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Quantitative Analysis of Two Genetically Modified Maize Lines by Real-Time PCR

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Abstract A quantitative analytical method to detect new lines of genetically modified (GM) maize, NK603 and TC1507, has been developed by using a real-time polymerase chain reaction (PCR). To detect these GM lines, two specific primer pairs and probes were designed. A plasmid as a reference molecule was constructed from an endogenous DNA sequence of maize, a universal sequence of a cauliflower mosaic virus (CaMV) 35S promoter used in most GMOs, and each DNA sequence specific to the NK603 and TC1507 lines. For the validation of this method, the test samples of 0, 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0% each of the NK603 and TC1507 GM maize were quantitated. At the 3.0% level, the biases (mean vs. true value) for the NK603 and TC1507 lines were 3.3% and 15.7%, respectively, and their relative standard deviations were 7.2% and 5.5%, respectively. These results indicate that the PCR method developed in this study can be used to quantitatively detect the NK603 and TC1507 lines of GM maize.

Key words: Real-time PCR, genetically modified (GM) maize, reference molecule

Genetically modified organisms (GMOs) have appeared as solutions to food shortage and to environmental destruction caused by pesticides or herbicides. A number of genetically modified (GM) crops have been developed worldwide through the recombinant DNA technology and commercialized by various agricultural biotechnological companies in recent years. The global area for GM crops continues to grow for the seventh consecutive year, from 1.7 million hectares in 1996 to 81.1 million hectares in 2004 [2]. GM maize was planted on 15.5 million hectares (23% of global area). Korea imported about 99.3% of its maize demand in 2002; however, consumers' concerns about GM crops have led Korea to require the labeling of GM agricultural products and foods since 2001. The thresholds for the unintentional mixing level of GM crops are defined as 0.9% in the European Union, 5% in Japan, and 3% in Korea.

Qualitative and quantitative analytical methods are needed for GMO monitoring of soybean and maize imports. Qualitative analytical methods have been developed to detect GM soybean and maize [5, 8, 9, 12, 14, 15], and two kinds of quantitative PCR methods are available for detecting GMOs. One is based on competitive PCR [4, 10, 11, 21, 25], and involves an inconvenient process and has the risk of cross contamination [6]. The other is the real-time PCR developed as a new quantitative technique replacing the competitive PCR [15, 22, 24].

At present, it is possible to qualitatively and quantitatively analyze five lines of GM maize: Mon810, Event176, Bt11, T25, and GA21 [16, 20]. Recently, two new maize lines, herbicide-tolerant NK603 and insect/herbicide-resistant TC1507, were added to the approved GM maize lines for commercialization and importation in Korea. Glyphosate-tolerant maize NK603 contains the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium tumefaciens* CP4 strain. The insect-resistant and glufosinate-tolerant maize TC1507 line contains two transgenic genes, Cry1Fa2 and PAT (phosphinothricin-N-acetyltransferase). The Cry1Fa2 gene, isolated from *Bacillus thuringiensis* (Bt) var. *aizawai*, produces the insect control protein Cry1F, a delta-endotoxin. The PAT gene, isolated from *Streptomyces viridochromogenes*, allows the use of phosphinothricin herbicides, including glufosinate ammonium. Glufosinate inhibits the glutamine synthetase of plant. The PAT enzyme catalyzes the acetylation of phosphinothricin, detoxifying it into an inactive compound [3].

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In this study, we developed specific primers, probes, and a standard plasmid for the quantitative detection of the two GM maize lines, and validated the specificity and applicability of the new quantitative method by real-time PCR.

MATERIALS AND METHODS

Maize and Other Crops

Ground seeds of two F_1 lines of GM maize, NK603 (DKC57-40) and TC1507 (Herculex I), were provided by Monsanto Company (St. Louis, U.S.A.) and Dow AgroSciences Company (Indianapolis, U.S.A.), respectively. Dow AgroSciences Company also provided the conventional non-GM (hybrid control) maize. Five lines of GM maize, Bt11, MON810, GA21, T25, and Event176, were used to study the specificity of the designed primer pairs. Other crops, a rice (*Oryza sativa*) variety, "Ilpoom," and a barley (*Hordeum vulgare*) variety, "Saessal," were obtained from National Institute of Crop Science(Suwon, Korea) and also used.

Oligonucleotide Primers and Probes

All oligonucleotide primers and probes were designed by using the Primer Express software (Applied Biosystems, USA). The primers were synthesized and purified on PAGE columns by Bioneer Company (Daejeon, Korea). The probes were synthesized by Applied Biosystems (Foster City, U.S.A.). They were 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 Fig. 1. Their nucleotide sequences are shown in Table 1.

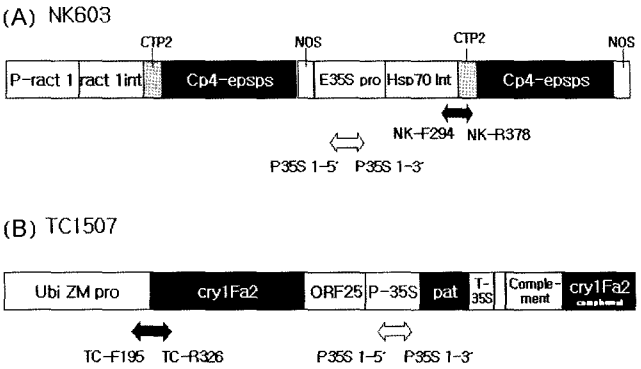

Fig. 1. Schematic diagrams of the designed PCR primer pairs to detect the two lines of GM maize, (A) NK603 and (B) TC1507. Closed arrows indicate the specific regions for detection of NK603 and TC1507 GM maize. Open arrows indicate the universal regions of p35S for GM maize.

SSIIb and P35S primers quantitatively detected the starch synthaseIIb (zSSIIb) gene of maize as an endogenous reference control and the region of CaMV 35S promoter gene as a positive control of the transgene in GM crops, respectively. They were synthesized from the DNA sequences of Kuribara *et al.* [15]. On the other hand, TC-F195/R326 primers were designed to detect the TC1507 line based on the junction region between the maize ubiquitin gene (MubG1, GenBank Accession No. U29159) and the Cry1F gene (GenBank Accession No. M73254). NK-F294/R378

Table 1. List of primers and probes for quantitative PCR.

Target	Name	**Sequence** (5'→3')	Specificity	Length (bp)
		Endogenous		
zSSIIb	SSbII1-5'	CTC CCA ATC CTT TGA CAT CTG C	zSSIIb	151
	SSbII1-3'	TCG ATT TCT CTC TTG GTG ACA GG	zSSIIb	
	SSbIITaq	FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA	zSSIIb	
		Universal		
CaMVp35S	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	
	P35S-Taq	FAM-CCC ACT ATC CTT CGC AAG ACC CTT CCT-TAMRA	p35S	
		Construct specific		
TC1507	TC-F195	CTG CCT TCA TAC GCT ATT TAT TTG C	ubiqutin	132
	TC-R326	GAC GCA CTG ATT CTG TAT GTT GTT CT	cry1F	
	TC-254-MGB	Fam-TGT TTG GTG TTA CTT CTG CAG-MGB	ubiqutin	
NK603	NK-F294	CAG ATA CCA AGC GGC CTC TAG A	hsp-70	85
	NK-R378	AGA GAT GGG TTC TGC ACA CCA	CTP2	
	NK-318-Taq	Fam- ATC CAG GAG CAA CCA TGG CGC A-TAMRA	CTP2	
	NK-F285[a]	ATT GTA ATG CAG ATA CCA AGC GG	hsp-70	109
	NK-R393[a]	GAG AGA TTG GAG ATA AGA GAT GGG TTC	CTP2	

[a]As a qualitative primer pair, it was specific to the NK603 line, and used in constructing the standard plasmid.

and NK-F285/R393 primers were designed to quantitatively detect the NK603 line, based on the junction region between the heat-shock protein 70 gene (GenBank Accession No. X03697) and the chloroplast transit peptide2 gene of EPSPS (GenBank Accession No. X06613). NK-F285/R393 primers were used in constructing the standard plasmid, and NK-F294/R378 primers were used as a quantitative primer pair (Fig. 4).

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) with the DNeasy Plant Maxi kit (Qiagen, Germany), according to the modified manufacturer's manual. The quality of the extracted DNA was monitored by UV spectrophotometer DU530 (Beckmann Coulter Inc., U.S.A.) and an agarose gel electrophoresis.

Qualitative PCR

For the specificity of the designed primer pairs, the qualitative PCR was carried out using a GeneAmp PCR System 9700 (Applied Biosystems, U.S.A.). A 25 μl of volume of reaction solution was composed of 50 ng of genomic DNA, 2.5 μl 10×PCR buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.5 μM each of primer, and 1.25 unit of AmpliTaqGold DNA polymerase (Applied Biosystems). The PCR conditions were as follows: 94°C for 10 min, and 40 cycles of 94°C for 30 sec, 60°C (69°C for NK-F294/R378 primers) for 30 sec, 72°C for 30 sec, and 72°C for 7 min. The PCR product was analyzed on 2% (w/v) agarose gel.

Standard Plasmid as a Reference Molecule

A standard plasmid, used as a reference molecule, was constructed based on a PCRII vector (Invitrogen, U.S.A.) integrated with four PCR amplicons. The four PCR amplicons were obtained from PCR amplifications by using primer pairs P35S1-5'/P35S1-3', SSIIb1-5'/SSIIb1-3', TC-F195/R326, and NK-F285/R393. The construction of the standard plasmid was performed according to Kuribara *et al.* [15].

The first of the two first PCRs was performed by a specific primer of each amplicon; the second by a connective primer 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 of 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 three times to connect the four amplicons. All PCRs were performed by a 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 sec, 60°C for 30 sec, 72°C for 30 sec, and 72°C for 7 min. The second PCR products were ligated into the pCRII plasmid vector by a 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 BstX1 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 a 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 50 ng of DNA sample, 0.5 μM primer pair, 0.1 μM probe, and 12.5 μl 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 sec, 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 the no template control (NTC). To calculate the GMO amounts (%), the conversion factor (C_f), which is the ratio of the copy numbers between an introduced gene and an endogenous gene in each genuine seed, was applied. According to the previous report [15], C_f was calculated by the following formula: C_f=[copies of recombinant DNA sequence extracted from GM seeds]/[copies of endogenous sequence in the DNA extracted from GM seeds].

In-House Validation

In order to validate the developed method, the samples were tested by mixing GM DNA from each line of GM

maize (NK603 and TC1507) with non-GM DNA from conventional maize according to the five 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 non-GM and NK603 or TC1507 GM maize lines. Copy numbers was measured by real-time PCR using the standard plasmid.

RESULTS AND DISCUSION

Specificity of the Designed Primer Pairs

It is necessary to develop the specific primer pair, probe, and reference molecule for quantitating a GMO. The primer pairs of SSIIb and CaMV 35S have already been developed and used for the first screening of GM maize [17, 23]. The SSIIb primer pair was used to differentiate maize from other plants. The CaMV 35S primer pair was used to differentiate GM maize from conventional maize (Figs. 2A, 2B). As shown in Fig. 2, new primer pairs of NK-F294/R378 and TC-F195/R326 were successful in amplifying the 85-bp and 132-bp products from each genomic DNA of NK603 and TC1507, respectively. No PCR products were observed from non-GM maize, other five lines of GM maize, rice, and barley, thus confirming that the two designed primer pairs have the specificity for each of the two GM maize lines.

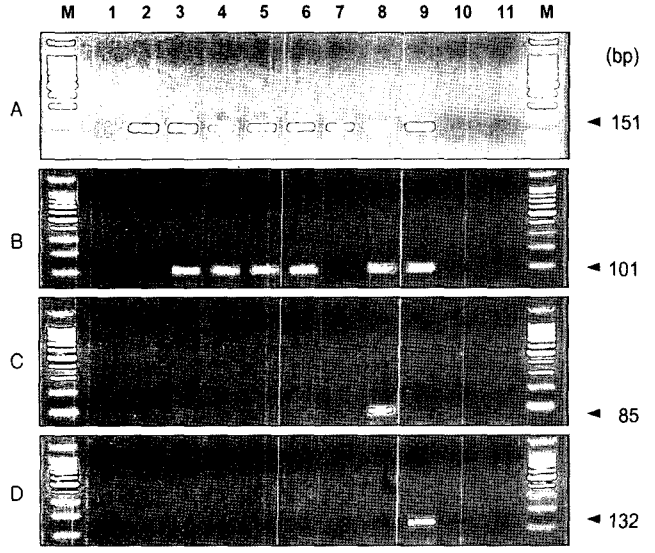

Fig. 2. Specificity of the designed primer pairs for two lines of GM maize.
PCR products were electrophoresed on 2% agarose gel. Arrowheads indicate the expected PCR amplification products. The respective primer pairs for detecting zSSIIb (A), p35S (B), NK603 (C), TC1507 (D), and GM maize were used. Template DNAs for each lane were as follows: Lanes 1–11, no template control, non-GM maize, MON810, Bt11, Event176, T25, GA21, NK603, TC1507, rice, and barley. M: 100-bp size ladder.

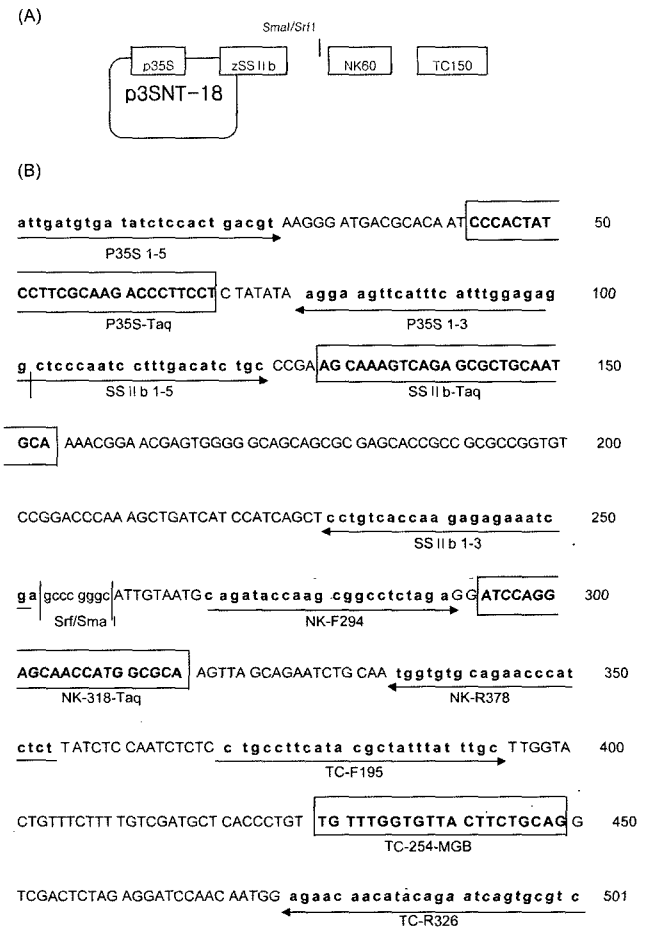

Fig. 3. Standard plasmid p3SNT-18 as a reference molecule.
Schematic diagram of p3SNT-18. SmaI/SrfI indicates a restriction site. Sequence of the integrated PCR amplicons in the p3SNT-18. The arrows indicate the location of primers with direction and the squared boxes indicate TaqMan probes.

Construction of a Standard Plasmid as a Reference Molecule

As a reference molecule, a plasmid p3SNT-18 was constructed by the integration of PCR amplicons for one endogenous gene and three recombinant genes into the pCRII vector (Invitrogen). Respective primer pairs for CaMV35S, zSSIIb, NK603, and TC1507 GM lines connected four PCR products. The sequences of the integrated PCR amplicons of p3SNT-18 are shown in Fig. 3.

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 [1], 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 NK603 or TC1507 lines was confirmed by quantitative PCR using the designed primer pairs, probes, and the standard plasmid, and calculated to be 0.999 of R^2 values (Fig. 4).

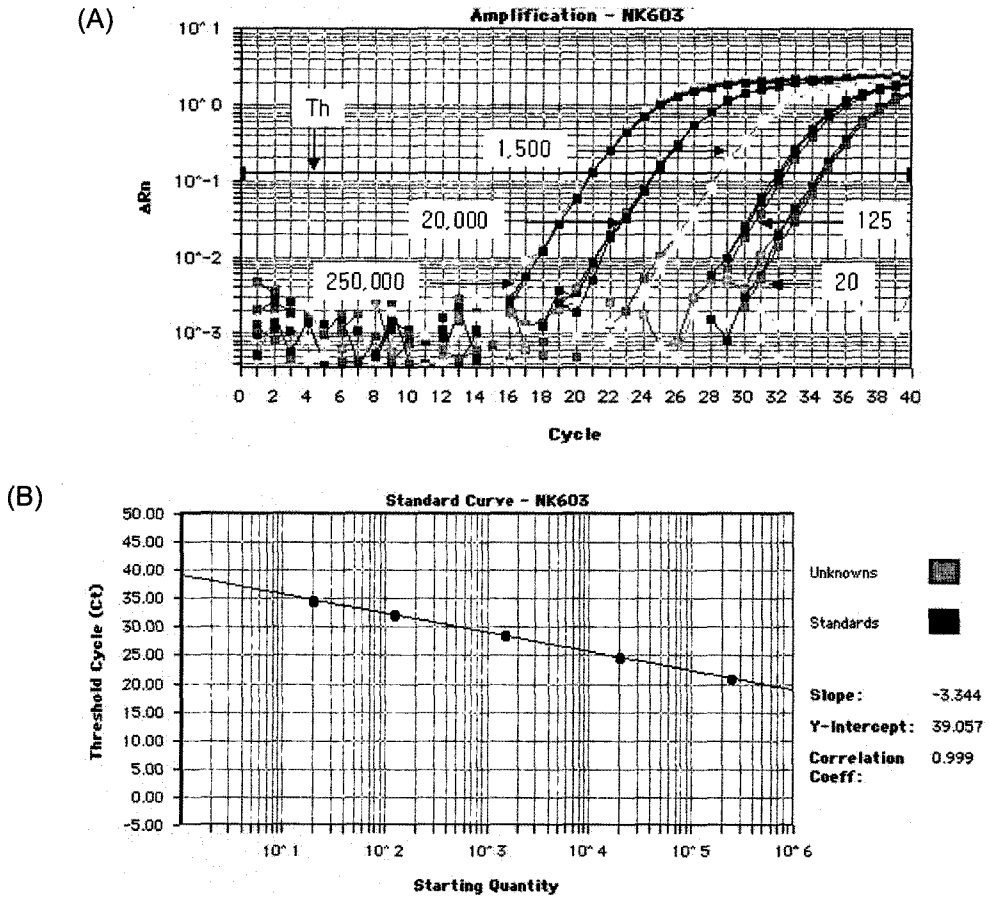

Fig. 4. Amplification plots and standard curve of real-time PCR.
(A) Amplification plots were generated from PCR of NK-F294/R378 primers and NK-318 Taq probe for the detection of NK603 GM maize by the six levels of p3SNT-18. (B) The standard curve from the data of the above amplification plots.

Under the five levels of the standard plasmid (p3SNT-18), 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 1.10% to 20.26% (Table 2). Although the larger RSD values were observed in the lower levels, most of the RSD values were below 10%. The variation within this range was not significant.

Table 2. Repeatability of the copy numbers of p3SNT-18.

Target	Copy No.		
	True value	Mean	RSD[a]
NK603	20	20.7	17.86
	125	124.3	10.11
	1,500	1,464.8	2.87
	20,000	20,222.8	2.30
	250,000	252,030.6	3.78
TC1507	20	20.7	20.26
	125	123.4	8.50
	1,500	1,502.1	4.12
	20,000	20,069.6	6.18
	250,000	251,508.7	1.10

[a]RSD=An average of the relative standard deviations of the triplicate reactions in a single experiment for each line, performed three times.

In general, the genomic DNAs from the seeds have been used as reference molecules. Recently, the new reference molecule, the standard plasmid by the recombinant DNA technology, replaced them. In comparison with the genomic DNA, the standard plasmid could be supplied in unlimited quantities with a consistent quality. Moreover, a single plasmid could be used for many GM varieties. Consequently, the result of this study led us to propose that the new standard plasmid be used as a reference molecule for quantitating the two GM maize lines.

Determination of Conversion Factors (C_fs)

Every GM line of maize has different copies of introduced target DNA; therefore, the original copy numbers of each GM line were required for quantitating the GM maize. The conversion factor (C_f) is a ratio of the copy numbers between an introduced gene and an endogenous gene in each GM maize line. The GMO amount in an unknown

Table 3. Conversion factors (C_fs) of quantitative PCR for two GM maize lines.

Target	Mean	SD[a]	RSD[b]
NK603	0.28	0.012	4.29
TC1507	0.30	0.006	2.00

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

sample can be calculated from the following formula: copy number of a foreign DNA/copy number of an endogenous DNA×1/C_f×100(%). All experiments were repeated three times, and the mean value was taken as C_f. Theoretically, the C_f value of F_1 seeds is either 0.4 or 0.6, depending on the parental reproductive types of GM maize, if the GM contains a single copy of transgene. The seed consists of the diploid embryo and the triploid endosperm. The genetic ratio of maternal-to-paternal genomes is 1:1 in diploid embryo and 2:1 in triploid endosperm. Therefore, the value is 0.4 if F_1 grain was derived from the male gamete of GMO and 0.6 if the grain was derived from the female gamete of GMO. The mean C_f value was 0.28 for NK603 and 0.30 for TC1507 (Table 3), suggesting that the F_1 corn flour of NK603 and TC1507 provided by the developer originated from the male gamete of GMO and female gamete of non-GMO, and both NK603 and TC1507 lines contained a single copy of the introduced gene. The C_f value for NK603 measured by Huang *et al.* is 0.73 [7]. In this case, the F_1 seed of NK603 may be originated from maternal GMO and paternal non-GMO. This suggests that the parent of origin-specific GMO affects the determination of the C_f value of F_1 seed. Thus, the only embryo of F_1 seed should be extracted and used to determine C_f. The embryo usually develops into seedling and forms various plant organs during vegetative development. The endosperm nourishes the embryo and subsequently degenerates. Since F_2 grains would ultimately be out of the market, a reproductive type of F_1 seeds need not to be considered.

Accuracy and Precision of the Quantitative PCR Method

To evaluate the methods accuracy and precision, the DNA test samples were prepared in five mixing levels (0.1%, 0.5%, 1.0%, 3.0%, 5.0%, and 10.0%). 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 (RSD). The thresholds for the unintentional mixing level of GMOs are 0.9% for the EU, 5.0% for Japan, and 3.0% for Korea. At the 1.0%, 3.0%, and 5.0% mixing levels of the NK603 line, the biases (mean vs. true value) were 2.0%, 3.3%, and 11.2%, respectively, and their RSDs were 9.8%, 7.2%, and 0.7%, respectively. The results were similar to the bias, ranging from −11.33% to 12.16%, published by previous studies, which detected the NK603 by real-time PCR [7]. In the case of the TC1507 line, the biases were 1.0%, 15.7%, and 11.6% at 1.0%, 3.0%, and 5.0% mixing levels, respectively, and their RSDs were 12.1%, 5.5%, and 1.4%, respectively. Therefore, we have proved that the values of the biases and RSDs were effective for the method's practical application [19, 20]. On the other hand, the limits of quantitation (LOQ) of the detection method for NK603 and TC1507 GM maize were all 0.5%. When the 0.1% samples were quantitated by this method, the copy numbers of the two GM maize lines were all below 20 copies of the lowest standard copy number. Therefore, the results could not be approved. Considering the low threshold (0.9%) of the EU, our method can be applicable worldwide to GMO labeling systems. In conclusion, the present real-time PCR

Table 4. Accuracy and precision of the quantitative PCR method.

GM line	True value (%)	Accuracy		Precision		Below 20 copies[c]
		Mean	Bias			
		GMO (%)	True value (%)	SD[a]	RSD[b]	
NK603	0.1	0.18	80.0	0.03	16.7	1/3
	0.5	0.54	8.0	0.06	11.1	0/3
	1.0	1.02	2.0	0.10	9.8	0/3
	3.0	2.90	-3.3	0.21	7.2	0/3
	5.0	4.44	-11.2	0.03	0.7	0/3
	10.0	9.13	-8.7	0.20	2.2	0/3
TC1507	0.1	0.10	0.0	0.01	10.0	3/3
	0.5	0.41	18.0	0.02	4.9	0/3
	1.0	0.99	-1.0	0.12	12.1	0/3
	3.0	2.53	-15.7	0.14	5.5	0/3
	5.0	4.42	-11.6	0.06	1.4	0/3
	10.0	9.25	-7.5	0.77	8.3	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.

quantitative method for the two lines of GM maize, NK603 and TC1507, was validated in-house, and is expected to be useful in practical GMO monitoring.

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