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Event-Specific Qualitative and Quantitative Polymerase Chain Reaction Analysis for Genetically Modified Canola T45

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Polymerase chain reaction (PCR) methods have been the main technical support for the detection of genetically modified organisms (GMOs). To date, GMO-specific PCR detection strategies have been developed basically at four different levels, such as screening-, gene-, construct-, and event-specific detection methods. Event-specific PCR detection method is the primary trend in GMO detection because of its high specificity based on the flanking sequence of exogenous integrant. GM canola, event T45, with tolerance to glufosinate ammonium is one of the commercial genetically modified (GM) canola events approved in China. In this study, the 5′-integration junction sequence between host plant DNA and the integrated gene construct of T45 canola was cloned and revealed by means of TAIL-PCR. Specific PCR primers and TaqMan probes were designed based upon the revealed sequence, and qualitative and quantitative TaqMan real-time PCR detection assays employing these primers and probe were developed. In qualitative PCR, the limit of detection (LOD) was 0.1% for T45 canola in 100 ng of genomic DNA. The quantitative PCR assay showed limits of detection and quantification (LOD and LOQ) of 5 and 50 haploid genome copies, respectively. In addition, three mixed canola samples with known GM contents were detected employing the developed real-time PCR assay, and expected results were obtained. These results indicated that the developed event-specific PCR methods can be used for identification and quantification of T45 canola and its derivates.

KEYWORDS: T45 canola; event specific; TAIL-PCR; qualitative and quantitative PCR.

INTRODUCTION

Since the first genetically modified (GM) tomato "FLAVR SAVR" was commercialized in the United States in 1994, many more GM plants have been developed and commercialized globally. In the past decade, the area of GM crops increased more than 53 fold, from 1.7 million hectares in 1996 to 90.0 million hectares in 2005, with an increasing proportion grown by developing countries, and the principal traits are herbicide tolerance and insect resistance (*1*).

Canola (*Brassica napus* L.), the main oil plant, is grown in over 30 countries, especially in China, Canada, and India. Canola oil has become widespread in the food industry as a vegetable oil in table spreads and cooking and as an ingredient in a range of mixed foods. In order to decrease the planted cost and improve the output of canola, the herbicide tolerance trait has been introduced into canola lines by addition of a bacterial gene, either *bar* or *pat*, to enable the canola plants to produce an enzyme, phosphinothricin acetyl transferase (PAT), which chemically inactivates the herbicide, phosphinothricin (*2*). Some GM canola varieties with herbicide tolerance trait have been developed and approved for commercialization in several countries, such as the United States, the European Union (EU), Canada, Japan, and China. In China, 18 imported GM crops have been approved for commercialization since 2004, including T45 canola developed by Bayer CropScience (*3*). T45 Canola was produced by *Agrobacterium*-mediated transformation of the canola cultivar AC Excel with the recombinant DNA molecule. The introduced recombinant DNA is based on the *PAT* gene which was originally isolated from a common soil bacterium, *Streptomyces viridochromogenes*, and encodes the enzyme, PAT. The gene introduced in the transformant, T45, was modified to optimize its expression in plants without altering the amino acid sequence it encodes. During transformation, only one single copy of the transferred DNA was incorporated into the host canola genome (*2*).

As more GM foods, food ingredients, and additives are being introduced into the market, more than 40 countries and areas

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Table 1. Primers and TaqMan Probes Used for PCR Systems

PCR system	name	sequence (5-3)	amplicon length (bp)	ref
TAIL-PCR	T1R	CCATCTTTGGGACCACTGTCG		this work
	T2R	CACATCAATCCACTTGCTTTGAAG		this work
	T3R	GCATCTTCAACGATGGCCTTTC		this work
	AD4	GTNCGASWCANAWGTT		(*33*)
conventional PCR	HMG-1F	GGTCGTCCTCCTAAGGCGAAAG	99	(*12*)
	HMG-2R	CTTCTTCGGCGGTCGTCCAC		
	C-1F	TCCCATTTATTTACGGTCAC	233	this work
	C-2R	CCATGGGAATTCATTTACAA		
real-time PCR	HMG-1F	GGTCGTCCTCCTAAGGCGAAAG	99	(*12*)
	HMG-2R	CTTCTTCGGCGGTCGTCCAC		
	HMG-p	HEX-CGGAGCCACTCGGTGCCGCAACTT-TAMRA		
	C-3F	TGCATATGGAATACAGTTGTAAATGAATT	113	this work
	C-4R	TCGTAAGAGACTCTGTATGAACTGTT		
	C-p	FAM CCAGTCTTTACGGCGAGTT (TAMRA) CTGTTAGGTCCTC		

have issued specific GMO labeling regulations and regulated the labeling threshold. For instance, the labeling threshold is defined as 0.9% in EU (*4*), 3% in Korea (*5*), and 5% in Japan (*6*). The labeling of GM foods is voluntary in the United States and Canada (*7*). In China, 17 foods derived from 5 different plants should be labeled, such as maize seeds, maize oil, tomato seeds, ketchup, soybean seeds, soybean oil, rapeseed seeds, cotton seeds, etc (*8*). Much effort has been expended to implement the GMO labeling regulations successfully in China, such as four novel endogenous reference genes (sucrose phosphate synthase, *SPS*, gene for rice; *LAT52* gene for tomato; stearoyl-ACP desaturase, *Sad1*, gene for cotton; and high-mobility-group proteins I/Y, *HMG I/Y*, gene for rapeseed) being developed for GMO detection (*9*−*12*). Qualitative and quantitative real-time PCR detection methods were well established for transgenic Huafan No.1 tomato, Bt cottons, MON863 maize, etc. (*13*−*16*).

To adapt these GMO labeling requirements, four general types of PCR detection strategies, i.e., screening-, gene-, construct-, and event-specific PCR detection methods, have been developed to discriminate between GM- and non-GM-derived DNA varieties (*17*). The screening method is associated with a particular risk of yielding false positives (*18*). Gene- and construct-specific methods are more specific but may cause false positives when the modified genes are used in other GMOs with variable copy number, and these methods cannot distinguish between different GMOs if the same construct has been integrated (*18*). To overcome these problems, the event-specific PCR must be performed. Until now, several event-specific quantitative PCR methods have been established for GTS 40-3-2 soybean (*18*−*21*), MON531 and MON1445 cotton (*22*), MON810 maize (*23*−*24*), Bt11 maize (*25*−*26*), GA21 maize (*27*), NK603 maize (*28*−*29*), T25 maize (*30*), MON863 maize (*15*−*16*), Event 176 maize (*31*), and CBH351 maize (*32*). However, until now, there has been no report on the event-specific PCR detection method for T45 canola.

In this study, the 5′ flanking sequence of the exogenous integration is cloned and identified using the thermal asymmetric interlaced (TAIL) PCR technique (*33*). On the basis of the revealed flanking sequences, qualitative and quantitative real-time PCR methods were developed. Furthermore, three mixed GM T45 canola samples were analyzed employing the developed TaqMan real-time PCR method.

MATERIALS AND METHODS

Materials and DNA Extraction. Genetically modified canola seeds (Ms8Rf3 and T45) were developed and supplied by Bayer Cropscience, GM canola leaves (Ms1Rf2 and Ms1Rf1) were developed by Aventis CropScience, and GM canola RT73 and GM soybean (GTS 40−3-2) seeds were developed and supplied by Monsanto. Non-transgenic canola seeds were purchased from local markets in Shanghai, China. Plant genomic DNA was extracted and purified using a Plant DNA Miniprep Kit (Shanghai Ruifeng Agro-tech Co., Ltd., Shanghai, China) according to the manufacturer's manual. The quantity of DNA in the samples was calculated using absorbance measurements at 260 nm, and its copy number was calculated from the quantity of DNA and canola genomic DNA average size (*34*).

Oligonucleotide Primers and Probes. Oligonucleotide primers and TaqMan fluorescent probes used in this study were designed using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA) and are listed in **Table 1**. The probe of endogenous *HMG I/Y* gene was labeled with the fluorescent reporter dye 5-hexachloro-fluorescein (HEX) on the 5′ end, while the probe for the exogenous gene was 5′ end-labeled with 6-carboxy-fluorescein (FAM). The fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) was attached at the 3′ end of the probes. Information on the transgene inserted in GM canola T45 was obtained from the Agbios website (www.agbios.com) as shown in **Figure 1A**. The specific primers T1R/2R/3R (**Table 1**) used in TAIL-PCR were designed based on the sequence of the CaMV35S promoter. Primer AD4 is an arbitraryily chosen primer also selected for use in TAIL-PCR. The event-specific PCR primer pairs (C-1F/2R and C-3F/4R) and probe (C-p) used for T45 canola detection were designed based on the 5′ junction region between host DNA and exogenous sequence originating from the CaMV35S promoter. Primers C-1F/2R were used in conventional PCR, while primers C-3F/4R combined with probe C-p were employed for TaqMan real-time PCR (**Table 1**). For total canola genome DNA detection, the *HMG I/Y* gene was selected as the endogenous reference gene (*12*), and the primers HMG-1F/2R and its combination of TaqMan probe HMG-p were used in conventional and real-time PCR, respectively. All primers and fluorescent probes were synthesized and purified by Shanghai BioAsia Co., Ltd. (Shanghai, China).

Determination of the Flanking DNA Sequence Using TAIL-PCR. The 5′ flanking sequence of the T45 exogenous insertion was cloned by means of TAIL-PCR, essentially as described by Liu et al. (*33*). PCR reactions were performed according to the following protocol. Briefly, the first TAIL-PCR amplification was performed in a total volume of 30 μL containing 1 × PCR buffer, 200 μM dNTPs, 0.5 μM primer T1R, 5 μM primer AD4, 2.5 U of *TaKaRa Ex Taq* HS DNA polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China) and 50 ng of T45 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: five cycles of 30 s at 94 °C and 6 min at 68 °C, one cycle of 30 s at 94 °C, 3 min at 45 °C, and 6 min at 68 °C, five cycles of 15 s at 94 °C, 30 s at 45 °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 45 °C, and 7 min at 68 °C. Secondary TAIL-PCR amplification was carried out in a total volume of 50 μL containing 1 × PCR buffer, 200 μM dNTPs, 0.2 μM primer T2R, 2 μM primer AD4, 5 U of *TaKaRa Ex Taq* HS DNA polymerase, and 1 μL of a 50-fold dilution of the

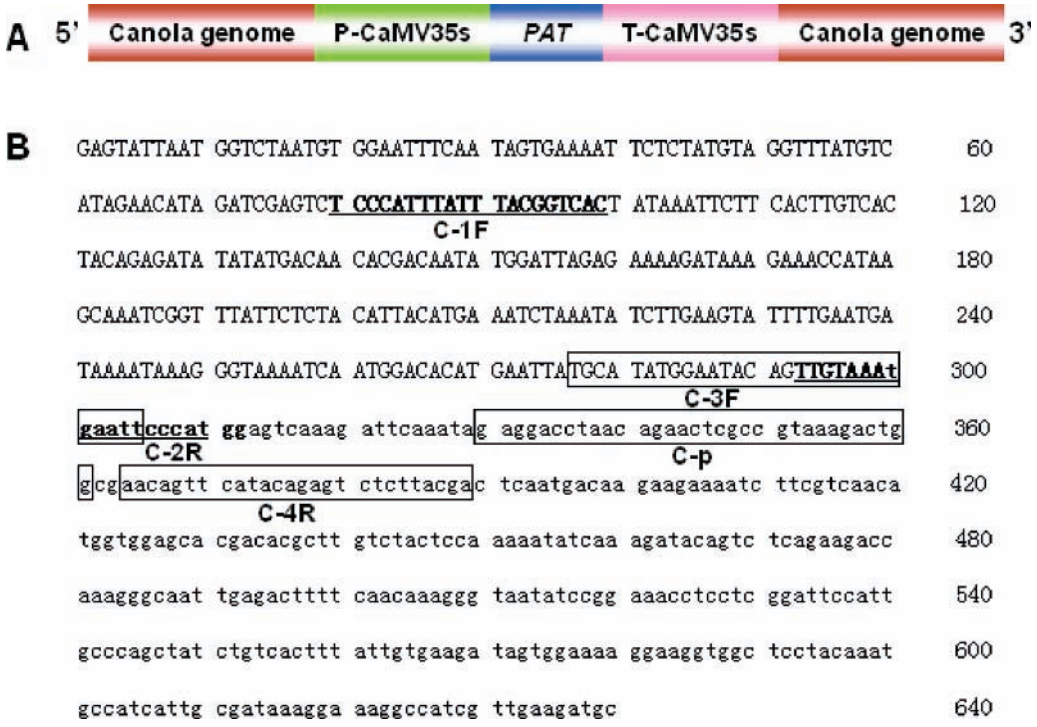

Figure 1. Organization of transgenic elements in T45 canola genome. (A) Schematic diagram of the integrated heterologous DNA in canola event T45. (B) 5′ junction sequence between the canola genome and the integrated heterologous DNA of T45 canola. Capital letters represent the flanking genomic sequence, and lowercase letters show the partial sequence of *CaMV35S* promoter. Primers used for conventional PCR amplification in Table 1 are underlined. Sequences in rectangles are the primers and probe used for real-time PCR and listed in Table 1.

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 45 °C, and 7 min at 68 °C. Tertiary TAIL-PCR amplification was carried out in a total volume of 50 μL containing 1 × PCR buffer, 200 μM dNTPs, 0.2 μM primer T3R, 2 μM primer AD4, 5 U of *TaKaRa Ex Taq* HS DNA polymerase, and 1 μL of a 50-fold dilution of the secondary PCR products. The tertiary TAIL-PCR program was 20 cycles of 30 s at 94 °C, 45 s at 68 °C, 6 min at 72 °C, and the last step of 5 min at 72 °C. All PCR reactions were carried out in a PTC-100 thermocycler (MJ Research, Waltham, MA). The second and tertiary PCR amplification products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining. The amplified fragments with similar size in these two PCR amplifications were purified with a Gel Extraction Mini Kit (Watson Biotechnologies, Inc, Shanghai, China) and ligated into pMD18-T vector (TaKaRa Biotechnology Co., Ltd.). Sequence analysis of the cloned DNA was performed using the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems Division of Perkin-Elmer Corp., Foster City, CA) by Shanghai BioAsia Biotechnology Co., Ltd. Parallel amplifications with non-transgenic canola genomic DNAs were carried out to identify T45-specific PCR products.

Conventional PCR. In conventional PCR assays, all amplifications were carried out in 30 μL volume reactions, with 100 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM of each dNTP, 0.8 μM of each primer, and 1.5 U of *Taq* DNA polymerase (TaKaRa biotechnology Co., Ltd, Dalian, China). The conventional PCR amplifications were performed with a PTC-100 Thermocycler (MJ Research) with the program as follows: one step of 7 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C; one step of 7 min at 72 °C. Amplification products were electrophoresised in 3% agarose gels for approximately 30 min at 100 V and stained with ethidium bromide for visualization. Each reaction of one test was repeated three times and each time with triple replication.

TaqMan Real-Time PCR Conditions. Real-time PCR reactions were carried out with a fluorometric thermal cycler (Rotor-Gene 3000; Corbett Research, Australia) in final volumes of 25 μL. Fluorescence was monitored during every PCR cycle at the annealing step. Reactions contained the following ingredients: 1 × PCR buffer, 400 μM each of dATP, dGTP, dCTP, 800 μM dUTP, 600 nM primers, 150 nM TaqMan probes, 1.5 U *Taq* DNA polymerase, 0.2 U Amperase Uracil N-glycosylase (UNG), and 6.5 mM $MgCl_2$. Real-time PCR reactions were carried out with the following program: 2 min at 50 °C and 10 min at 95 °C, 50 cycles of 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C. Data were analyzed with Rotor gene 3000 software version 6.0 (Corbett Research, Australia). All PCR reagents were purchased from Bio-color biotechnology Co., Ltd. (Shanghai, China) except for primers and probes.

Construction of the Standard Curves. After optimization of magnesium and primer/probe concentrations, series of T45 canola genomic DNA dilutions were used for quantification, reproducibility, repeatability test, and determination of the limits of detection and quantification (LOD and LOQ). Genomic DNA isolated from powdered 100% T45 canola was serially diluted with 0.1 × TE buffer to final concentrations equivalent to 10 000, 1000, 100, 10, and 1 copies haploid genome/μL considering 1.3 pg per haploid genome in the case of canola according to Weng et al. (*12*). In each reaction, 5 μL of diluted DNA samples was added, and all reactions were repeated three times and each time with triple replicates for each template DNA.

RESULTS AND DISCUSSION

Cloning and Sequencing of the 5′ Flanking Region of Exogenous Integration in T45 Canola. On the basis of the preliminary schematic map of the exogenous insertion used in T45 released from the Agbios web site (**Figure 1A**), three appropriate specific primers (T1R/2R/3R) were designed based on the *CaMV35S* promoter to clone the 5′ flanking sequences by TAIL-PCR method. Combination of the arbitrary primer AD4 and the specific primer T3R in tertiary amplification reactions amplified a discrete product about 700 bp in size. Using the genomic DNA from non-transgenic canola as the template, no amplification product was observed in the parallel experiments. The TAIL-PCR amplicon was cloned into the pMD18-T vector, and eight individual clones were selected and sequenced. The result of sequence analysis indicated that one 640 bp fragment

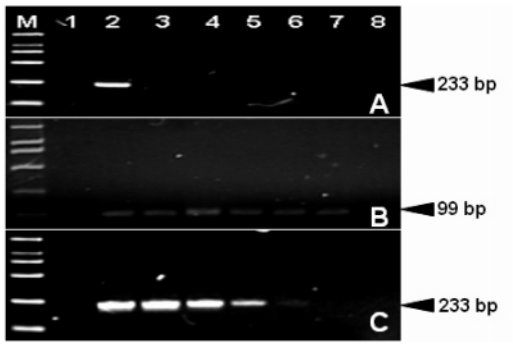

Figure 2. Three percent agarose gel electrophoresis of PCR products amplified with the T45 event-specific primers C-1F/2R and *HMG I/Y* gene primes HMG-1F/2R. (A) T45 event-specific PCR amplified results. (B) *HMG I/Y*-specific PCR amplified results. Lane 1: NTC (no template control). Lanes 2–8: amplification of T45 canola, Ms8Rf3 canola, Ms1Rf1 canola, Ms1Rf2 canola, RT73 canola, non-GM canola, and GM soybean (GTS 40-3-2). Lane M: DL2000 DNA marker. (C) Sensitivity test of T45 event-specific assay. PCR products were amplified from mixed GM T45 canola DNAs with different GM content levels. Lane 1: NTC. Lanes 2–8: amplification of mixed GM maize DNAs with 10.0%, 5.0%, 3.0%, 1.0%, 0.1%, 0.05%, and 0.01% GM contents, respectively. Lane M: DL2000 DNA marker.

encompassing the 5′ junction region was obtained of which 299 bp originated from *Brassica napus* genomic DNA and 341 bp originated from the cauliflower mosaic virus *35S* DNA (**Figure 1B**).

Conventional PCR Detection for T45 Canola Event. Event-specific primers (C-1F/2R) were designed based on the revealed 5′ flanking sequence above and employed to develop the conventional PCR assay for T45 canola (Figure 1). The primer C-1F was located at the canola genome and the primer C-2R at the *CaMV35S* promoter. The canola *HMG I/Y* gene was selected as endogenous reference gene, and the primer pair HMG-1F/2R was employed for canola identification. As expected, in the established PCR assay, one single 233 bp DNA fragment was obtained from T45 canola, and no fragment was detected in other GM canola lines (Ms8Rf3, Ms1Rf2, Ms1Rf1, and RT73), GM soybean (GTS 40-3-2), non-transgenic canola, and no template control (NTC) (**Figure 2**A). The target fragment of *HMG I/Y* gene (99 bp) was detected in all GM canola and non-transgenic canola samples except for GM soybean and NTC (**Figure 2B**). These data confirmed that the obtained DNA fragment is from the T45 event-specific region between the 5′ flanking sequence of transgene and canola genomic DNA.

Since DNA is often degraded during food processing and the DNAs with smaller size derived from GMOs often occur in practical detection, consequently this means that one more sensitive PCR detection system is important and necessary. In qualitative PCR, test sensitivity is measured by the limit of detection (LOD). To test the limit of detection (LOD) of the established event-specific PCR assay, the DNA mixture was prepared from T45 canola line and non-GM canola line at various levels such as 0.01%, 0.05%, 0.1%, 1.0%, 3.0%, 5.0%, and 10%. The test results showed that the target fragment was detected from all the levels tested except for the 0.01% and 0.05% levels, which means that the lowest tested level was 0.1% in 100 ng of genomic DNA (**Figure 2C**).

TaqMan Real-Time PCR Assay Based on the 5′ Flanking Sequence of T45 Canola. Through analyzing the 5′ end insertion sequences corresponding to the unique insertion event in T45, the event-specific quantitative detection method based on TaqMan real-time PCR was developed. Primers (C-3F/4R) and TaqMan probe C-p were designed based on the revealed flanking sequence. Primer C-3F was located on the canola genomic DNA, while primer C-4R is located on the *CaMV35S* promoter. Probe C-p contains the annealing sequences for both the *CaMV35S* promoter sequence and the canola genomic DNA. For the total canola quantification, the *HMG I/Y* real-time PCR assay employing primers (HMG-1F/2R) and probe HMG-p was also established.

Construction of the Standard Curves. After optimization of the magnesium and primer/probe concentrations, the standard curves of the event-specific and endogenous *HMG I/Y* real-time PCR assays were constructed employing the T45 genomic DNA ranging from 50 000, 5000, 500, 50, and 5 copies haploid genome. The high PCR reaction efficiencies for both *HMG I/Y*-specific and event-specific assays (0.90 for the event-specific PCR assay and 1.04 for *HMG I/Y*) were obtained. These results demonstrated that GM contents could be calculated employing

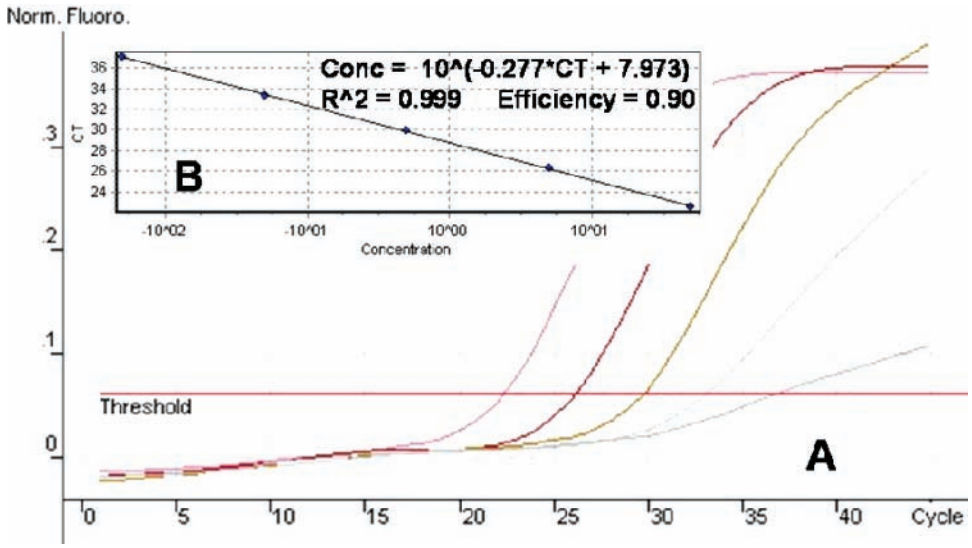

Figure 3. Amplification plots and standard curve for T45 event-specific real-time PCR assay. (A) Amplification curves (5 serial dilutions, corresponding to 50 000, 5000, 500, 50, and 5 copies of the T45 haploid genome per reaction) were generated for T45 canola quantification. The horizontal line indicates the threshold line determined for drawing up the standard curve. (B) Parameters of the regression line through data points are indicated within the plot.

Table 2. Repeatability and Reproducibility of the Developed T45 Event-Specific and *HMG I/Y* PCR Assays

target copies	Ct values 1	2	3	mean	mean copy number	SD^r	SD^R	RSD^r (%)	RSD^R (%)
event-specific assay									
50 000	22.58	22.61	22.7	22.63	53942.37	0.05	0.05	0.22	0.32
5000	26.23	26.25	26.18	26.22	4947.89	0.03	0.03	0.11	0.24
500	29.99	29.83	29.7	29.84	444.88	0.12	0.07	0.40	0.36
50	32.81	33.12	32.93	32.95	56.04	0.13	0.16	0.39	0.73
5	36.69	36.85	37.04	36.86	4.16	0.14	0.18	0.38	0.84
HMG I/Y assay									
50 000	21.29	21.34	21.27	21.3	48927.52	0.03	0.07	0.14	0.34
5000	24.24	24.31	24.36	24.30	5045.60	0.05	0.05	0.21	0.27
500	27.43	27.28	27.41	27.37	494.73	0.07	0.12	0.26	0.72
50	30.45	30.73	30.69	30.62	42.33	0.12	0.14	0.39	0.58
5	33.74	33.61	33.45	33.60	4.45	0.12	0.17	0.36	0.81

Table 3. Amplification Data Used To Determine the Absolute LOD and LOQ

template copies	signal rate (positive signals)	mean Ct values	SD	RSD (%)
50 000	9/9	22.68	0.07	0.31
5000	9/9	26.15	0.09	0.34
500	9/9	29.82	0.07	0.23
50	9/9	33.03	0.12	0.36
5	7/9	36.87	0.16	0.43

these two PCR assays by means of the relative quantitative method. The square regression coefficients (R^2) were 0.998 and 0.999 for the *HMG I/Y*- and event-specific PCR assay, respectively. The good linearity between DNA quantities and fluorescence values (Ct) indicated that these assays were well suitable for quantitative measurements (**Figure 3**).

Repeatability and Reproducibility of the PCR Assays. Repeatability and reproducibility were both determined and calculated using the above-described standard DNA dilutions. The standard deviation (SD^r) and relatively standard deviation (RSD^r) of repeatability and standard deviation (SD^R) and relatively standard deviation (RSD^R) of reproducibility were calculated from the data of triplicate reactions and three replications. The SD^r values of T45 PCR assay ranged from 0.03 to 0.14 and 0.03 to 0.12 for the *HMG I/Y* PCR assay. The RSD^r values of T45 PCR assay ranged from 0.11% to 0.40% and 0.14% to 0.39% for the *HMG I/Y* PCR assay (**Table 2**). The SD^R values of T45 PCR assay ranged from 0.03 to 0.18 and 0.05 to 0.17 for the *HMG I/Y* PCR assay. The RSD^R values of T45 PCR assay ranged from 0.24% to 0.84% and 0.27% to 0.81% for the *HMG I/Y* PCR assay (**Table 2**). The above results indicated that these quantitative PCR assays were stable and reliable in GM canola quantification.

Determination of the Limit of Detection (LOD) and Limit of Quantitation (LOQ). There are three different ways for expressing detection and quantitation limits, although they all refer to the lowest quantity of the target that can be reliably detected and quantified with a probability of ≥95% (*19*, *35*). The absolute limit is the lowest number of initial template copies that can be detected and quantified. The relative limit refers to the lowest percentage of GMO relative to the species that can be detected and quantified, and the practical limit is the functional limit of the sample during the practical analysis. In order to determine the LOD and LOQ of the established event-specific real-time PCR assay, a series of T45 genomic DNA dilutions prepared above were tested in three parallel reactions and repeated three times (**Table 3**). As expected, the ability to detect T45 canola decreased with decreasing genomic DNA copy numbers. T45 genomic DNA could be detected seven in nine reactions down to five copies. These results indicated that the LOD value was about five copies. The data also showed that the SD values of the nine reactions with same template concentration increased with decreasing copy number. To obtain reliable quantization results under ideal conditions, approximately 50 initial template copies were required, and we concluded that the LOQ of the event-specific real-time PCR assay is 50 copies haploid genome.

Quantitative Analysis of the Practical T45 Canola Samples Employing the Developed Event-Specific PCR Assay. Three T45 canola samples, i.e., S1, S2, and S3 with 5%, 3%, and 1%, were artificially prepared by mixing the pure dried T45 seed flours with non-GM dried rapeseed flours on a wt/wt basis and quantified based on the standard curve of T45 event-specific PCR assay. As shown in **Table 4**, the mean quantitative results of these three samples were 4.63%, 3.18%, and 1.12%, respectively. The quantified bias values of these three samples were 7.48%, 5.89%, and 11.57%, respectively. It is important to note that the mixing of the samples was based on a wt/wt ratio not the genome/genome ratio. Thus, this small bias of the quantified result may partially result from the possible differences in the genome/weight ratios of the two canola materials (T45 and non-GM canola).

In short, we revealed the 5′ integration junction sequence between host plant DNA and the integrated gene construct of T45 canola, and event-specific qualitative and quantitative PCR assays were developed in this paper. The limit of detection (LOD) for qualitative PCR was 0.1% for T45 canola, and the limits of detection and quantification (LOD and LOQ) of quantitative PCR assay were 5 and 50 haploid genome copies, respectively. These results indicate that the quantitative system described in this paper was easily and reliably applied to various food products, even for samples with a low quantity of DNA.

Table 4. Quantitative Analysis of T45 Canola Content in Three Mixed Samples

sample	Ct mean 1	mean 2	mean 3	mean of all Ct values	SD	RSD (%)	calculated DNA amounts (pg)	GM content (%)	bias (%)
HMG I/Y PCR assay									
S1 (5%)	20.31	20.26	20.29	20.29	0.02	0.10	105304.9		
S2 (3%)	20.19	20.21	20.28	20.23	0.04	0.20	110194.4		
S3 (1%)	20.27	20.25	20.18	20.23	0.04	0.20	109640.1		
T45 event-specific PCR assay									
S1 (5%)	26.19	26.25	26.29	26.24	0.04	0.15	4871.7	4.63	7.48
S2 (3%)	26.87	26.64	26.71	26.74	0.10	0.37	3500.6	3.18	5.89
S3 (1%)	28.42	28.33	28.21	28.32	0.09	0.32	1223.3	1.12	11.57

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