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ARTICLES

Qualitative and Quantitative Polymerase Chain Reaction Analysis for Genetically Modified Maize MON863

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Qualitative and quantitative analytical methods were developed for the new event of genetically modified (GM) maize, MON863. One specific primer pair was designed for the qualitative polymerase chain reaction (PCR) method. The specificity and sensitivity of the designed primers were confirmed. PCR was performed on genomic DNAs extracted from MON863, other GM events, and cereal crops. Single PCR product was obtained from MON863 by the designed primer pair. Eight test samples including GM maize MON863 were prepared at 0.01∼10% levels and analyzed by PCR. Limit of detection of the method was 0.01% for GM maize MON863. On the other hand, another specific primer pair and probe were also designed for quantitative method using a real-time polymerase chain reaction. As a reference molecule, a plasmid was constructed from a taxon-specific DNA sequence for maize, a universal sequence for a cauliflower mosaic virus (CaMV) 35S promoter used in most genetically modified organisms, and a construct-specific DNA sequence for the MON863 event. Six test samples of 0.1, 0.5, 1.0, 3.0, 5.0 and 10.0% of GM maize MON863 were quantitated for the validation of this method. At the 3.0% level, the bias (mean vs true value) for MON863 was 3.0%, and its relative standard deviation was 5.5%. Limit of quantitation of the method was 0.5%. These results show that the developed PCR methods can be used to qualitatively and quantitatively detect GM maize MON863.

KEYWORDS: Genetically modified (GM) maize; limit of detection (LOD); real-time polymerase chain reaction (RT-PCR); reference molecule; limit of quantitation (LOQ)

INTRODUCTION

In recent years, a number of genetically modified (GM) crops have been developed and commercialized by many agricultural biotechnological companies. Genetically modified organisms, or GMOs, have a foreign gene inserted into them that creates one or more traits. Most GMOs offer a variety of benefits. GMOs first appeared as herbicide-tolerant or insect-resistant crops for producers. Now they are being developed as functionally nutritious GMOs for consumers.

The global area for GM crops has continued to grow for the ninth consecutive year, from 1.7 million hectares in 1996 to 81.0 million hectares in 2004 (*1*). Maize (*Zea mays* L.) is the world's third leading cereal crop, following wheat and rice, and also the most commercialized GM crop. GM maize has been planted on 19.3 million hectares (23% of global biotech crop area) in 2004 (*1*). Korea imported about 99.3% of its maize demand in 2002. Accordingly, with the increasing importation of GM grains, a GMO labeling system has become a requirement. The purpose of the labeling system is to inform the consumers of the presence of GMOs in the product, thus providing the consumers with the option to select their preferred products. Thus, consumers' concerns about GM crops have led Korea to require the labeling of GM agricultural products and foods since 2001. Many countries have established labeling systems based on their own criteria, with thresholds for unintentional mixing of GM crops defined as 0.9% in the EU (*2*), 3% in Korea (*3*), and 5% in Japan (*4*).

Qualitative and quantitative methods are needed for GMO monitoring of maize imports. The polymerase chain reaction (PCR) is one of the most widely used methods for detecting the presence of GMOs. The qualitative PCR method has been developed to detect GM maize (*5*−*8*). Qualitative PCR analysis detects whether the test sample contains GMO, which kinds of GM lines, and whether the GMO is approved. Two kinds of quantitative PCR methods are available for detecting GMOs. One is based on competitive PCR (*9*−*13*). It involves an inconvenient process and has the risk of cross contamination

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Figure 1. Schematic diagram of the designed PCR primer pairs to detect GM maize MON863. Solid arrows indicate the specific primers for qualitative and quantitative detection of GM maize MON863.

Table 1. List of Primers and Probes for Qualitative and Quantitative PCRs

target	name	sequence (5′ → 3′)	specificity	length (bp)
		endogen ous		
zSSIIb-1	SSIIb -5′	CTC CCA ATC CTT TGA CAT CTG C	zSSIIb	151
	SSIIb-3′	TCG ATT TCT CTC TTG GTG ACA GG	zSSIIb	
	SSIIb Taq	FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA	zSSIIb	
zSSIIb-3	SSIIb 3-5′	CCA ATC CTT TGA CAT CTG CTC C	zSSIIb	114
	SSIIb 3-3′	GAT CAG CTT TGG GTC CGG A	zSSIIb	
	SSIIb Taq	FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA	zSSIIb	
		universal		
CaMVp 35S	p35S 1-5′	ATT GAT GTG ATA TCT CCA CTG ACG T	p35S	101
	p35S 1-3′	CCT CTC CAA ATG AAA TGA ACT TCC T	p35S	
tNOS	tNOS 2-5′	GTC TTG CGA TGA TTA TCA TAT AAT TTC TG	TNOS	151
	tNOS 2-3′	CGC TAT ATT TTG TTT TCT ATC GCG T	TNOS	
		construct specific		
MON863	CRTA 1-F	GGT TCT CGG GTG ATA AGA ATG AAC T	Cry3Bb1	271
	CRTA 4-R	CTG TTG GCG ATT AGC CGA TTA	tahsp17	
	CRTA 7-F	CAA GAT CGA GTT CAT CCC CGT	Cry3Bb1	111
	CRTA 7-R	TCG CAC ACA CAT CAA CCA AAT T	tahsp17	
	CRTA 7-2 Taq	FAM- CGT TTG GAC GTA TGC TCA TTC AGG TTG G –TAMRA	tahsp17	

(*14*). The other is the real-time PCR developed as a new quantitative technique replacing the competitive PCR (*15*−*17*).

At present, it is possible to qualitatively and quantitatively analyze seven lines of GM maize: MON810, Event176, Bt11, T25, GA21, NK603, and TC1507 (*17*−*19*). Recently, a new GM maize line, insect-resistant MON863, was added to the approved GM maize lines for commercialization and importation in Korea.

GM maize MON863 was produced using recombinant DNA techniques to express the *cry3Bb1* gene encoding a Coleopteran-specific insecticidal protein from *Bacillus thuringiensis* (subsp. *kumamotoensis*) in order to control infestation with corn root worm (CRW; *Diabrotica sp*.). The *cry3Bb1* gene encodes the insect control protein Cry3Bb1, a δ-endotoxin. Cry proteins, of which Cry3Bb1 is only one, act by selectively binding to specific sites localized on the lining of the midgut of susceptible insect species. Following binding, pores are formed that disrupt midgut ion flow, causing gut paralysis and eventual death due to bacterial sepsis. Cry3Bb1 is lethal only when eaten by Coleopteran species, including corn root worm, and its specificity of action is directly attributable to the presence of specific binding sites in the target insects. There are no binding sites for the δ-endotoxin of *B. thuringiensis* on the surface of mammalian intestinal cells; therefore, livestock animals and humans are not susceptible to these proteins (*20*).

In this study, we reported two kinds of specific primers, a probe, and a standard plasmid for the qualitative and quantitative detection of GM maize MON863. We have also confirmed the applicability of the new quantitative method by real-time PCR.

MATERIALS AND METHODS

Maize and Other Crops. Ground F1 seeds of GM maize event, MON863 (TP5504-TD), were provided by Monsanto Company (St. Louis, MO). To study the specificity of the designed primer pairs as a conventional non-GM maize, Hi-II line was directly imported from the United States. Three events of GM maize, which are Bt11, MON810, and Event176, were purchased from the IRMM (Retieseweg, Belgium). Two events of GM maize, which are GA21 and T25, were directly imported from the United States. Ground seeds of NK603 and TC1507 events were provided by developers. Roundup Ready soybean was directly imported from the United States. Other crops, a soybean (*Glycine max*) variety, "Taekwang", a rice (*Oryza sativa*) variety, "Ilpoom", and a barley (*Hordeum vulgare*) variety, "Saessal", were used.

Oligonucleotide Primers and Probe. All oligonucleotide primers and probe were designed by using the Primer Express software (Applied Biosystems, U.S.A.). The primers were synthesized and purified on PAGE columns by Genotech Company (Daejeon, Korea). The probe was synthesized by Applied Biosystems (Foster City, U.S.A.). It was labeled with 6-carboxy-fluorecein and 6-carboxyteramethyl-rhodamine at the 5′ and 3′ ends, respectively. The sites of primers and probes are shown in **Figure 1**. Their nucleotide sequences are shown in **Table 1**.

SSIIb-1 and -3 primer pairs detected the maize starch synthase IIb (zSSIIb) gene as a taxon-specific reference control. The p35S and tNOS primer pairs detected the widely used genes in GM crops, CaMV 35S promotor gene and nopaline synthase, respectively. These primer pairs were synthesized based on the DNA sequences previously reported by Kuribara et al. and Yoshimura et al.

On the other hand, CRTA 1F/4R primers were designed to qualitatively detect the MON863 event. CRTA 7F/R primers and a CRTA 7-2 probe were also designed to detect for the MON863 event, based on the junction region between the Cry3Bb1 gene (GenBank Accession No. M89794) and the wheat 17.3 kD heat shock protein (*tahsp17*) gene (EMBL Accession No. X13431) (**Figure 1**).

DNA Extraction. Maize and other crop samples were ground by an electric mill (Fritsch pulverisette 14, Germany). Genomic DNA was extracted from the ground sample (about 1 g) using the DNeasy Plant Maxi kit (Qiagen, Germany) according to the modified manufacturer's manual. The incubation time was extended from 10 min to 1 h after the addition of buffer AP1 and RNase A solution to ground samples in the first extraction step, and genomic DNA was eluted with water

incubated at 65 °C. The quality of the extracted DNA was monitored by a UV spectrophotometer DU530 (Beckmann Coulter Inc., U.S.A.) and an agarose gel electrophoresis. In the standard of the purity of DNA, the absorption ratios at 260/280 nm and 260/230 should be more than 1.7. For the concentration of DNA, the average of triplicate measurements was used for the calculation. These DNAs were used in the subsequent PCR.

PCR Inhibition Check. PCR inhibition tests on the extracted DNAs were performed by two ways (data not shown). One was checked by qualitative PCR, the other was tested by quantitative PCR. In qualitative PCR, the duplicate samples per one genomic DNA were prepared, and two PCRs per one sample were performed in the presence and absence of the known positive control (spike) DNA to see if the extract DNA inhibits amplification. On the other hand, in case of quantitative PCR, two levels of concentration (50 ng/μL and 10 ng/μL) for MON863 genomic DNA were prepared, and they were quantitated by SSIIb-3 and MON863 primers/probe sets, respectively, to see if the extracted MON863 DNA contains PCR inhibitors by comparison with copy numbers between two concentrations.

Qualitative PCR. PCR was performed to confirm the specificity of the designed primers on genomic DNAs extracted from non-GM maize, GM maize events including MON863, non-GM soybean, RR soybean, rice, and barley. To determine the sensitivity of the qualitative PCR method, the DNA mixture was prepared for GM maize MON863 and non-GM maize events at the following levels: 0.01, 0.05, 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0% (v/v).

The qualitative PCR was carried out using a GeneAmp PCR System 9700 (Applied Biosystems, U.S.A.). A 25 μL volume of reaction solution was composed of 50 ng of genomic DNA, 2.5 μL of 10×PCR buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 1.25 unit of AmpliTaqGold DNA polymerase (Applied Biosystems, U.S.A.). The PCR conditions were as follows: 94 °C for 10 min, and 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and 72 °C for 7 min. The PCR product was analyzed on 2% (w/v) agarose gel.

Sequence Analysis. All PCR products were subcloned into TOPO TA cloning vector (Invitrogen, U.S.A.). DNA sequencing was performed by CoreBioSystems (Seoul, Korea).

Standard Plasmid as a Reference Molecule. A standard plasmid, used as a reference molecule, was constructed based on a PCR2.1 vector (Invitrogen, U.S.A.) integrated with five PCR amplicons. The five PCR amplicons were obtained from PCR amplifications by using primer pairs for CaMVp35S, zSSIIb, NK603, TC1507, and MON863. The construction of the standard plasmid was performed according to Kuribara et al.

The two first PCRs were performed by a specific primer of each amplicon; next, by connective primers between two amplicons. The designed primers were used as specific primers. The connective primers were made by combining specific primers between two amplicons. The connective primers' lengths of sequences were from 47 to 54 nucleotides. A 50 μL volume of reaction solution was composed of 5 μL of 10×PCR buffer, 0.2 mM dNTP, 1 mM $MgSO_4$, 0.3 μM primer pair, 1 unit KOD-Plus-DNA polymerase (Toyobo Co., Japan), and 2 ng of plasmid DNA as a reaction template. The second PCR was performed to connect two of the first PCR amplicons. A 25 μL reaction solution was composed of 2.5 μL of 10×PCR buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.5 μM primer pair, 1.25 unit of AmpliTaqGold DNA polymerase, and 2 μL of the two first PCR solutions as a reaction template. The first and second PCRs were done four times for connecting the five amplicons. All PCRs were performed bythe GeneAmp PCR system 9700 (Applied Biosystems, U.S.A.) according to the following PCR program: 94 °C for 10 min, and 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and 72 °C for 7 min. The second PCR products were ligated into the pCR2.1 plasmid vector by the TOPO TA cloning kit (Invitrogen, U.S.A.). The recombinant plasmid transformed *Escherichia coli* strain TOP10 cells (Invitrogen, U.S.A.). The cloned DNA was selected by EcoR1 digestion (Roche Applied Science, Germany) and confirmed by sequencing analysis. DNA sequencing was performed by CoreBio Systems (Seoul, Korea).

The cloned DNA was extracted and purified by the Qiagen Plasmid Midi kit (Qiagen). The purified plasmid DNA was cut by SmaI restriction endonuclease (Roche) and separated on 2% agarose gel. The linearized plasmid DNA was purified again by the QIA Quick Gel Extraction kit (Qiagen). The concentration of the plasmid DNA was measured by the UV spectrophotometer DU-530 (Beckmann Coulter Inc.). The standard plasmid was serially diluted with salmon testis DNA (5 ng/μL, Sigma Chemical Co., U.S.A.) solution to 2×10, 1.25×10^2, 1.5×10^3, 2×10^4, and 2.5×10^5 copies per 2.5.μL.

Quantitative PCR. Real-time PCR was carried out using three wells in triplicates for a sample DNA by ABI PRISM 7700 (Applied Biosystems, U.S.A.). In the case of one well, a 25 μL volume of the reaction solution was composed of a 50 ng sample of DNA, 0.5 μM primer pair, 0.1 μM probe, and 12.5 μL of Universal Master Mix (Applied Biosystems, U.S.A.). The PCR conditions were as follows: uracil-N-glycosylate treatment at 50 °C for 2 min, denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 30 s, and annealing and extension at 59 °C for 1 min.

For the standard curve, five concentrations of standard plasmid DNA, 2×10, 1.25×10^2, 1.5×10^3, 2×10^4, and 2.5×10^5 copies per reaction, were used as a reference molecule. Salmon testis DNA (5 ng/μL) was used as no template control (NTC). To calculate the GMO amounts (%), the conversion factor (Cf), which is the ratio of the copy numbers between an introduced gene and an endogenous gene, was applied.

In-House Validation. To validate the developed method, the test samples were prepared by mixing GM DNA from GM maize MON863 and non-GM DNA from conventional maize according to the six levels of 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0%. Before mixing the DNAs, we adjusted the copy numbers of the endogenous gene (zSSIIb) to be similar between GM maize MON863 and non-GM maize events. The measurement of copy numbers was performed by real-time PCR using the standard plasmid.

RESULTS AND DISCUSSION

Specificity of the Designed Primer Pairs. The primer pairs of zSSIIb-3, CaMVp35S, and tNOS were used for the primary screening of GM maize (*21*, *22*). The SSIIb-3 primer pair was designed to differentiate maize from other grasses such as soybean, rice, barley, and wheat (*23*). CaMVp35S and tNOS are universal primer pairs that can distinguish GM maize from conventional non-GM maize. The construct of GM maize MON863 contained both CaMV35S promoter and NOS terminator genes (**Figure 1**). Although these primer pairs can screen out GMOs from test samples of corn grains, they cannot identify each line of GM maize mixed in the test samples. The results confirmed that CaMVp35S and tNOS primer pairs could not screen all GM lines, suggesting the need for a qualitative detection of GMOs to differentiate GMOs from non-GMOs and identify each GM event.

Amplified fragments of 271 and 111 bp were observed when MON863 DNA was amplified as a template, respectively, whereas no amplifications were observed from the non-GM maize, the other seven events of GM maize, non-GM soybean, RR Soybean, rice, and barley (**Figure 2**). These results demonstrated that the two primer pairs were specific for MON863 GM maize event in PCR analysis. This specificity is basically attributable to the specific primer pairs designed to amplify the region containing both a trait gene and a regulatory gene, such as a terminator. The specificity results suggested that the developed qualitative PCR detection method for the MON863 GM maize event was suitable for practical use in the monitoring and identification of GMO.

Sensitivity of the Primer Pairs Designed for GM Maize. In this study, we selected one primer pair among two kinds of specific primer pairs for the qualitative PCR method; the other primer pair was used in quantitative PCR method. The sensitivity test was performed to determine the limit of detection (LOD) of the new qualitative PCR method by three times repeat. The

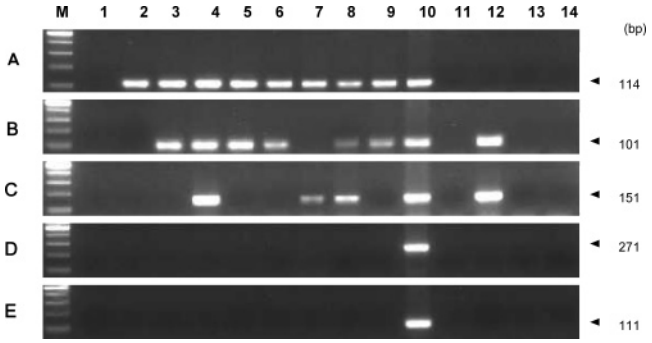

Figure 2. Specificity of the designed primer pairs for GM maize MON863. PCR products were electrophoresed on 2% agarose gel. Arrowheads indicate the expected PCR amplification products. The respective primer pairs for detecting zSSIIb-3 (**A**), p35S (**B**), tNOS (**C**), and MON863 (**D**, **E**) were used. Template DNAs for lanes 1–14 were as follows: no template control, non-GM maize, MON810, Bt11, Event176, T25, GA21, NK603, TC1507, MON863, non-GM soybean, RR soybean, rice, and barley. M: 100 bp size ladder.

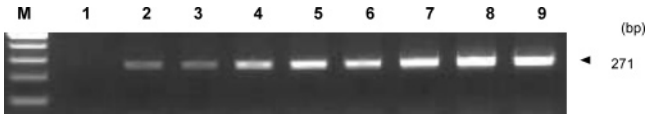

Figure 3. Sensitivity of the designed primer pair for GM maize MON863. PCR products were electrophorese on 2% agarose gel. Arrowhead indicates the expected PCR amplification product. The primer pair for detection of GM maize MON863 was used. Template mixing DNAs for lanes 1–9 were as follows: 0, 0.01, 0.05, 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0%, respectively. M: 100 bp size ladder.

test DNA samples were prepared into eight mixing levels of 0.01, 0.05, 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0% (v/v) for GM maize MON863.

The LOD for MON863 GM maize was 0.01% (**Figure 3**). This sensitivity level was similar to the qualitative detection levels of other GM maize lines, GM soybean, and GM potatoes. Considering the lowest threshold value, which is 0.9% of EU, the results of the sensitivity test demonstrated that the developed qualitative PCR method can meet the requirements for monitoring GMO labeling systems worldwide.

Construction of a Standard Plasmid as a Reference Molecule. As a reference molecule, a plasmid p3SNTM-9 was constructed by the integration of PCR amplicons of one endogenous gene and four introduced genes into the pCR2.1 vector (Invitrogen). Five PCR products were connected by the respective primer pairs for CaMVp35S, zSSIIb, NK603, TC1507, and MON863 GM events. The sequences of the integrated PCR amplicon for MON863 of p3SNTM-9 are shown in **Figure 4**.

For real-time PCR, five levels of standard plasmid concentration were set to 2×10, 1.25×10^2, 1.5×10^3, 2×10^4, and 2.5×10^5 copies per reaction. In consideration of the genomic size of maize (*24*), it was sufficient to quantitate 0.5∼100% of GMO using 50 ng of genomic DNA for one reaction ranging from 2×10 to 2.5×10^5 copies. The linearity of the standard curves for MON863 line was confirmed by quantitative PCR using the designed primer pairs, probe, and the standard plasmid, and calculated to 0.999 of R^2 value (**Figure 5**).

Under the five levels of the standard plasmid (p3SNTM-9), the repeatability of the standard plasmid's copy numbers was calculated from the data of triplicate reactions. The values of relative standard deviation (RSD) of the triplicate reactions ranged from 0.68 to 19.27% (**Table 2**). Although the larger RSD

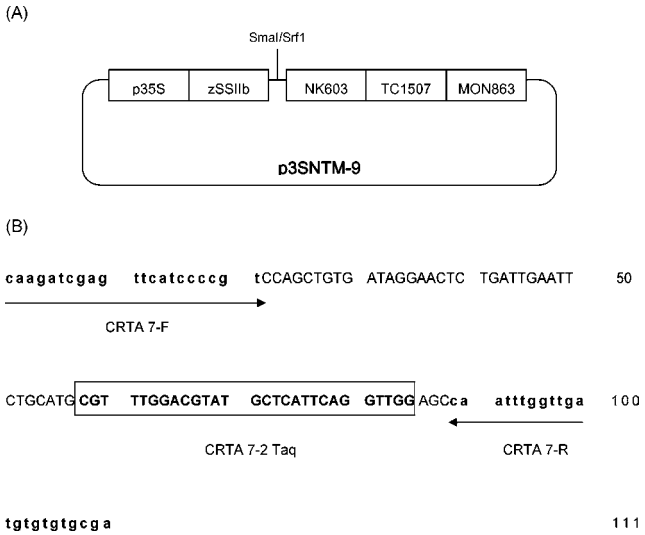

Figure 4. Standard plasmid p3SNTM-9 as a reference molecule. (**A**) Schematic diagram of p3SNTM-9. SmaI/Srf1 indicates a restriction site. (**B**) Sequence of the integrated PCR amplicon for GM maize MON863 in the p3SNTM-9. The arrows indicate the location of primers with direction and the boxes indicate TaqMan probes.

values were observed in the lower levels, all of the RSD values were below 20%. The variation within this range was not significant.

In general, the genomic DNAs from the seeds have been used as reference molecules. Recently, the new reference molecule, the standard plasmid using the recombinant DNA technology, replaced them. In comparison with the genomic DNA, the standard plasmid can be supplied in unlimited quantities with a consistent quality. Moreover, a single plasmid can be used for many GM varieties. As a result of this study, we propose that the new standard plasmid can be used as a reference molecule for quantitating the new GM maize event.

Determination of Conversion Factor (Cf). Every GM event of maize has different copies of introduced target DNA, and so the original copy numbers of each GM event were required for quantitating the GM maize. The conversion factor (Cf) is a ratio of the copy numbers between an introduced gene and a taxon-specific gene in each GM maize event. The GMO amount in an unknown sample can be calculated from the following formula: copy numbers of a construct-specific recombinant DNA sequence/copy numbers of a taxon-specific DNA sequence $\times$ 1/Cf $\times$ 100(%). All experiments were repeated three times, and the mean value was chosen to be Cf. Theoretically, the Cf value of F1 seed is either 0.4 or 0.6, depending on parental reproductive types of GM maize if the GM contains a single copy of transgene. The value is 0.4 if F1 seed was derived from male gamete of GMO and 0.6 if the seed derived from female gamate of GMO. The mean Cf value is 0.54 for MON863 (**Table 3**). The results suggest that the F_1 ground seeds of MON863 provided by the developer originated from the male gamate of non-GMO and female gamate of GMO, and MON863 contains a single copy of the introduced gene.

However, in case of maize, the Cf values from the F1 seeds were not considered to correspond to those of F2 grains. To get more practical GM amount, the Cf values determined from F_2 grains should be applied because practical samples are usually from F2 grains. But, it could be difficult to get the Cf values from F2 grains because of the contamination that occurs during cultivation in farmland and the different seed genotypes as progenies of F1 seed. Accordingly, now the current GM

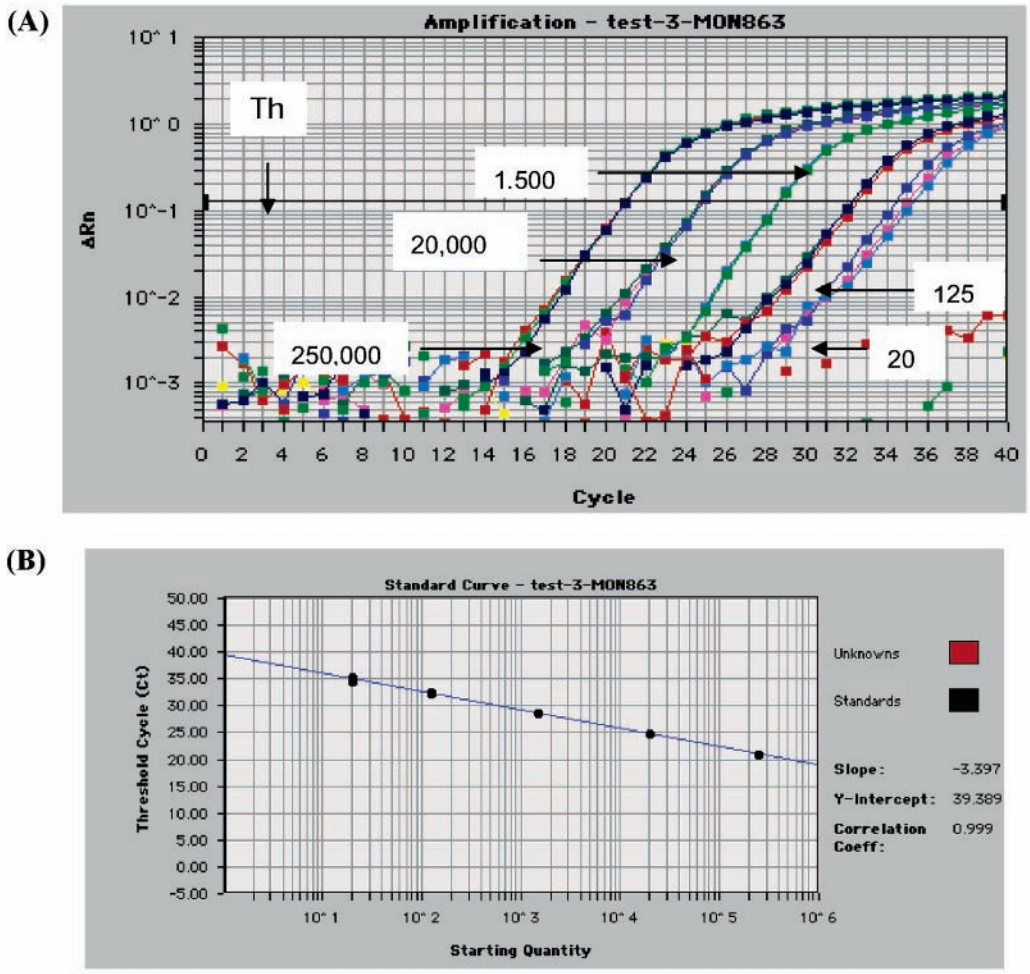

Figure 5. Amplification plots and standard curve of real-time PCR. (**A**) Amplification plots were generated from PCR of CRTA7-F/R primers and CRTA7-2 Taq probe for the detection of GM maize MON863 by the six levels of p3SNTM-9. (**B**) The standard curve from the data of the above amplification plots.

Table 2. Repeatability of the Copy Numbers of p3SNTM-9

	copy no.		
target	true value	mean	RSD[a]
SSIIb-3	20	20.5	16.15
	125	128.8	16.55
	1500	1420.0	5.12
	20000	19751.8	2.67
	250000	256568.0	1.02
MON863	20	18.3	19.27
	125	142.5	17.51
	1500	1517.1	4.55
	20000	19910.7	2.58
	250000	244856.6	0.68

[a] RSD = an average of the relative standard deviations of the triplicate reactions in a single experiment for MON863 line.

quantifying method using Cf value from F1 seed is the most suitable method for quantification (*23*).

Accuracy and Precision of the Quantitative PCR Method. To evaluate accuracy and precision, the DNA samples in six mixing levels, 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0%, were tested. The accuracy of the method was evaluated as the bias (%) of the experimental mean value from the theoretical value. The precision was evaluated by the relative standard deviation.. The thresholds for the unintentional mixing level of GMOs are 0.9% for the EU, 3.0% for Korea, and 5.0% for Japan. At the 1.0, 3.0, and 5.0% mixing levels of the MON863 event, the biases

Table 3. Conversion Factor (Cf) of Quantitative PCR for MON863

target	mean	SD[a]	RSD[b]
MON863	0.54	0.026	4.81

[a] SD = standard deviation. [b] RSD (relative standard deviation) was calculated by dividing the standard deviation by the mean value and is given in percent. Experiments were performed three times each.

Table 4. Accuracy and Precision of the Quantitative PCR Method

		accuracy		precision		
GM line	true value (%)	mean GMO (%)	bias true value (%)	SD[a]	RSD[b]	below 20 copies[c]
MON863	0.1	0.12	20.0	0.03	25.0	2/3
	0.5	0.56	12.0	0.05	8.9	0/3
	1.0	1.08	8.0	0.10	9.3	0/3
	3.0	3.09	3.0	0.17	5.5	0/3
	5.0	5.29	5.8	0.12	2.3	0/3
	10.0	10.88	8.8	0.69	6.3	0/3

[a] SD = standard deviation. [b] RSD = relative standard deviation. Experiments were performed three times. [c] Below 20 copies = the number of experiments below 20 copies/total number of experiments.

(mean vs true value) were 8.0, 3.0, and 5.8%, respectively, and their RSDs were 9.3, 5.5, and 2.3%, respectively (**Table 4**).

We have proved that the values of the biases and RSDs were effective for the method's practical application (*25*, *26*). On the

other hand, the limit of quantitation of the detection method for GM maize MON863 was 0.5%. When the 0.1% sample was quantitated by this method, the copy numbers of two-thirds were below 20 copies of the lowest standard copy number. Therefore, the results could not be approved. In consideration of the lowest threshold, 0.9% of the EU, our method can be applicable to GMO labeling systems in the world. In this study, we concluded that the real-time PCR quantitative method for the MON863 event was validated in house, and we confirmed it to be useful in practical GMO monitoring.

ABBREVIATIONS

4-AS1, four tandem repeats of activating sequence-1; wtCAB, wheat chlorophyll a/b binding protein; ract 1 intron, rice actin 1 gene.

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