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Event Specific Qualitative and Quantitative Polymerase Chain Reaction Detection of Genetically Modified MON863 Maize Based on the 5′-Transgene Integration Sequence

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Because of the genetically modified organisms (GMOs) labeling policies issued in many countries and areas, polymerase chain reaction (PCR) methods were developed for the execution of GMO labeling policies, such as screening, gene specific, construct specific, and event specific PCR detection methods, which have become a mainstay of GMOs detection. The event specific PCR detection method is the primary trend in GMOs detection because of its high specificity based on the flanking sequence of the exogenous integrant. This genetically modified maize, MON863, contains a *Cry3Bb1* coding sequence that produces a protein with enhanced insecticidal activity against the coleopteran pest, corn rootworm. In this study, the 5′-integration junction sequence between the host plant DNA and the integrated gene construct of the genetically modified maize MON863 was revealed by means of thermal asymmetric interlaced-PCR, and the specific PCR primers and TaqMan probe were designed based upon the revealed 5′-integration junction sequence; the conventional qualitative PCR and quantitative TaqMan real-time PCR detection methods employing these primers and probes were successfully developed. In conventional qualitative PCR assay, the limit of detection (LOD) was 0.1% for MON863 in 100 ng of maize genomic DNA for one reaction. In the quantitative TaqMan real-time PCR assay, the LOD and the limit of quantification were eight and 80 haploid genome copies, respectively. In addition, three mixed maize samples with known MON863 contents were detected using the established real-time PCR systems, and the ideal results indicated that the established event specific real-time PCR detection systems were reliable, sensitive, and accurate.

KEYWORDS: MON863 maize; 5′-transgene integration sequence; event specific; qualitative and quantitative PCR; TAIL-PCR

INTRODUCTION

During the 9 year period from 1996 to 2004, the global area of genetically modified (GM) crops increased more than 47-fold, from 1.7 million hectares in 1996 to 81.0 million hectares in 2004, with an increasing proportion grown by developing countries, and the principal traits are herbicide tolerance and insect resistance (*1*). Corn (*Zea mays L.*), the world's third leading cereal crop, following wheat and rice, is grown commercially in over 25 countries, especially in the United States and China. However, corn yields are negatively impacted by a number of insect pests, especially by the corn rootworm (*2*). Thus, the genetic engineering method to improve corn insect resistance is widely applied in corn planting, and several insect resistant corn varieties, i.e., MON810, MON863, Bt11, and Bt176, have been produced and approved for commercialization (*3*). In China, 17 imported GM crops were approved for commercialization in 2004, including MON863 maize developed by Monsanto Company. MON863 was obtained by direct transformation of a plasmid PV-ZMIR13 containing two linked exogenous gene cassettes, *Cry3Bb1* gene cassette and neomycin phosphotransferase type II (*NptII*) gene cassette (*2*, *4*). The

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Table 1. Primers and TaqMan Probes Used for PCR Systems

PCR system	name	sequence (5′-3′)	amplicon	ref
TAIL-PCR	L1R	CCATCTTTGGGACCACTGTCG		this work
	L2R	CACATCAATCCACTTGCTTTGAAG		this work
	L3R	GATAGTGGGATTGTGCGTCATCC		this work
	AD20	TCTTICGNACITNGGA		*28*
conventional PCR	Z1F	CGGTGGATGCTAAGGCTGATG	88	this work
	Z2R	AAAGGGCCAGGTTCATTATCCTC		
	M1F	GCACTCAAAGACCTGGCGAATGA	411	this work
	M2R	CCATCTTTGGGACCACTGTCG		
real-time PCR	Z1F	CGGTGGATGCTAAGGCTGATG	88	this work
	Z2R	AAAGGGCCAGGTTCATTATCCTC		
	Zp	HEX TAAGGAGCACTCGCCGCCGCATCTG TAMRA		
	M3F	CCTACTTGTTCGGATGGGTGTT	90	this work
	M4R	CTTCCTTTTCTACTGTCCTTTTGATGA		
	Mp	FAM AGTGTACCAAGCT (TAMRA) TTCCGATCCTACCTGTCA		

Cry3Bb1 coding sequence was under the control of 5′-noncoding elements consisting of four repeats of activating sequence-1 (*AS1*), a single portion of the *35S* promoter, a 5′-untranslated leader sequence from wheat chlorophyll a/b binding protein (*wtCAB*), and the first intron of the rice *actin 1* sequence (*ract1*). The 3′-nontranslated region of wheat heat shock protein 17.3 (*tahsp 3′*) gene was inserted at the 3′-end of *Cry3Bb1*. The *NptII* cassette contains *NptII* coding sequence under the control of a *CaMV35S* promoter and the nopaline synthase 3′-nontranslated sequence (*NOS 3′*) of *Agrobacterium tumefaciens* (*2*, *4*). During transformation, only one copy of the intact construct was inserted into the inbred *Z. mays* line A634 (*2*, *4*).

As more genetically modified organism (GMO) foods, food ingredients, and additives are introduced to the markets, nearly 40 countries and areas have issued GMO labeling regulations; for instance, the labeling threshold is defined as 0.9% in the European Union (*5*), 3% in Korea (*6*), and 5% in Japan (*7*). The labeling of GM foods is not compulsory in the United States and Canada (*8*). In China, 17 foods derived from five different plants should be labeled, such as maize seeds, maize oil, tomato seeds, ketchup, soybean seeds, soybean oil, rapeseed seeds, and cotton seeds, etc. (*9*). Much effort has been expended to implement the GMO labeling successfully in China. Four novel endogenous reference genes (*SPS* gene for rice, *LAT52* gene for tomato, *Sad1* gene for cotton, and *HMG I/Y* for rapeseed) were used for GMO detection (*10*−*13*); also, the qualitative and quantitative real-time polymerase chain reaction (PCR) detection methods for transgenic Huafan no. 1 tomato, GK19 cotton, SGK321 cotton, and MON531 cotton, etc. are well-established (*14*, *15*).

To adapt these labeling requirements, four different PCR detection strategies, i.e., screening and gene, construct, and event specific PCR detection methods, have been developed to discriminate between GM- and non-GM-derived DNA varieties (*4*). The screening method is associated with a particular risk of yielding false positives (*16*). Gene specific and construct specific methods are more specific but may cause false positives when the modified genes are used in other GMOs with variable copy numbers, and these methods cannot distinguish between different GMOs if the same construct has been integrated (*17*). To overcome these problems, a line or transformation event specific PCR should be performed. Until now, several event specific quantitative PCR methods have been established for GTS 40-3-2 soybean (*18*−*20*), MON531 and MON1445 cotton (*21*), MON810 maize (*22*, *23*), Bt11 maize (*17*), Starlink maize (*24*), GA21 maize (*25*), NK603 maize (*26*), T25 maize (*27*), and Event176 maize (*28*). However, till now, there is no report on a MON863 maize event specific PCR detection method.

In this study, we have identified the maize genomic sequences flanking the 5′-site of the MON863 event integration locus by means of the thermal asymmetric interlaced (TAIL)-PCR technique (*29*) and developed the qualitative and quantitative real-time PCR methods to identify and quantify this transformation event. Three mixed GM MON863 samples were also quantitatively analyzed using the established real-time PCR method.

MATERIALS AND METHODS

Materials and DNA Extraction. GM maize lines (MON863, MON810, and GA21), GM soybean (GTS 40-3-2), and GM canola (GT73) were developed and supplied by Monsanto Company. GM maize lines, Event176 and Bt11, were developed and supplied by Syngenta Seeds, Inc. The GM maize line T25 was developed and supplied by Bayer CropScience. GM maize TC1507 was developed and supplied by Mycogen. Nontransgenic maize was purchased from a local market in Shanghai, China. Plant genomic DNA was extracted and purified using a Plant DNA Mini-prep Kit (Shanghai Ruifeng Agrotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. Ground plant material (100 mg) mixed with 500 μL of buffer A was transferred to a 1.5 mL tube and incubated for 10 min at 65 °C and then centrifuged at 13400*g* for 10 min, and the resultant supernatant was collected, precipitated with 500 μL of buffer B, mixed for 30 s, and then centrifuged at 13400*g* for 10 min. The deposit was dissolved in 100 μL of buffer C at 37 °C over 5 min, and then, the solution was transferred to a silica-based DNA binding column. The column was centrifuged at 5900*g* for 1 min, and then, the silica column was washed in turn with wash buffer I and wash buffer II twice, respectively. At the last step, genomic DNA was eluted with water from the centrifugal column. The quantity of DNA in the samples was calculated using absorbance measurements at 260 nm wavelength, and its copy number was calculated from the quantity of DNA and maize genomic DNA average sizes.

Oligonucleotide Primers and Probes. Sequences of oligonucleotide primers and TaqMan fluorescent probes employed in this study are listed in **Table 1** and were designed using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA). The probe of endogenous *zSSIIb* gene was labeled with the fluorescent reporter dye 5-hexachloro-fluorescein (HEX) on the 5′-end, and the exogenous gene was labeled with 6-carboxy-fluorescein (FAM) on the 5′-end. The fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) was located on the 3′-end of the probes. The specific primers L1R/2R/3R (**Table 1**) used in TAIL-PCR were designed based on the sequence of the *CaMV35S* promoter, and the arbitrary primer AD20 was also selected and used in TAIL-PCR. The event specific PCR primer pairs (M1F/2R and M3F/4R) and probe (Mp) used for MON863 maize detection were designed based on the junction region between the host DNA and the exogenous sequence originating from the *CaMV35S* promoter, the primers M1F/2R were employed for conventional PCR, and the primers M3F/4R combined with probe Mp were

employed for TaqMan real-time PCR (**Table 1**). For the total maize DNA detection, the maize z*SSIIb* gene was selected as an endogenous reference gene (*30*), and the primers Z1F/2R and its combination of TaqMan probe Zp were used in conventional and real-time PCR, respectively. All of the primers and fluorescent probes were synthesized and purified by Shanghai BioAsia Co. Ltd. (Shanghai, China).

Determination of Flanking DNA Sequence Using TAIL-PCR Method. The 5′-flanking sequence of the MON863 exogenous insertion was determined using the TAIL-PCR method, essentially as described by Liu et al. (*29*). PCR reactions were performed according to the protocol of Liu et al. described with slight modifications. The first TAIL-PCR amplification was performed in a total volume of 30 μL containing the following: 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$), 200 μM dNTPs, 0.5 μM primer L1R, 5 μM primer AD20, 2.5 U of *TaKaRa Ex Taq* HS DNA polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China), and 50 ng of MON863 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 the following: 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$), 200 μM dNTPs, 0.2 μM primer L2R, 2 μM primer AD20, 5 U of *TaKaRa Ex Taq* HS DNA polymerase (TaKaRa Biotechnology Co., Ltd.), and 1 μL of a 50-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 45 °C, and 7 min at 68 °C. Tertiary TAIL-PCR amplification was carried out in a total volume of 50 μL containing the following: 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$), 200 μM dNTPs, 0.2 μM primer L3R, 2 μM primer AD20, 5 U of *TaKaRa Ex Taq* HS DNA polymerase (TaKaRa Biotechnology Co., Ltd.), 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, and 6 min at 72 °C and the last step of 5 min at 72 °C. All PCR reactions were carried out in PTC-100 thermocycler (MJ Research, Waltham, MA). The second and tertiary PCR amplification products were analyzed by agarose gel electrophoresis with ethidium bromide staining, and the amplified fragments with similar sizes in these two PCR amplifications were purified with Gel Extraction Mini Kit (Watson Biotechnologies, Inc., Shanghai, China) and ligated into pMD18-T vector (TaKaRa Biotechnology Co., Ltd.). Sequencing analysis of the cloned DNA was performed using the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems Division of Perkin-Elmer Corp.) by Shanghai BioAsia Biotechnology Co., Ltd. Parallel amplifications with wild-type isogenic DNA were carried out to identify MON863 specific PCR products.

Conventional PCR Conditions. In conventional PCR assays, all of the 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 each dNTP, 0.8 μM each primer, and 2.5 U of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd.). The multiplex PCR conditions were the same as described above, except for the primer concentration as 0.8 μM M1F/2R and 0.4 μM Z1F/2R instead. All of the conventional PCR amplifications were performed in PTC-100 Thermalcycler (MJ Research, Watertown, MA) 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 electrophoresed in 2% 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 assays were carried out in a fluorometric thermal cycler (Rotor-Gene 2000; Corbett Research, Australia) with a final volume 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, and dCTP, 800 μM dUTP, 600 nM primers, 150 nM TaqMan probes, 1.5 U of *Taq* DNA polymerase, 0.2 U of Amperase Uracil N-glycosylase (UNG), and 6.5 mM MgCl2. Real-time PCR reactions were carried out with the following procedures: 2 min at 50 °C and 10 min at 95 °C, 50 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. Data were analyzed with Rotor gene 2000 software version 5.0 (Corbett Research). All of the PCR reagents were purchased from Roche Molecular Biochemicals (Shanghai, China) except for primers and probes.

Construction of the Standard Curves. After optimization of magnesium and primer/probe concentrations, one series of standard MON863 genomic DNA dilutions was used for quantification, the reproducibility test, and determination of the limits of detection and quantification (LOD and LOQ). Genomic DNA isolated from powdered 100% MON863 maize was serially diluted to final concentrations equivalent to 80000, 8000, 800, 80, and eight copies haploid genome/μL (amounts of DNA per reaction tube ranged from 80000, 8000, 800, 80, and eight copies haploid genome) considering 2504 Mb per haploid genome in the case of maize according to the report of Arumuganathan and Earle (*31*).

RESULTS AND DISCUSSION

Cloning and Sequencing of 5′-Flanking Region in MON863 Maize. According to the released information from the Agbios web site (*32*), we obtained the sequence of MON863 (**Figure 1A**) and three appropriatespecific primers (L1R/2R/3R) were designed based on the obtained *CaMV35S* promoter sequence to amplify the 5′-flanking sequences by means of the TAIL-PCR technique. The combination of the arbitrary primer AD20 and the specific primer L3R in tertiary amplification reactions amplified a discrete product of about 0.9 kb. Using the genomic DNA from wild-type isogenic maize as the template, no amplification product was observed in parallel experiments. The TAIL-PCR product was cloned into the pMD18-T vector, and 10 individual clones were selected and sequenced. The result of sequence analysis indicated that one 850 bp fragment encompassing the 5′-junction region was obtained, of which 713 bp originated from endogenous DNA of *Zea mays* strain NB mitochondrion and 137 bp originated from the cauliflower mosaic virus *35S* DNA (**Figure 1B**). This 713 bp DNA fragment showed 97% homology with the sequence of *Z. mays* NADH dehydrogenase subunit 4 (*nad4*) gene (Genbank accession number AY506529).

Development of the Qualitative Event Specific PCR Detection for MON863 Maize. Event specific primers (M1F/2R) were designed based on the 5′-flanking sequence described above and employed to establish the qualitative event specific PCR assay for MON863 maize (**Figure 1B**). The primer M1F was located at the maize genome, and the primer M2F was located at the *CaMV35S* promoter. The maize z*SSIIb* gene was selected as an endogenous reference control, and the primer pair Z1F/2R was employed in maize identify PCR assay. As expected, in the established qualitative PCR assay, one single 411 bp DNA fragment was obtained using the MON863 maize DNA as template, and no fragment was detected in other GM maize lines (MON810, GA21, Bt11, Event176, T25, and TC1507), GM soybean (GTS 40-3-2), GM canola (GT73), and no template control (NTC) (**Figure 2A**). The target fragments of z*SSIIb* gene (88 bp) were detected in all GM maizes and nontransgenic maize except for GM soybean, GM canola, and NTC (**Figure 2B**). These data confirmed that the obtained DNA fragment was a MON863 event specific region between the 5′-flanking sequence of transgene and the maize genomic DNA.

Because the degradation of low quantity DNAs derived from GMOs often occurs in practical detection, a more sensitive PCR detection system is important and necessary. In qualitative PCR,

Figure 1. Organization of transgenic elements in MON863 maize genome. (**A**) Schematic diagrams of the integrated heterologous DNAs in maize event MON863. The abbreviations are described in the Abbreviations Used. (**B**) TAIL-PCT amplified 5′-junction sequence. Capital letters represent the flanking genomic sequence, and lowercase letters show the 3′-end sequence of *CaMV35S* promoter. Primers used for PCR amplifications detailed in **Table 1** are underlined.

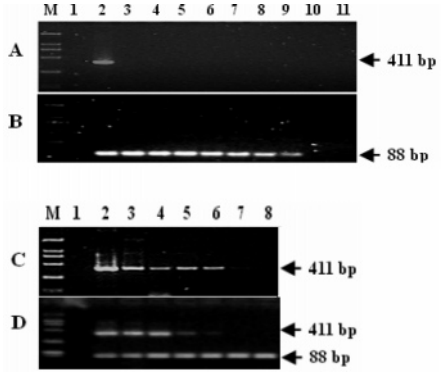

Figure 2. Three percent agarose gel electrophoresis of PCR products amplified with the MON863 event specific primers M1F/2R and maize *zSSIIb* gene primes Z1F/2R. (**A**) MON863 event specific results. (**B**) Maize endogenous gene, *zSSIIb*, specific results. Lane 1, NTC (no template control); lanes 2–11, amplification of MON863 maize, MON810 maize, GA21 maize, Bt11 maize, Event176 maize, T25 maize, TC1507 maize, Non-GM maize, GM soybean (GTS 40-3-2), and GM canola (GT73); and lane M, DL2000 DNA marker. (**C**) Sensitivity test of MON863 event specific assay. (**D**) Sensitivity test of the multiplex PCR assay employing primers Z1F/2R and M1F/2R. PCR products were amplified from mixed GM MON863 maize 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, 0.5, 0.1, and 0.05% GM contents, respectively; and lane M, DL2000 DNA marker.

the test sensitivity is measured by the LOD. To test the LOD of the established event specific PCR assay, the DNA mixture was prepared from MON863 maize line and non-GM maize line at various levels such as 0.05, 0.1, 0.5, 1.0, 3.0, 5.0, and 10%. The test results showed that the target fragment was detected from all of the levels tested except for the 0.05% level, which meant that the lowest tested level was 0.1%, which approximates to 40 haploid genome copies according to the genome sizes of maize (**Figure 2C**) (*31*). These LODs were similar to those of GM soybean and GM maize (*33*), which indicated that the established qualitative event specific PCR detection system of MON863 maize was suitable for the practical detection of GM maize samples.

In addition, the multiplex PCR assay for MON863 maize was also established, which could improve the detection efficiency and save time and reagents. In this assay, primer pairs M1F/2R and Z1F/2R were amplified in one tube. The amplified results indicated that two target fragments for MON863 5′-integration flanking sequence and *zSSIIb* gene, respectively, were detected in MON863 maize, whereas only one z*SSIIb* fragment was detected in non-GM maize and no fragments in NTC (data not shown). We also estimated the LOD of the multiplex PCR assay using the above-prepared DNA mixtures. The amplified results indicated that MON863 event specific fragment was obtained except for 0.1 and 0.05% levels (**Figure 2D**), which meant that the LOD of the multiplex assay was 0.5% or about 200 haploid genome copies, which were calculated from the genome sizes of maize. Concluded from the results, we believe that the established multiplex PCR assay is suitable for the practical GMOs detection with high throughout and little labor.

Development of TaqMan Real-Time PCR Assays Based on the 5′-Flanking Sequence. Through analyzing the 5′-end insertion sequences corresponding to the unique insertion event in MON863, the specific detection and quantification methods based on TaqMan real-time quantitative PCR were developed. The primers (M3F/4R) and TaqMan probe Mp were designed based on the obtained flanking sequence. The primer M3F was located on the maize genomic DNA, the primer M4R was located on the *CaMV35S* promoter of *NptII* cassette, and the probe Mp contained the annealing sequences for both the *CaMV35S* promoter sequence and the maize genomic DNA. For the total maize quantification, the *zSSIIb* real-time PCR assay employing primers (Z1F/2R) and probe Zp was also established.

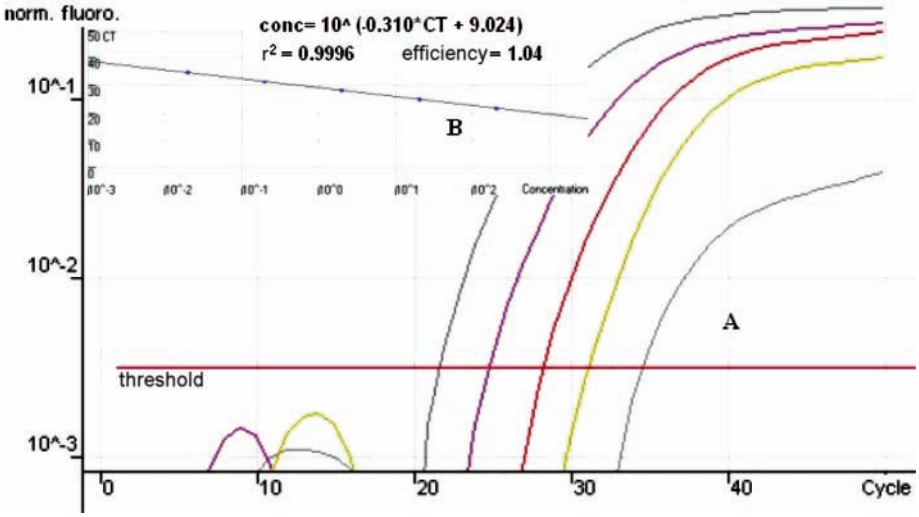

Figure 3. Amplification plots and standard curve for MON863 event specific real-time PCR assay. (**A**) Amplification curves (five serial dilutions, corresponding to 80000, 8000, 800, 80, and 8 copies of the MON863 haploid genome per reaction) were generated for detection of MON863 maize. The horizontal 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. Reproducibility of the C_t Measurements of Replicate Standards from 80000 to 8 Copies Haploid Genomic DNA of MON863 Maize

target copies	C_t values			mean C_t	SD
	1	2	3		
		event specific assay			
80000	20.97	20.89	20.91	20.92	0.03
8000	23.77	23.88	23.92	23.86	0.06
800	26.99	26.95	26.86	26.93	0.05
80	30.08	30.14	29.97	30.06	0.07
8	33.19	33.11	33.36	33.22	0.10
		zSSIIb assay			
80000	22.24	22.38	22.19	22.27	0.08
8000	25.4	25.38	25.43	25.40	0.02
800	28.64	28.62	28.57	28.61	0.03
80	31.59	31.72	31.67	31.66	0.06
8	34.65	34.79	35.03	34.82	0.16

Construction of the Standard Curves. After optimization of magnesium and primer/probe concentrations, the standard curves of the event specific and endogenous *zSSIIb* real-time PCR assays were constructed using the standard MON863 genomic DNA dilutions ranging from 80000, 8000, 800, 80, and eight copies haploid genome. The similar PCR reaction efficiencies between z*SSIIb* assay and event specific assay (1.04 of event specific PCR assay and 1.07 of z*SSIIb*) indicated that GM contents could be calculated using these two PCR assays by means of the relative quantitative method. The square regression coefficients (R^2) were 0.9994 and 0.9996 for the *zSSIIb* and event specific PCR assay, respectively. The good linearity between DNA quantities and fluorescence values (C_t) (**Figure 3**) indicated that these assays were well-suited for quantitative measurements.

Reproducibility of the TaqMan Assays. Reproducibility of the threshold of detection (C_t) measurements was determined using the above standard DNA dilutions repeated in triplicate (**Table 2**). TaqMan assays for z*SSIIb* gene detection gave mean C_t values varying from 20.89 to 33.36 cycles in each standard dilution with a standard deviation (SD) value of 0.03−0.10. The event specific PCR assay gave mean C_t values varying from 22.19 to 35.03 with an SD value of 0.02−0.16. These results indicated that these quantitative PCR assays were stable and reliable.

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

template copies	signal rate (positive signals)	means (C_t)	SD
80000	9/9	20.94	0.04
8000	9/9	23.87	0.05
800	9/9	26.93	0.04
80	9/9	30.04	0.09
8	8/9	33.25	

Determination of the LOD and the LOQ. There are at least three different ways for expressing detection and quantitation limits, although they all refer to the lowest quantity of the target that reliably can be detected and quantified with a probability of ≥95% (*34*). 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 (e.g., maize) that can be detected and quantified, and the practical limit is the functional limit of the sample during the practical analysis. To determine the LOD and LOQ of the established event specific real-time PCR assay, a series of DNA dilutions containing an estimated average of 80000, 8000, 800, 80, and eight copies of the MON863 haploid genome per reaction were tested in three parallel reactions and repeated three times (**Table 3**). As expected, the ability to detect MON863 decreased with decreasing copy numbers. The MON863 maize DNA could be detected in all of the nine reactions down to eight copies. These results indicated that the LOD value was about eight copies. The data also showed that the SD values of the nine reactions with the same template concentration increased with decreasing copy number, especially for the dilution with eight copies of the haploid genome. To obtain reliable quantization results under ideal conditions, approximately 80 initial template copies were required, and we concluded that the LOQ of the event specific real-time PCR assay was 80 copies of the haploid genome.

Quantification of the Samples with Known MON863 Maize Content Using Screening and Event Specific Real-Time PCR Assays. On the basis of the standard curve, three

Table 4. Quantification of MON863 Maize Content in Three Mixed Samples

sample	probe dye	C_t mean 1	C_t mean 2	C_t mean 3	mean of all C_t values	SD	calcd DNA amounts	GM content (%)
				zSSIIb PCR assay				
S1 (5%)	HEX	23.22	23.31	23.18	23.24	0.05	95.93	
S2 (3%)	HEX	23.19	23.12	23.26	23.19	0.06	99.26	
S3 (1%)	HEX	23.2	23.36	23.11	23.22	0.10	96.87	
				event specific PCR assay				
S1 (5%)	FAM	25.69	25.93	25.72	25.78	0.11	5.01	5.22
S2 (3%)	FAM	26.39	26.56	26.63	26.53	0.10	2.86	2.88
S3 (1%)	FAM	27.99	27.91	28.17	28.02	0.11	0.93	0.96

samples, i.e., S1, S2, and S3 with 5, 3, and 1% of MON863 content, were prepared by mixing the pure dried MON863 maize flours with non-GM dried maize flours on a wt/wt basis, and then, three samples were used for quantification. As shown in **Table 4**, the quantitative results of these three samples were 5.22, 2.88, and 0.96%, respectively. We observed that the quantified results deviated slightly from the actual contents of the MON863 in these three samples. It is important to note that the mixing of the samples was based on a wt/wt ratio, not the genome/genome ratio. So this small deviation of the quantified result may partially result from the possible differences in the genome/weight ratios of the two maize materials (MON863 and non-GM maize). According to Peccoud and Jacob, the quantitative uncertainty in PCR reactions is mainly due to molecular fluctuations with low numbers of initial template copies in simulation studies by varying numbers of template copies and amplification efficiencies (*35*). In the quantitative PCR detection of GM samples with low DNA levels, the quite large deviations in the quantified results were possible and acceptable on the premise that the endogenous PCR and exogenous PCR reactions have high PCR amplification efficiencies. These results showed 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.

ABBREVIATIONS USED

GM, genetically modified; 4-AS1, a promoter that contains four copies of AS-1 element and a part from cauliflower mosaic virus (CaMV); wt CAB, 5′-terminal untranslated region of wheat chlorophyll a/b binding the expression of target genes; ract1, intron of rice actin gene that activates the expression of target genes; *Cry3Bb1*, the gene that encodes modified Cry3Bb1 protein of *Bacillus thuringiensis*; tahsp 17 3′, 3′-terminal untranslated region of wheat heat shock protein 17.3; NOS 3′, 3′-nontranslated polyadenylation signal of *Agrobacterium tumefaciens* nopaline synthase gene; *NptII*, nopaline synthase gene from *A. tumefaciens*; 35S, cauliflower mosaic virus 35S promoter; UNG, *Amperase uracil N-glycosylase* gene; LOD, limit of detection; LOQ, limit of quantification; SD, standard deviation.

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