JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLES

Event-Specific Quantitative Detection of Nine Genetically Modified Maizes Using One Novel Standard Reference Molecule

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With the development of genetically modified organism (GMO) detection techniques, the Polymerase Chain Reaction (PCR) technique has been the mainstay for GMO detection, and real-time PCR is the most effective and important method for GMO quantification. An event-specific detection strategy based on the unique and specific integration junction sequences between the host plant genome DNA and the integrated gene is being developed for its high specificity. This study establishes the event-specific detection methods for TC1507 and CBH351 maizes. In addition, the event-specific TaqMan real-time PCR detection methods for another seven GM maize events (Bt11, Bt176, GA21, MON810, MON863, NK603, and T25) were systematically optimized and developed. In these PCR assays, the fluorescent quencher, TAMRA, was dyed on the T-base of the probe at the internal position to improve the intensity of the fluorescent signal. To overcome the difficulties in obtaining the certified reference materials of these GM maizes, one novel standard reference molecule containing all nine specific integration junction sequences of these GM maizes and the maize endogenous reference gene, *zSSIIb*, was constructed and used for quantitative analysis. The limits of detection of these methods were 20 copies for these different GM maizes, the limits of quantitation were about 20 copies, and the dynamic ranges for quantification were from 0.05 to 100% in 100 ng of DNA template. Furthermore, nine groups of the mixed maize samples of these nine GM maize events were quantitatively analyzed to evaluate the accuracy and precision. The accuracy expressed as bias varied from 0.67 to 28.00% for the nine tested groups of GM maize samples, and the precision expressed as relative standard deviations was from 0.83 to 26.20%. All of these indicated that the established event-specific real-time PCR detection systems and the reference molecule in this study are suitable for the identification and quantification of these GM maizes.

KEYWORDS: Genetically modified maize; event-specific; real-time PCR; standard reference molecule; integration junction sequence

INTRODUCTION

In the past decade, biotechnology has been widely used in modern agriculture and related industries, and hundreds of genetically modified (GM) plants have been approved for commercialization worldwide. From 1996 to 2005, the global area dedicated to GM crops increased >53-fold, from 1.7 million hectares in 1996 to 90.0 million hectares in 2005, with an increasing proportion located in developing countries (*1*). In 2005, the planted area of GM maize (*Zea mays* L.) was 21.2 million hectares with the ratio of 24% in global biotechnology area; the principal traits of GM maizes are herbicide tolerance (B16, T25, NK603, and GA21, etc.), insect resistance (MON863,

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10.1021/jf0615754 CCC: $37.00
Published on Web 12/14/2006

MON802, and MON810, etc.), and the combined traits of herbicide tolerance and insect resistance (Bt176, Bt11, CBH351, and TC1507, etc.) (*1*). In 2004 and 2005, 18 GM events from the United States and Canada were approved for commercialization in China, that is, one GM soybean (GTS 40-3-2), eight GM maizes (Bt11, Bt176, GA21, T25, NK603, MON810, MON863, and TC1507), seven GM canolas (Ms1Rf1, Ms1Rf2, Ms8Rf3, GT73, T45, Oxy235, and Topas 19/2), and two GM cottons (MON531 and MON1445) (*2*).

However, as more GM foods, food ingredients, and additives are introduced from the field to the market and table, several controversial issues are being discussed, such as food safety, environment risk, and ethical concerns, resulting in more than 40 countries and areas have issued GMO labeling regulations for protecting the consumers' authority. For instance, the labeling threshold was defined as 0.9% in the European Union (*3*), 3% in Korea (*4*), and 5% in Japan (*5*, *6*). In China, 17 GM products must be labeled, such as maize seeds, maize oil, tomato seeds, ketchup, soybean seeds, soybean oil, rapeseed seeds, and cotton seeds (*7*). To date, much effort has been expended to implement the GMO labeling requirement in China: for example, the validation of four novel endogenous reference genes, the *sucrose phosphate synthase* (*SPS*) gene for rice, the *LAT52* gene for tomato, the *stearoyl-acyl carrier protein desaturase* (*Sad1*) gene for cotton, and the *high-mobility-group protein I/Y* (*HMG I/Y*) for rapeseed (*8*−*11*) and the construct-specific and even-specific detection methods for transgenic Huafan No. 1 tomato, MON863 maize, GK19 cotton, SGK321 cotton, MON1445 cotton, and MON531 cotton, etc. (*12*−*16*).

To execute the labeling requirements, the Polymerase Chain Reaction (PCR) technique has become the main technique for genetically modified organism (GMO) detection, and the PCR detection strategies which discriminate the GM- and non-GM-derived DNA varieties are divided into four levels, that is, screening and gene-, construct-, and event-specific PCR detection. The screening method is associated with a particular risk of yielding false positives. Gene-specific and construct-specific methods are more specific, but may cause false positives when the same gene or construct is integrated into other GMOs with variable copy number. To overcome this, a line or transformation event-specific PCR method should be performed (*17*, *18*). Up to now, some event-specific quantitative PCR methods for a few GM crop events, compared with the total commercialized GM events, have been developed, such as GTS 40-3-2 soybean (*19*−*21*), MON531 and MON1445 cotton (*14*), GT73 canola (*22*), MON810 maize (*23*, *24*), Bt11 maize (*25*, *26*), GA21 maize (*22*, *27*), MON863 maize (*15*, *16*), Bt176 maize (*22*), T25 maize (*28*), NK603 (*29*), and CBH351 (*30*).

Moreover, the standard reference molecule has been proved to be a good alternate for reference materials in GMO detection (*14*, *31*). However, few standard reference molecules containing the integration junction sequences of GM maizes have been reported. In this study, the event-specific real-time PCR methods of nine GM maize events were systematically developed. Also, a novel standard reference molecule with maize endogenous reference gene and the specific integration junction sequences of these nine GM maizes was constructed and proved to be a valid substitute for the certified positive reference materials in GM maize quantification.

MATERIALS AND METHODS

Materials. Genuine seeds of GM maize lines MON863, MON810, GA21, and NK603, GM canola (GT73), GM cotton (MON531), and GM soybean (GTS 40-3-2) were developed by Monsanto Co. Genuine seeds of GM maize lines Bt11 and Bt176 were developed by Syngenta Seeds, Inc. Genuine seeds of GM maize lines CBH351 and T25 were developed by Aventis CropScience Co. Genuine seeds of TC1507 maize were developed by Mycogen. The GM maize samples MON810, Bt176, Bt11, GA21, NK603, and MON863 were purchased from Fluka Co., and the other GM maize samples (T25, CBH351 and TC1507) were supplied by the developers. Nontransgenic maize was purchased from a local market in Shanghai, China.

DNA Isolation and Purification. Maize and other crop genomic DNAs were extracted and purified using the Plant DNA Mini-Prep kit (Ruifeng Agro-tech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. Briefly, 100 mg of ground plant material mixed with 500 μL of buffer A was transferred to a 1.5-mL tube, 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. Plasmid DNAs were isolated and purified using the Plasmid Mini Kit (Watson Biotechnologies, Inc., Shanghai, China). The quality and quantity of DNA samples were evaluated and calculated using absorbance measurements at 260 and 280 nm wavelengths and 1% agarose gel electrophoresis. The copy number of genomic DNA samples was measured as to the DNA quantity and maize genomic DNA average size.

Oligonucleotide Primers and Probes. The oligonucleotide primers and TaqMan fluorescent probes used in this study were designed using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA). The fluorescent reporter [5-hexachlorofluorescein (HEX) or 6-carboxyfluorescein (FAM)] was dyed on the 5′ end of the probe. To increase the fluorescent intensity, the fluorescent quencher [6-carboxytetramethylrhodamine (TAMRA)] was dyed on the T-base of the probes within internal positions according to the description of Proudnikov et al. (*32*).

Our previous data indicated that the quantitative detection results of GMOs with less bias could be obtained with similar and shorter lengths of endogenous and exogenous amplicons, especially for the detection of the processed food samples (data not shown). To accurately quantify these GM maizes, we designed the amplicons of the endogenous and exogenous target sequences to be <100 bp in size except those for the GA21 and NK603 maizes. The amplicons of NK603 and GA21 maizes were about 110 bp in size due to the difficulty in designing primers and probes with the integration junction sequence. The primers and probe for TC1507 were designed for amplification of the 83-bp fragment on the basis of the 3′ junction fragment of TC1507 maize between the partial *Pat* gene and maize genome disclosed in one U.S. patent (*33*). The primers and probe for CBH351 were designed on the basis of the reported event-specific sequence (Genbank No. AJ506040). Primers and probe for GA21 maize with amplification of the 112-bp specific 5′ junction fragment of GA21 maize between the maize genome and rice *actin1* promoter were used based on the previous paper (*34*). The TaqMan primers and probes of another six GM maize lines, that is, Bt11, Bt176, MON810, MON863, NK603, and T25, were designed on the basis of their specific integration junction sequences and the detailed information about the integration of nine GM maize lines and are shown in **Figures 1** and **2** and **Table 1**. All of the primers and fluorescent probes were synthesized and purified by Shanghai BioAsia Co. Ltd. (Shanghai, China).

Construction of the Standard Reference Molecule. To overcome the difficulty in obtaining the certified positive and negative reference maize materials, one novel plasmid named pMD-ZM was constructed and used in GMO quantitation. The fragments with nine 3′ or 5′ integration junction sequences from the GM maize lines and the specific fragment of the *zSSIIb* gene were cloned using overlapping PCR and the standard subcloning methods (*35*). The primer pairs for the *zSSIIb* gene and the transgene integration fragments of the GM maizes are listed in **Table 2** and **Figure 1**. After amplification of these 10 DNA fragments independently, we first combined 3 DNA fragments, that is,

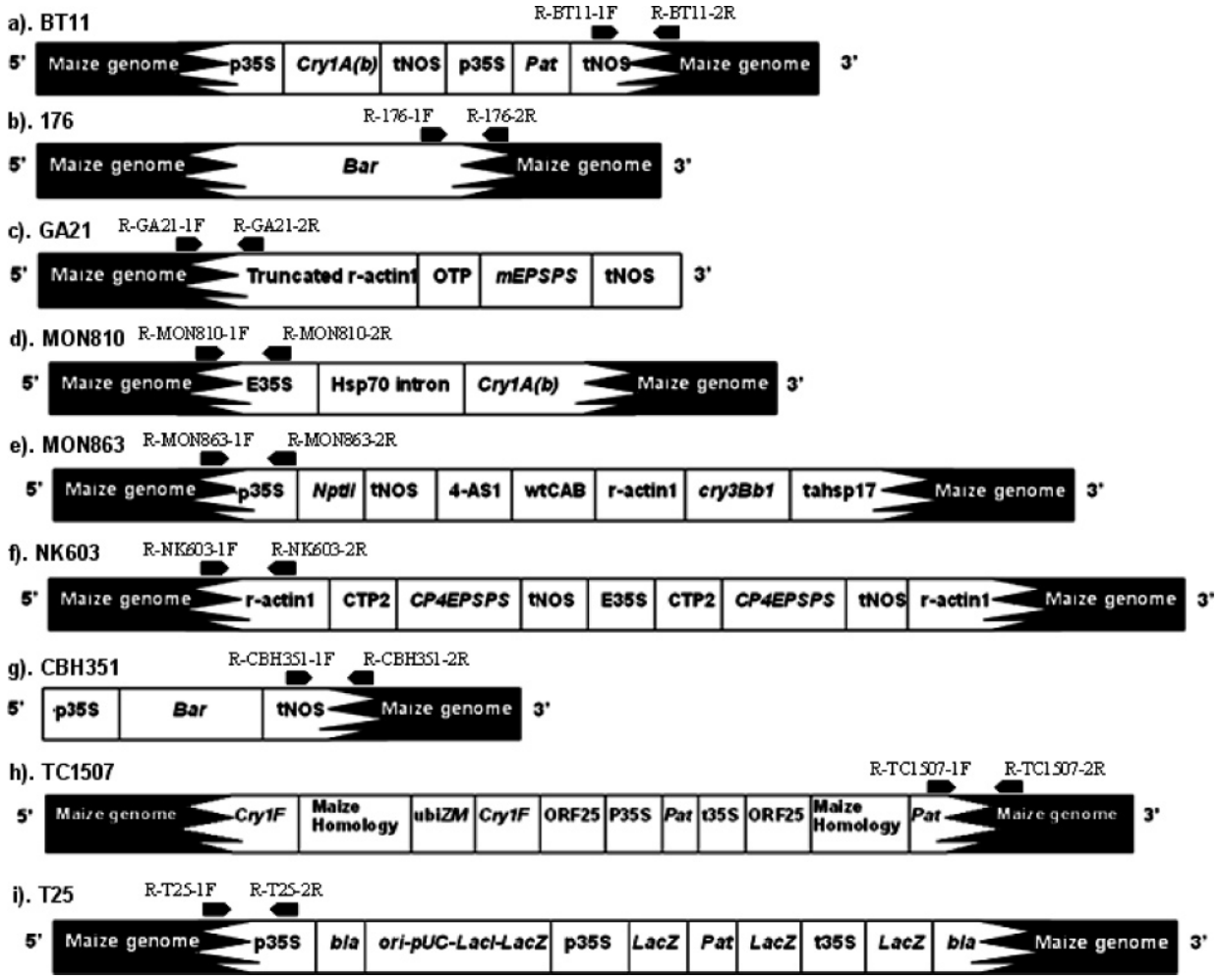

Figure 1. Schematic diagrams of the integrated heterologous DNAs in GM maize events, such as Bt11, Bt176, GA21, MON810, MON863, NK603, TC1507, CBH351, and T25 maize..

fragment A with the flanking sequences of Bt11, MON810, MON863, and T25, fragment B with the flanking sequences of NK603 and CBH351, and fragment C with the flanking sequences of TC1507, Bt176, and GA21, using overlapping PCR strategy (*14*). Then, the three new fragments and fragment D (z*SSIIb*) were cloned into the cloning vector pMD18-T through a molecular manipulation method, and the resulting plasmid, pMD-ZM, was confirmed by sequencing analysis using the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems) by Shanghai BioAsia Biotechnology Co., Ltd. (Shanghai, China).

Qualitative PCR Conditions. In qualitative 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 of each dNTP, 0.4 μM of each primer, and 2.5 units of Taq DNA polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China). All of the qualitative PCR amplifications were performed in a PTC-100 Peltier thermal cycler (MJ Research, Waltham, 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 on 3.0% agarose gels in 0.5× TBE buffer for approximately 30 min at 100 V and stained with EtBr for visualization. The reproducibility of PCR patterns was verified by triple replicate experiments.

Quantitative PCR Conditions. TaqMan real-time PCR assays were carried out in a fluorometric thermocycler (Rotor-Gene 3000A, Corbett Research, Australia) with a final volume of 25 μL. Fluorescence was monitored during every PCR cycle at the annealing step. Reactions contained either 300−600 nM endogenous gene or exogenous gene primers and 150−450 nM endogenous or exogenous probes for real-time assays, 1× PCR buffer, 400 μM each of dATP, dGTP, dCTP, 800 μM dUTP, 1.5 units of Taq DNA polymerase, 0.2 unit of Amperase uracil *N*-glycosylase (UNG), and 6.5 mM $MgCl_2$. Real-time PCR reactions ran with the following procedures: 2 min at 50 °C and 10 min at 95 °C, 45 cycles of 30 s at 95 °C, 60 s at 58 °C. Data were analyzed with Rotor gene 2000 software version 6.0.25 (Corbett Research). All of the PCR reactants were purchased from Roche Molecular Biochemicals (Shanghai, China) except for primers and probes.

A serially diluted plasmid pMD-ZM DNA, that is, 2×10^0, 2×10^1, 2×10^2, 2×10^3, 2×10^4, 2×10^5, and 2×10^6 copies, was used as standard reference molecule per reaction to the construct standard curve. Salmon testis DNA (10 ng/μL) was used as no-template control (NTC). All of the real-time PCR was repeated three times, and each time was triple-replicated for each template DNA (i.e., NTC, standard plasmid DNA, and sample DNA).

In the tests of the repeatability and reproducibility of these real-time PCR assays, six diluted concentrations of standard plasmid DNA, that is, 2×10^1, 2×10^2, 2×10^3, 2×10^4, 2×10^5, and 2×10^6 copies, were used per reaction, respectively. Each reaction of one concentration was repeated three times and each time with triple replication.

Quantitative Analysis of Mixed GM Maize Samples. Standard curves for real-time PCR assays were obtained according to the methods described by Kuribara et al. (*31*). GMO amounts (percent) were calculated by the ratios of the copy numbers of transgene DNA and an endogenous gene in GM maize samples with application of coefficient values (CVs), according to the method described previously (*14*, *31*).

RESULTS

Specificity Test of the Event-Specific Quantitative PCR Systems. To set up the event-specific real-time PCR systems of nine GM maize lines, the specificity of the designed TaqMan

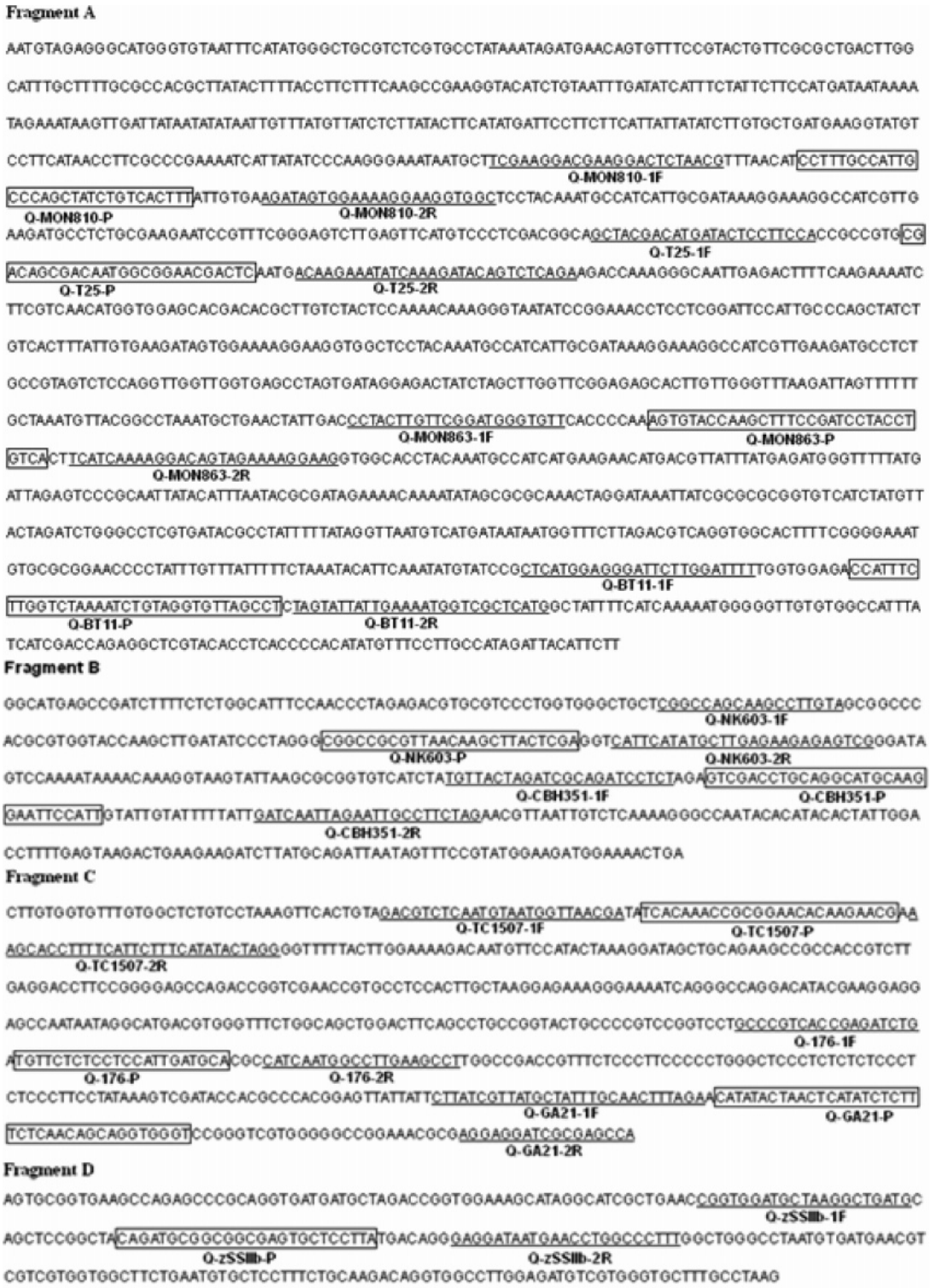

Figure 2. Sequences of the integrated DNAs in the standard plasmid pMD-ZM. The underline and boxes indicate the sequences of TaqMan primers and probes, respectively, designed for nine GM maize events and the maize *zSSIIb* gene. Fragment A includes the specific flanking sequences of MON810, T25, MON863, and Bt11 maize; fragment B includes the specific flanking sequences of NK603 and CBH351 maize; fragment C includes the specific flanking sequences of TC1507, Bt176, and GA21 maize; fragment D is the specific sequence of maize endogenous reference gene, *zSSIIb*.

primers and probes was tested by means of qualitative PCR analysis. Nine qualitative PCR systems, named A−I, specific to the flanking sequences of GA21, NK603, Bt176, Bt11, MON863, TC1507, T25, CBH351, and MON810 maizes, were checked using the genomic DNAs from the nine GM maize lines and non-GM maize, as well as a set of other transgenic plants (GTS40-3-2 soybean, MON531 cotton, and GT73 canola). In qualitative PCR system A employing primer pair Q-GA21−1F/2R, the target fragment 112 bp in size was obtained from only GA21 maize, and no amplified fragments were obtained from the other eight GM maize lines, non-GM maize, and other GM crops (GTS40-3-2 soybean, MON531 cotton, and GT73 canola) (**Figure 3**). Similarly, the qualitative PCR primer pairs, that is, Q-NK603−1F/2R, Q-Bt11−1F/2R, Q-MON863−1F/

Table 1. Primers and Probes for Real-Time PCR Analysis

target	primer name	sequence (5′–3′)	specificity	amplicon (bp)	ref
Bt11	Q-Bt11-1F	CTCATGGAGGGATTCTTGGATTTT	tNOS/maize genome	96	Genbank AY123624
	Q-Bt11-2R	CATGAGCGACCATTTTCAATAATACTA			
	Q-Bt11-P	FAM CCATTTCTTGGTCT(TAMRA)AAAATCTGTAGGTGTTAGCCT			
Bt176	Q-Bt176-1F	GCCCGTCACCGAGATCTG	bar/maize genome	64	Genbank AJ878607
	Q-Bt176-2R	AGGCTTCAAGGCCATTGATG			
	Q-Bt176-P	FAM TGTTCTCTCCT(TAMRA)CCATTGATGCA			
GA21	Q-GA21-1F	CTTATCGTTATGCTATTTGCAACTTTAGA	maize genome/ r-actin1	112	*30*
	Q-GA21-2R	TGGCTCGCGATCCTCCT			
	Q-GA21-P	FAM CATATACTAACTCAT(TAMRA)ATCTCTTTCTCAACAGCAGGTGGGT			
MON810	Q-MON810-1F	CGAAGGACGAAGGACTCTAACG	maize genome/ p35S	91	Genbank AF434709
	Q-MON810-2R	GCCACCTTCCTTTTCCACTATCT			
	Q-MON810-P	FAM CCTTTGCCATTGCCCAGCT(TAMRA)ATCTGTCACTTT			
MON863	Q-MON863-1F	CCTACTTGTTCGGATGGGTGTT	maize genome/ p35S	90	*15*
	Q-MON863-2R	CTTCCTTTTCTACTGTCCTTTTGATGA			
	Q-MON863-P	FAM AGTGTACCAAGCT(TAMRA)TTCCGATCCTACCTGTCA			
NK603	Q-NK603-1F	CGGCCAGCAAGCCTTGTA	maize genome/ r-actin1	110	Genbank AF434709
	Q-NK603-2R	CGACTCTCTTCTCAAGCATATGAATG			
	Q-NK603-P	FAM CGGCCGCGTT(TAMRA)AACAAGCTTACTCGA			
CBH351	Q-CBH351-1F	TGTTACTAGATCGCAGATCCTCT	tNOS/maize genome	96	Genbank AJ506040
	Q-CBH351-2R	CTAGAAGGCAATTCTAATTGATC			
	Q-CBH351-P	FAM GTCGACCT(TAMRA)GCAGGCATGCAAGGAATTCCATT			
TC1507	Q-TC1507-1F	GACGTCTCAATGTAATGGTTAACGA	pat/maize genome	83	*31*
	Q-TC1507-2R	CCTAGTATATGAAAGAATGAAAAGGTGCTT			
	Q-TC1507-P	FAM TCACAAACCGCGGAACACAAGAACG TAMRA			
T25	Q-T25-1F	GCTACGACATGATACTCCTTCCA	maize genome/ p35S	90	*27*
	Q-T25-2R	TCTGAGACTGTATCTTTGATATTTCTTGT			
	Q-T25-P	FAM CGACAGCGACAAT(TAMRA)GGCGGAACGACTC			
zSSIIb	Q-zSSIIb-1F	CGGTGGATGCTAAGGCTGATG		88	*15*
	Q-zSSIIb-2R	AAAGGGCCAGGTTCATTATCCTC			
	Q-zSSIIb-P	HEX TAAGGAGCACT(TAMARA)CGCCGCCGCATCTG			

Table 2. Primers for the Construction of the Standard Reference Molecule

target	primer name	sequence (5′–3′)	specificity	amplicon (bp)
Bt11	R-Bt11-1F	CCTACAAATGCCATCATGAAGAACATGACGTTATTTATGAGATGGGTT	tNOS/maize genome	471
	R-Bt11-2R	AAACCATGGTTTCGGCGGAAGAATGTAATCTATGGCAAGG		
Bt176	R-Bt176-1F	GAAGGAGGAGCCAATAATAGGCATGACGTGGGTTTCTG	bar/maize genome	208
	R-Bt176-2R	AATAGCATAACGATAAGAATAATAACTCCGTGGGCGTGGTAT		
GA21	R-GA21-1F	CGCCCACGGAGTTATTATTCTTATCGTTATGCTATTTGCAACTTTAGA	maize genome/r-actin1	112
	R-GA21-2R	AAACCATGGTGGCTCGCGATCCT		
MON810	R-MON810-1F	TACGTAAATGTAGAGGGCATGGGTG	maize genome/p35S	485
	R-MON810-2R	CAAGACTCCCGAAACGGATTCTTCGCAGAGGCATCTTCAACG		
MON863	R-MON863-1F	GTTGAAGATGCCTCTGCCGTAGTCTCCAGGTTGGTTGGTGAG	maize genome/p35S	231
	R-MON863-2R	TCTCATAAATAACGTCATGTTCTTCATGATGGCATTTGTAGGTG		
NK603	R-NK603-1F	AAACCGCCGGGCATGAGCCGATCTTTT	maize genome/r-actin1	202
	R-NK603-2R	TAGTAACATAGATGACACCGCGCTTAATACTTACCTTTGTTTTATTTTGGAC		
CBH351	R-CBH351-1F	GTCCAAAATAAAACAAAGGTAAGTATTAAGCGCGGTGTCATCTATGTTACTA	tNOS/maize genome	224
	R-CBH351-2R	AACCATGGTCAGTTTTCCATCTTCCATA		
TC1507	R-TC1507-1F	AAACCATGGCTTGTGGTGTTTGTGGCTCT	pat/maize genome	279
	R-TC1507-2R	CCCACGTCATGCCTATTATTGGCTCCTCCTTCGTATGT		
T25	R-T25-1F	CGTTGAAGATGCCTCTGCGAAGAATCCGTTTCGGGAGTCTTG	maize genome/p35S	351
	R-T25-2R	CTCACCAACCAACCTGGAGACTACGGCAGAGGCATCTTCAAC		
zSSIIb	R-zSSIIb-1F	GGATCCAGTGCGGTGAAGCCAGAGC	*zSSIIb* gene	255
	R-zSSIIb-2R	TCTAGACTTAGGCAAAGCACCCACGA		

2R, Q-TC1507−1F/2R, Q-T25−1F/2R, Q-CBH351−1F/2R, and Q-MON810−1F/2R, used in the qualitative PCR gave rise to expected specific DNA fragments as shown in **Figure 3**. In PCR system J, the 88-bp endogenous fragment, *zSSIIb*, was obtained in all maize DNA samples using Q-zSSIIb−1F/2R, and no fragment was obtained in NTC and other GM crops (**Figure 3**). All of these indicated that all 10 designed primer pairs have high specificity and are suitable for further quantitative analysis of GM maize samples.

Construction of the Standard Reference Molecule, pMD-ZM. The reference molecule could be easily produced in bacteria with high quality and quantity; the standard dilution

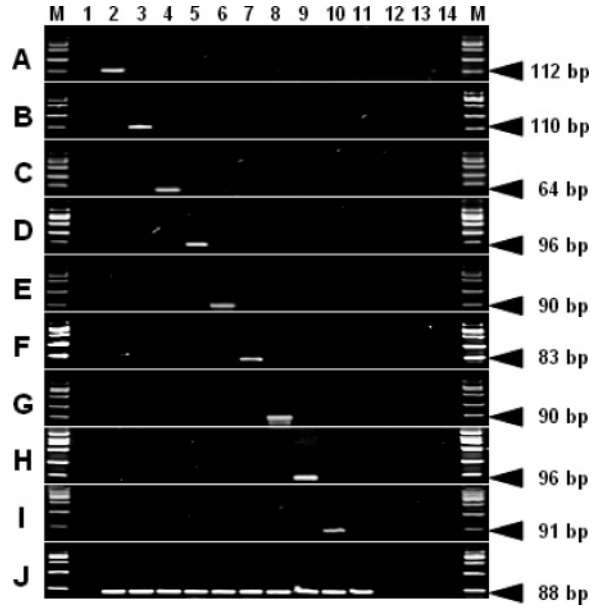

Figure 3. Specificity tests of the designed TaqMan primers for the specific flanking sequence of nine GM maize events and maize endogenous reference gene. 2.5% agarose gel electrophoresis of the amplification products of PCR systems correspond to the 112 bp GA21 (**A**), 110 bp NK603 (**B**), 64 bp Bt176 (**C**), 96 bp CBH351 (**D**), 90 bp MON863 (**E**), 83 bp TC1507 (**F**), 90 bp T25 (**G**), 96 bp Bt11 (**H**), 91 bp MON810 (**I**), and 88 bp *zSSIIb* gene (**J**). Arrowheads indicate the expected PCR amplified products. Lane 1, NTC (no template control); lanes 2–14, amplification of GM maize GA21, NK603, Bt176, CBH351, MON863, TC1507, T25, Bt11, MON810, non-GM maize, GM soybean GTS40-3-2, GM canola GT73, and GM cotton mon531, respectively; lane M, DL2000 DNA marker.

procedure was simplified with few error rates occurring, and many target genes from different GM lines might be integrated into one reference molecule, which decreased the requirement of the standard GM plants (*14*). On the basis of the method described above, the reference molecule, pMD-ZM, which contained the 10 specific fragments (9 GM maize event-specific fragments and *zSSIIb*), was constructed. Totally, this combined DNA fragment was 2969 bp in length and inserted by *Bam*HI and *Xba*I restriction enzyme sites into pMD18-T vector; this fused fragment was also subjected to sequencing confirmation (**Figure 4**). For further use of this plasmid, we produced the pMD-ZM in *E. coli* according to the routine molecular method, and the purified plasmid DNA was linearized with an enzyme, *Sca*I, located outside the integrated fragments; the resulting plasmid DNA was diluted to 2×10^0, 2×10^1, 2×10^2, 2×10^3, 2×10^4, 2×10^5, and 2×10^6 copies per microliter on the basis of the plasmid pMD-ZM size (*24*).

Quantitative PCR Analysis of Nine GM Maizes Using the Standard Reference Molecule. To test the applicability of the reference plasmid pMD-ZM for GM maize detection instead of positive GM reference materials, we optimized the magnesium and primer/probe concentrations, and highly sensitive and quantitative event-specific real-time PCR assays for the accurate measurement of the nine GM maize lines using plasmid pMD-ZM as reference molecule were established separately.

In the case of the quantitative range, seven gradual levels of this reference molecule were set for calibration of the nine GM maizes detection, that is, 2×10^0, 2×10^1, 2×10^2, 2×10^3, 2×10^4, 2×10^5, and 2×10^6 copies for per reaction. On the basis of the genome sizes of the constructed reference molecule pMD-ZM (7380 bp) and the maize (*24*, *36*), we deduced that the copy number range was from 2×10^0 to 2×10^6 of the

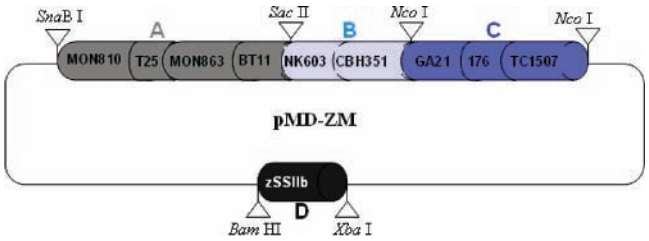

Figure 4. Schematic diagram of the integrated fragments in the constructed pMD-ZM. MON810, five specific flanking fragments of MON810 maize; T25, five specific flanking fragments of T25 maize; MON863, five specific flanking fragments of MON863 maize; Bt11, three specific flanking fragments of Bt11 maize; Bt11, three specific flanking fragments of Bt11 maize; NK603, five specific flanking fragments of NK603 maize; CBH351, three specific flanking fragments of CBH351 maize; GA21, five specific flanking fragments of GA21 maize; Bt176, three specific flanking fragments of Bt176 maize; TC1507, three specific flanking fragments of TC1507 maize; zSSIIb, specific sequence of maize *zSSIIb* gene. Fragment A contained MON810, T25, MON863, and Bt11; fragment B contained NK603 and CBH351; fragment C contained GA21, Bt176, and TC1507; fragment D was zSSIIb.

reference molecule, which was sufficient to quantify GMOs from 0.05 to 100% in 100 ng of template for one reaction. Also, this range was supposed to be sufficient to meet the needs of quantifying GMOs in several countries and areas.

On the basis of the calibration curves constructed by the reference molecule for nine GM maizes and the endogenous *zSSIIb* gene, the limits of detection (LODs) and limits of quantitation (LOQs) of the established event-specific real-time PCR assays were analyzed. The LOD and LOQ refer to the lowest quantity of the target that reliably can be detected and quantified with a probability of ≥95% (*37*). In the constructed calibration curves, the square regression coefficients (R^2) were 0.9990, 0.9981, 0.9999, 0.9997, 0.9998, 0.9993, 0.9981, 0.9998, 0.9991, and 0.9992 for Bt11, Bt176, GA21, MON810, MON863, NK603, TC1507, CBH351, T25, and *zSSIIb* amplicons, respectively. Good linearity between copy number and fluorescence values (Ct) visualized in the calibration curves indicated that these 10 real-time PCR assays combined with reference molecule established in this study were well suited for quantitative measurements. In the testing of LOD and LOQ for all 10 PCR assays, the ability to detect event-specific sequence decreased with decreasing copy numbers. The target event-specific sequences could be detected in all nine amplification reactions down to 20 copies. From these results, we estimated the LODs of Bt11, Bt176, GA21, MON810, MON863, NK603, TC1507, CBH351, T25, and *zSSIIb* PCR assays were low to 20 copies. These data also showed that the SD values of the nine reactions with the same template concentration increased with decreasing copy number, especially for the target molecule of 20 copies. To obtain reliable quantified results under ideal conditions, approximately 20 initial template copies were required, and we concluded that the LOQs of the event-specific real-time PCR assay were 20 copies of the haploid genome. All of the results indicated that these real-time PCR systems were suitable for the detection of transgenic maize samples.

To validate the reproducibility and repeatability of these real-time PCR assays, six reference molecule dilutions (corresponding to 2×10^1, 2×10^2, 2×10^3, 2×10^4, 2×10^5, and 2×10^6 copies for each reaction) were employed in the established 10 PCR assays and performed three times with triplicate reactions each time. The repeatability standard deviation (SD^r) and reproducibility standard deviation (SD^R) of the reference

molecule pMD-ZM were calculated from the data of triplicate reactions and three replications. The SD^r values of these 10 PCR assays ranged from 0.04 to 0.53 (**Table 3**), and the SD^R values ranged from 0.02 to 0.29 (**Table 3**). All of the above results indicated that the pMD-ZM could be successfully used as standard material for quantitation of all nine GM maizes.

Measurement of Coefficient Values Using Genuine Seeds of Nine GM Maizes. According to the previous paper, the ratios of introduced DNA and endogenous sequence in each genuine seed were calculated and defined as coefficient value (CV) (*36*) and used for GMO amount (percent) calculation of unknown sample. In the quantitative analysis of GM maize samples using cloned standard reference plasmid, the CV was applied to normalize the ratio of GM and reference target copies in genomic DNA samples. Also, the conversion factor (Cf) term was sometimes used in GMO analysis to normalize the ratio of GM and reference target copies in genomic DNA samples in other papers with the same meaning of CV (*40*, *41*).

We determined the CVs of all nine GM maizes three times, each time with triple-replicated reactions, and then the mean value was deduced as the CV. **Table 4** shows the CVs of the tested nine GM maizes, and the values were 1.23, 0.88, 0.78, 1.12, 1.08, 0.98, 0.87, 1.10, and 0.82 for Bt11, Bt176, GA21, MON810, MON863, NK603, TC1507, CBH351, and T25 maize, respectively. These results reflect that the CVs were proportional to the copy number of target transgene in the genome. Because the event-specific fragments of the nine GM maizes were unique in the haploid maize genome, the ideal CVs of all nine GM maizes should be 1.0. The discrepancy between experimental and theoretical values might be generated by different PCR efficiencies that resulted from the amounts of the nontargeted sequences in the plasmid and genomic DNA.

Quantitative Analysis of the Nine Groups of GM Maize Samples Using the Reference Molecule pMD-ZM. Nine groups of GM maize mixtures were prepared by mixing the pure dried GM maize flours with non-GM dried maize flours on a weight/weight basis and used to evaluate the accuracy and precision of the established real-time PCR methods in this study. Each group of GM maize mixtures contained only one GM maize line (Bt11, Bt176, GA21, MON810, MON863, NK603, TC1507, CBH351, or T25 maize) with five different GM contents (0, 0.5, 1.0, 3.0, and 5.0%). One hundred nanograms of DNAs extracted from each maize mixture was used as template in one reaction, and each template was analyzed in three parallels; the quantitative estimates were calculated by the defined formula, that is, (copy number of a foreign DNA in GM sample × 100)/(copy number of an endogenous DNA in GM sample × CV) (*14*).

Accuracy of the quantitative method was measured as bias (percent), which was calculated as the equation |1 − calculated GM contents/known GM contents|. As shown in **Table 5**, as to the samples containing different GM contents of nine GM maizes, the bias ranged from 0.67 to 28.00%. The bias existing in our experiment was also similar to those of the previous studies on maize and soybean samples (*14*, *31*). In addition, the experimental mean value was directly proportional to the true value, as shown in **Figure 5**; all of the R^2 values of nine group GM maize samples were above 0.990, and the slope values were about 1.0. The above results indicated that the accuracy of this measurement was ideal and credible.

Estimation of precision was shown as relative standard deviation (RSD) and standard deviation (SD). The RSD and SD values were calculated from the quantified results of nine group GM maize samples; the RSD values of all the mixed

Table 3. Repeatability and Reproducibility of pMD-ZM[a]

	Ct values						
true copy no.	1	2	3	mean	mean copy no.	SD^r	SD^R
			Bt11				
2000000	16.14	16.28	16.19	16.20	2104764.00	0.07	0.05
200000	19.81	19.93	19.96	19.90	165158.10	0.08	0.03
20000	22.73	22.68	22.89	22.77	22949.15	0.11	0.11
2000	26.36	26.29	26.18	26.28	2047.75	0.09	0.06
200	29.38	29.57	29.47	29.47	226.71	0.10	0.06
20	33.17	33.27	33.19	33.21	17.31	0.06	0.04
			Bt176				
2000000	19.67	19.81	19.76	19.75	2267845.00	0.07	0.06
200000	22.68	22.49	22.89	22.69	236403.20	0.20	0.10
20000	25.97	26.01	25.86	25.95	19266.97	0.08	0.03
2000	28.96	29.12	29.01	29.03	1798.79	0.08	0.06
200	32.23	32.16	32.38	32.26	150.41	0.11	0.04
20	34.81	34.59	34.61	34.67	23.51	0.12	0.16
			GA21				
2000000	16.27	16.19	16.29	16.25	1914256.00	0.05	0.03
200000	19.49	19.53	19.56	19.53	211452.70	0.04	0.02
20000	23.08	23.01	23.13	23.073	19479.90	0.06	0.03
2000	26.57	26.68	26.41	26.55	1876.84	0.14	0.08
200	29.91	30.03	29.96	29.97	189.12	0.06	0.06
20	33.19	33.24	33.36	33.26	20.61	0.09	0.03
			MON810				
2000000	16.28	16.15	16.32	16.25	2258136.00	0.09	0.03
200000	19.87	19.81	19.96	19.88	188642.70	0.08	0.02
20000	23.32	23.37	23.13	23.27	18527.64	0.13	0.05
2000	26.79	26.57	26.87	26.74	1726.75	0.16	0.05
200	29.99	30.02	29.85	29.95	192.25	0.09	0.03
20	33.01	33.06	33.42	33.16	21.40	0.22	0.10
			MON863				
2000000	17.79	17.81	17.89	17.83	2171201.00	0.05	0.04
200000	21.24	21.16	21.27	21.22	192649.00	0.06	0.03
20000	24.48	24.49	24.24	24.40	19905.21	0.14	0.05
2000	27.61	27.69	27.55	27.62	2008.32	0.07	0.09
200	30.34	30.67	30.91	30.64	232.06	0.29	0.10
20	34.27	34.43	34.09	34.26	17.47	0.17	0.18
			NK603				
2000000	17.51	17.78	17.61	17.63	1800943.10	0.14	0.12
200000	20.57	20.49	20.62	20.56	215575.80	0.07	0.04
20000	23.91	23.93	23.83	23.89	19259.72	0.05	0.02
2000	27.28	27.19	27.35	27.27	1655.39	0.08	0.04
200	30.21	30.34	30.03	30.19	199.11	0.16	0.16
20	33.63	33.08	33.02	33.24	21.79	0.34	0.14
			CBH351				
2000000	17.16	17.23	17.09	17.16	2541441.00	0.07	0.02
200000	21.99	21.94	21.89	21.94	187163.00	0.05	0.03
20000	26.38	26.41	26.32	26.37	16684.38	0.05	0.04
2000	30.62	30.38	30.56	30.52	1732.85	0.12	0.05
200	34.25	34.38	34.81	34.48	199.64	0.29	0.23
20	37.86	37.56	38.21	37.88	31.28	0.33	0.29
			TC1507				
2000000	20.45	20.53	20.61	20.53	1958845.00	0.08	0.04
200000	23.83	23.61	23.68	23.71	218273.00	0.11	0.04
20000	27.2	27.27	27.31	27.26	18749.95	0.06	0.07
2000	30.36	30.49	30.61	30.49	2018.37	0.13	0.06
200	33.49	33.74	33.97	33.73	214.29	0.24	0.12
20	36.92	37.98	37.35	37.42	16.83	0.53	0.13
			T25				
2000000	17.49	17.53	17.57	17.53	1746305.00	0.04	0.03
200000	20.39	20.49	20.59	20.49	232252.30	0.10	0.06
20000	23.78	23.81	24.01	23.87	23252.34	0.13	0.11
2000	27.48	27.38	27.03	27.30	2244.85	0.23	0.12
200	31.09	31.13	31.01	31.08	170.73	0.06	0.04
20	33.79	33.92	34.68	34.13	21.31	0.48	0.27
			zSSIIb				
2000000	19.57	19.49	19.61	19.56	1800667.00	0.06	0.03
200000	22.63	22.57	22.45	22.55	212569.00	0.09	0.06
20000	25.79	25.71	25.62	25.71	22332.29	0.09	0.05
2000	28.71	28.94	28.91	28.85	2363.02	0.13	0.04
200	32.67	32.58	32.35	32.53	170.87	0.17	0.09
20	35.38	35.78	35.83	35.66	18.30	0.25	0.11

[a] SD^r, repeatability standard deviation; SD^R, reproducibility standard deviation.

Table 4. CVs of Nine GM Maize Event-Specific PCR Systems Using Genuine GM Maize Seeds

event	mean event copies	mean zSSIIb copies	mean CVs	SD	RSD
Bt11	58140.73	47141.13	1.23	0.10	8.28
Bt176	44236.69	50268.97	0.88	0.08	9.30
GA21	42110.91	53988.34	0.78	0.07	8.41
MON810	43672.56	38877.65	1.12	0.09	8.08
MON863	46543.82	42963.53	1.08	0.20	18.00
NK603	50518.02	51724.94	0.98	0.15	15.37
CBH351	49730.09	57161.02	0.87	0.13	15.07
TC1507	41140.98	37514.57	1.10	0.15	13.44
T25	33376.81	40869.56	0.82	0.11	12.86

GM maize samples were from 0.83 to 26.20% (**Table 5**). The SD values were from 0.03 to 0.30 in the nine GM maize mixed samples (**Table 5**). By comparison with the RSD values previously reported from maize, soybean, cotton, and potato samples (*14*, *31*, *38*, *39*), the resulting RSD values of tested samples were accepted except for two tested samples with the slightly higher RSD values of 24.49 and 26.20%, respectively. These two higher RSD values might be generated from the low DNA level of GM maizes derived from tested samples with low reproducibility; another possible reason was the low level of GM contents in the samples.

In fact, GMO quantification accuracy depends on the nature of the reference material and the nature of the transgenic material present in the analyzed sample (*42*). Although the genome compositions of maize teguments, embryo, and endosperm vary in maize materials, these differences could be corrected and the mixed GM maize samples might be accurately quantified using a standard reference molecule (*42*). From these quantitatively analyzed results, we believe that the established event-specific quantitative PCR systems for nine GM maizes in this paper could be used for the detection of these nine GM maizes.

DISCUSSION

The event-specific PCR method with high specificity has been widely used for GM event detection because of its high specificity. In this study, the TaqMan real-time PCR systems for CBH351 and TC1507 maize events were first reported and established on the basis of the sequences of integration junctions.

In addition, the quantitative event-specific PCR detection systems for another seven GM maize events (Bt11, Bt176, GA21, MON810, MON863, NK603, and T25) were optimized and developed systematically. In these systems, we improved the design of TaqMan probes, and the quencher molecule, TAMRA, was dyed on the T-base of the probes with the internal position. This improvement can increase the quenching efficiency of TAMRA, the intensity of the fluorescent signal, and the PCR detection limit, which has been demonstrated by Proudnikov et al. (*32*). In the established real-time PCR systems for nine GM maizes, the low LODs and LOQs of 20 copies relied on the novel design of the TaqMan probe to a degree.

The specific amplicons with improved TaqMan primers and probes of GM maize events and endogenous gene were shorter and <100 bp except for GA21 and NK603 maize events, which was due to the relatively accurate quantitative analysis of GM maizes and their processed products according to our results (unpublished data) and the report of Yoshimura et al. (*41*). In the practical detection of GM maize samples using these established systems, the bias of quantitative results was <20.00% except for only two samples, which indicated that the systems established in this study are suitable for quantitative analysis of the GM samples with low contents. Compared with our unpublished results and the previous paper (*41*), the quantitative results with slight bias obtained in our study are ideal and relatively accurate.

A novel standard reference plasmid containing the integration junction sequences of nine GM maize events and an endogenous gene sequence was constructed and used as standard reference molecule in GM maize event-specific quantitative analysis

Table 5. Accuracy and Precision Statistics for Quantitative Methods

		event copies/ zSSIIb copies			accuracy		precision	
true value (%)	coefficient value	mean 1	mean 2	mean 3	mean GMO (%)	bias (%)	SD	RSD[a] (%)
				Bt11				
5.0		6.54	6.74	6.91	5.47	9.40	0.15	2.74
3.0		4.05	3.60	3.51	3.02	0.67	0.23	7.75
1.0	1.23	1.02	1.12	0.97	0.84	16.00	0.06	7.25
0.5		0.47	0.36	0.50	0.36	28.00	0.06	17.35
0		0	0	0	0			
				Bt176				
5.0		4.3	4.22	4.32	4.86	2.80	0.06	1.32
3.0		2.46	2.19	2.36	2.65	11.67	0.15	5.72
1.0	0.88	0.92	0.84	0.8	0.97	3.00	0.07	7.43
0.5		0.33	0.36	0.43	0.42	16.00	0.06	14.43
0		0	0	0	0			
				GA21				
5.0		3.66	3.7	3.79	4.76	4.80	0.09	1.83
3.0		2.44	2.54	2.46	3.18	6.00	0.06	1.96
1.0	0.78	0.85	0.9	0.81	1.1	10.00	0.06	5.50
0.5		0.33	0.36	0.28	0.41	18.00	0.05	12.18
0		0	0	0	0			
				MON810				
5.0		5.73	5.76	5.91	5.18	3.60	0.09	1.68
3.0		3.28	3.23	3.18	2.88	4.00	0.05	1.56
1.0	1.12	1.03	0.91	0.93	0.85	15.00	0.06	6.87
0.5		0.39	0.55	0.65	0.47	6.00	0.12	24.49
0		0	0	0	0			
				MON863				
5.0		5.32	5.27	5.24	4.89	2.20	0.04	0.83
3.0		3.34	3.47	3.42	3.16	5.33	0.06	1.94
1.0	1.08	0.93	0.9	1.04	0.88	12.00	0.07	7.71
0.5		0.73	0.62	0.66	0.62	24.00	0.06	8.98
0		0	0	0	0			
				NK603				
5.0		5.07	5.13	5.26	5.26	5.20	0.10	1.95
3.0		2.85	2.77	2.83	2.88	4.00	0.04	1.45
1.0	0.98	1.22	1.07	1.02	1.12	12.00	0.10	9.27
0.5		0.55	0.62	0.6	0.6	20.00	0.04	6.01
0		0	0	0	0			
				CBH351				
5.0		4.53	4.49	4.43	5.15	3.00	0.06	1.17
3.0		2.76	2.69	2.57	3.07	2.33	0.11	3.63
1.0	0.87	0.83	1.2	1.08	1.19	19.00	0.22	18.43
0.5		0.30	0.34	0.42	0.40	20.00	0.07	17.59
0		0	0	0	0			
				TC1507				
5.0		4.72	5.31	4.75	4.48	10.40	0.30	6.77
3.0		4.08	3.88	3.67	3.53	17.67	0.19	5.25
1.0	1.10	1.24	1.3	1.4	1.19	19.00	0.07	5.95
0.5		0.43	0.45	0.5	0.42	16.00	0.03	7.33
0		0	0	0	0			
				T25				
5.0	0.82	4.58	4.38	4.75	5.57	11.40	0.23	4.05
3.0		2.86	2.72	2.8	3.41	13.67	0.09	2.50
1.0		0.68	0.78	0.71	0.88	12.00	0.06	6.92
0.5		0.29	0.48	0.34	0.45	10.00	0.12	26.20
0		0	0	0	0			

[a] RSD (relative standard deviation) values were calculated by dividing the standard deviation by mean value.

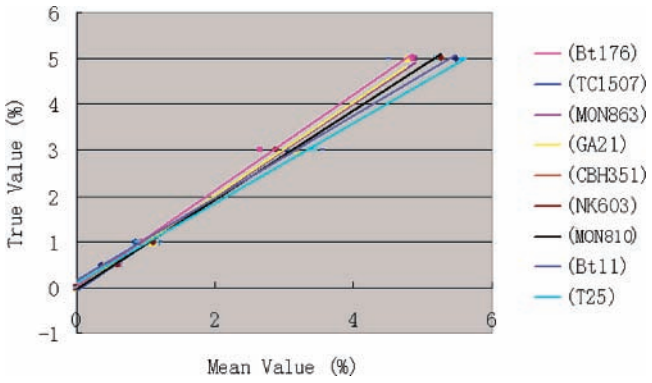

Figure 5. Correlation between the tested and true values of nine GM maize samples employing the standard reference molecule pMD-ZM and the established GM maize quantitative PCR systems.

instead of standard reference materials. In this novel reference plasmid, 10 specific DNA fragments including 9 event-specific fragments and 1 specific fragment of maize endogenous reference gene, *zSSIIb*, were cloned into one plasmid. Compared with the previous reports, only the reference molecules containing the gene- or construct-specific sequences of several GM maize events have been constructed, and these are not suitable for event-specific GMO detection. Furthermore, nine event-specific sequences of GM maizes in one plasmid can be easily used for a stacked GM maize event and mixed GM maize sample quantification. In stacked GM maize and mixed samples, more than two transformed events coexisted in one combined-trait GM maize and mixed samples; each of these GM events must be quantified separately using several different certified reference materials to construct the standard curves, and the results and PCR efficiency can be easily influenced by the different reaction backgrounds with different certified reference material genomic DNAs. However, this factor could be solved in these quantitative systems using one standard reference molecule, pMD-ZM. In addition, the reference plasmid pMD-ZM could be easily produced and quantified.

In conclusion, the novel constructed standard plasmid and the established nine GM maize event detection systems are suitable for the identification and quantification of nine GM maize events, and also the novel constructed standard plasmid might be used for further study of multiplex quantitative detection of nine GM maize events and their processed food and feed samples.

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Received for review June 6, 2006. Revised manuscript received October 23, 2006. Accepted November 2, 2006. This work was supported by the Fund of National Key Basic Research Developments Program of the Ministry of Science and Technology, People's Republic of China (2001CB109002), National Natural Science Foundation of China (30370893), the Program for New Century Excellent Talents in University (NCET-04-0403), the ShuGuang Scholarship (04SG15), the Shanghai Municipal Committee of Science and Technology (05DZ19101), and the Shanghai Standard special fund (05dz05004).

JF0615754