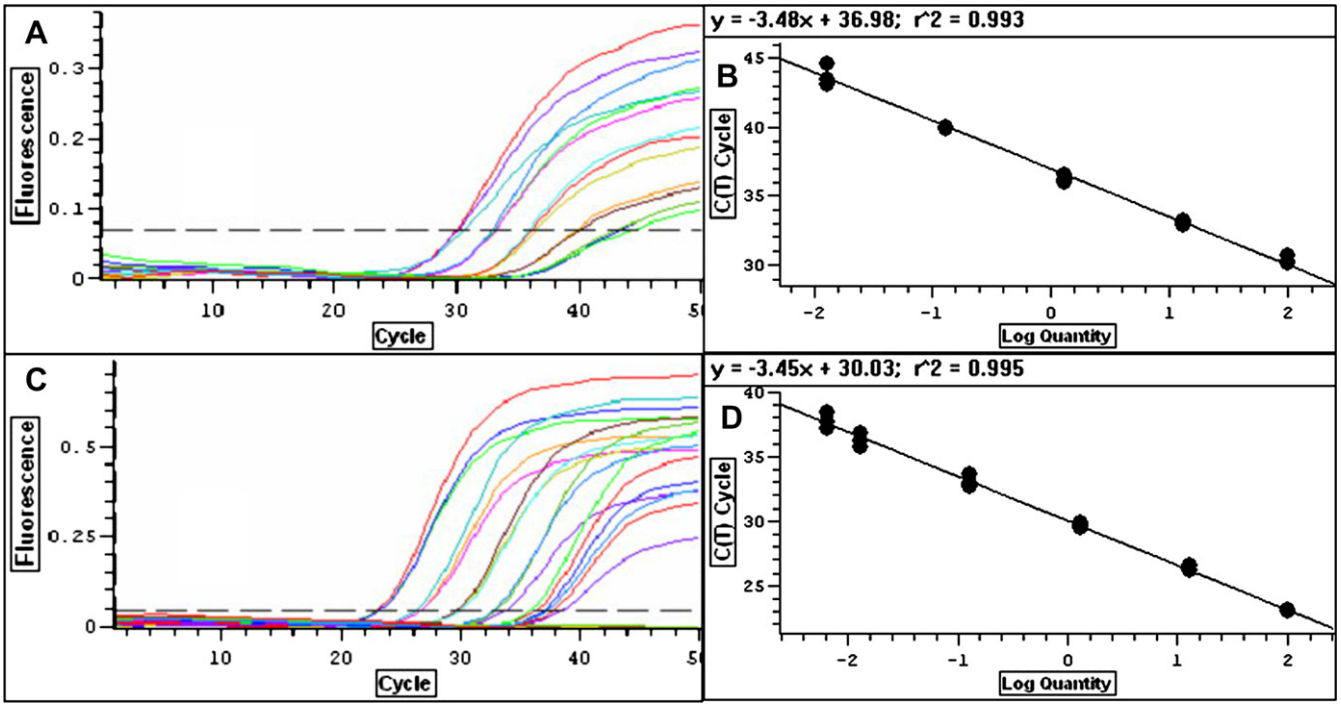

Fig. 3. Amplification and standard curves for the Topas 19/2 event using gradient-diluted Topas 19/2 genomic DNA as the template. A: Amplification graph for the *HMG-I/Y* gene assay. B: Standard curve for the *HMG-I/Y* gene assay, analyzed using Opticon Monitor® 2 Version 2.02. The quantities of rapeseed genome in each dilution were 100, 13, 1.3, 0.13, and 0.013 ng per reaction, respectively. C: Amplification graph for the Topas 19/2 event-specific assay. D: Standard curve for the Topas 19/2 event-specific assay, analyzed using Opticon Monitor® 2 Version 2.02. The quantities of the Topas 19/2 genome in each dilution were 100, 13, 1.3, 0.13, 0.013, and 0.0065 ng per reaction, respectively.

significantly higher than the minimum requirement of 0.98 (Taverniers et al., 2004), indicating that the real-time assay established in this research was suitable for the quantitation of Topas 19/2 DNA.

The repeatability of the real-time methods was analyzed using the serially diluted standards as described above. The standard deviation (SD) and relative standard deviation (RSD) of 3 replicates, and the standard deviation (SD^r) and relative standard deviation (RSD^r) of repeatability were calculated based on the data from three replicate reactions and three repeats of the assays. With the reduction of template weight from 100 ng to 0.013 ng, the average *Ct* values of the event-specific assay increased from 23.025 to 36.453 with RSD values of three replicate reactions from 0.260% to 1.541% (Table 2). At the same time the RSD^r value of three replications ranged from 0.181% to 0.826% (Table 2). It was concluded that the quantitative PCR assays were established with good repeatability and are suitable for the practical measurement of Topas 19/2 DNA from GM samples.

In order to determine the limit of detection (LOD) and limit of quantitation (LOQ) of the quantitative PCR method, the data from nine replicate reactions of each sample was analyzed. As shown in Table 2, Topas 19/2 genomic DNA was detected in all nine reactions at template levels as low as 0.013 ng. DNA was detected in eight samples when the template level was as little as 0.0065 ng and the corresponding copy number of the haploid genome was 5. However, when the template copy number was 1, the amplicons could only be detected in three reactions, and the *Ct* values of these samples deviated from the standard curve significantly. This phenomenon can be explained with the Poisson distribution model. Based on these data the 95% confidence interval of the detection limits was calculated to be 2.5–5 copies for the event-specific quantitative assay established in this study. From this result, the LOD for the Topas 19/2 event was estimated to be 5 copies, or 5 copies of Topas 19/2 haploid genomic DNA. Although all the data ranging from 100 ng to 0.0065 ng was found to be concordant with the standard curve, the SD value increased with the reduction of the content of template. When the template content was as low as 0.013 ng, the *Ct* values of three replicates showed a deviation exceeding 1 (Table 2). It was estimated that LOQ of the event-specific quantitative detection of the 3′ end junction of the Topas 19/2 event was 0.065 ng or 50 copies. Taking all of this into account, it is safe to conclude that as little as 0.065% of Topas 19/2 genomic DNA in 100 ng of total rapeseed genomic DNA samples could be detected and quantified by the method presented in this paper.

The LOD and LOQ of the real-time PCR assay of *HMG-I/Y* were reported to be 0.0013 ng and 0.013 ng, respectively (Weng et al., 2005). Obviously, those of the event-specific method of Topas 19/2 established in this study were much higher. Combining the limits of the event-specific quantitative detection method and the reference gene we conclude that as little as 0.0065 ng Topas 19/2 genomic DNA in rapeseed genomic DNA samples could be detected and as little as 0.065 ng could be quantified.

3.4. Validation of the quantitative real-time PCR method

Using the quantitative method based on the 3' end junction of the Topas 19/2 event, no amplification was obtained using the DNA from non-GM *B. napus* cv. 821 as template. For sample S1 the estimated proportion of Topas 19/2 DNA ranged from 1.43% to 1.62% with the mean 1.54 and SD 0.10, and for S2 the estimates ranged from 0.42% to 0.66% with the mean 0.56% and SD 0.13 (Table 3).

However, from the results cited above, biases of the quantified results from the theoretically true value were observed for every sample. These small deviations could be explained by uncertainty in the genome size of rapeseed. The uncertainty in the DNA purity and quantitation after extraction (Corbisier et al., 2005), and the molecular fluctuations with low copy numbers of initial template are also thought to contribute to the quantitative uncertainty in

Table 2
Repeatability of real-time PCR assays employing Topas 19/2 DNA as reference

DNA amount (ng)	Copy number[a]	Repeat	*Ct* value			Mean of *Ct* Values	SD	RSD (%)	Mean of All *Ct* Values	SD^r	RSD^r (%)
			1	2	3						
100	78000	1	22.979	23.050	23.098	23.042	0.060	0.260	23.025	0.042	0.181
		2	23.203	22.842	22.888	22.978	0.196	0.855			
		3	23.066	23.156	22.944	23.055	0.106	0.462			
13	10000	1	26.513	26.534	26.132	26.393	0.226	0.857	26.433	0.105	0.398
		2	26.344	26.524	26.193	26.354	0.166	0.629			
		3	26.492	26.775	26.39	26.552	0.199	0.751			
1.3	1000	1	29.592	29.527	29.881	29.667	0.188	0.635	29.572	0.124	0.419
		2	29.702	29.462	29.692	29.619	0.136	0.458			
		3	29.546	29.355	29.395	29.432	0.101	0.342			
0.13	100	1	32.879	33.429	32.763	33.024	0.356	1.077	33.049	0.208	0.630
		2	32.643	32.861	33.059	32.854	0.208	0.633			
		3	33.394	33.224	33.188	33.269	0.110	0.331			
0.013	10	1	36.178	35.723	36.834	36.245	0.559	1.541	36.453	0.301	0.826
		2	36.167	36.016	36.766	36.316	0.397	1.092			
		3	37.153	36.594	36.648	36.798	0.308	0.838			
0.0065	5	1	38.421	37.177	37.672	37.757	0.626	1.659	37.886	0.545	1.440
		2	38.848	37.109	39.234	38.397	1.132	2.948			
		3	37.558	37.066	–	37.312	0.348	0.932			
0.0013	1	1	40.594	–	–	40.594	–	–	40.496	–	–
		2	39.979	40.916	–	40.448	0.663	1.638			
		3	–	–	–	–	–	–			

[a] Calculated based on an estimated genome size of 1187Mb.

Table 3
Accuracy and precision statistics for quantitative method

Sample	Theoretical (%)	Experimental (%)			Mean (%)	SD (%)	Bias (%)
		1	2	3			
S1	1.5	1.43	1.62	1.56	1.54	0.10	2.44
S2	0.5	0.66	0.61	0.42	0.56	0.13	12.67

PCR reactions (Peccoud & Jacob, 1996). Generally speaking, the lower the template level results in greater uncertainty because of the stochastic variations of the amplifications because of calibration curves properties and the stochastic variations of amplifications (Estalilla, Medeiros, Manning, & Luthra, 2000; Fieller, 1940; Foy & Parkes, 2001). In this research, the copy number of the Topas 19/2 event was about 1200 in 100 ng 1.5% Topas 19/2 mixed sample, and 400 in the 0.5% mixed sample. According to the analysis above, the observed deviations are acceptable.

In conclusion, we established the event-specific qualitative and quantitative PCR detection methods of Topas 19/2 rapeseed, based on the 3′ end junction sequence spanning the integrated transgenic construct and plant DNA in the transgenic rapeseed event Topas 19/2.

These assays were optimized for use of the equipments and chemicals described in this paper, especially for the MJ Research (now Bio-Rad) fluorometric thermal cycler DNA Engine Opticon® 2. As far as we know, other systems and chemicals may be used, but reaction conditions should be verified and re-optimized. For example, 5-Carboxy-X-rhodamine (ROX) may be needed in reaction mixture for the real-time assay on a charge-coupled device (CCD) camera based fluorometric detection system such as ABI 7000, 7300, Bio-Rad MyiQ, iQ5 etc. The thermal cycle program of both qualitative and quantitative PCR should be modified when the hot start Taq polymerase is changed. When a capillary based real-time machine such as Roche LightCycler is used, both the program and reaction mixture components should be modified according to the manufacturer's advices.

Over all, this paper provided the 3′ end fragment sequences and a method for the event-specific detection of Topas 19/2 though its fitness for purpose in other laboratories still need inter-laboratories validation to establish the reliability, accuracy and reproducibility to meet the requirement of ISO 5725 standard (ISO, 1994).

Acknowledgements

This research was supported by a grant from the Special Foundation of Social Benefits of China (Grant No. 2005DIA2J026) and the National High Technology Research and Development Program of China (Grant No. 2006AA10Z444).

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