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Detection Methods for Biotech Cotton MON 15985 and MON 88913 by PCR

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Plants derived through agricultural biotechnology, or genetically modified organisms (GMOs), may affect human health and ecological environment. A living GMO is also called a living modified organism (LMO). Biotech cotton is a GMO in food or feed and also an LMO in the environment. Recently, two varieties of biotech cotton, MON 15985 and MON 88913, were developed by Monsanto Co. The detection method is an essential element for the GMO labeling system or LMO management of biotech plants. In this paper, two primer pairs and probes were designed for specific amplification of 116 and 120 bp PCR products from MON 15985 and MON 88913, respectively, with no amplification from any other biotech cotton. Limits of detection of the qualitative method were all 0.05% for MON 15985 and MON 88913. The quantitative method was developed using a TaqMan real-time PCR. A synthetic plasmid, as a reference molecule, was constructed from a taxon-specific DNA sequence of cotton and two construct-specific DNA sequences of MON 15985 and MON 88913. The quantitative method was validated using six samples that contained levels of biotech cotton mixed with conventional cotton ranging from 0.1 to 10.0%. As a result, the biases from the true value and the relative deviations were all within the range of ±20%. Limits of quantitation of the quantitative method were all 0.1%. Consequently, it is reported that the proposed detection methods were applicable for qualitative and quantitative analyses for biotech cotton MON 15985 and MON 88913.

KEYWORDS: Biotech cotton; genetically modified organism (GMO); living modified organism (LMO); limits of detection (LODs); real-time PCR; reference molecule; limits of quantitation (LOQs)

INTRODUCTION

Since the first biotech tomato in 1994, many biotech crops have been developed and commercialized every year. The global area devoted to biotech crops has also continuously grown for the 10th consecutive year, from 1.7 million hectares in 1996 to 90 million hectares in 2005 (*1*).

As the global hectares of biotech crops have increased, consumers' concerns have also increased. Therefore, a number of countries have implemented their own labeling system for the biotech products. The purpose of the labeling system is to inform consumers of the presence of the biotech products in the crops or derived products and, therefore, to help the consumers choose their preferred products. Many countries have established labeling systems based on their own criteria, with thresholds for the adventitious presence (AP) of biotech crops defined as 0.9% in the European Union (EU) (*2*), 3% in South Korea (*3*), and 5% in Japan (*4*). In South Korea, mandatory labeling on the biotech-agricultural products and foods has been performed since 2001. In addition, living genetically modified organisms (GMOs) are also treated as living modified organisms (LMOs) in the environment according to the Cartagena Protocol on Biosafety (BSP). According to the BSP, there are specific provisions for the handling, transport, packaging, and identification of LMOs between countries. As a management tool for these systems, detection methods for GMOs or LMOs have been required by regulatory authorities in many countries.

The detection method is an essential element for the GMO labeling system or LMO management for biotech plants. The Polymerase Chain Reaction (PCR) method is currently the most commonly used DNA-based detection method for the identification of biotech plants. Qualitative methods using conventional PCR have been developed to detect the presence of the biotech plants (*5*−*9*). Quantitative methods using real-time PCR have also been developed to analyze the content of biotech plants (*10*−*14*). There are several varieties of biotech cotton in the world: MON 531, MON 1445, MON 15985, and MON 88913 from Monsanto Co.; LLcotton25 from Bayer CropSciences; 281-24-236/3006-210-23 from Dow AgroSciences; and some events from China. Detection methods for some biotech cotton products have been previously developed (*15*, *16*).

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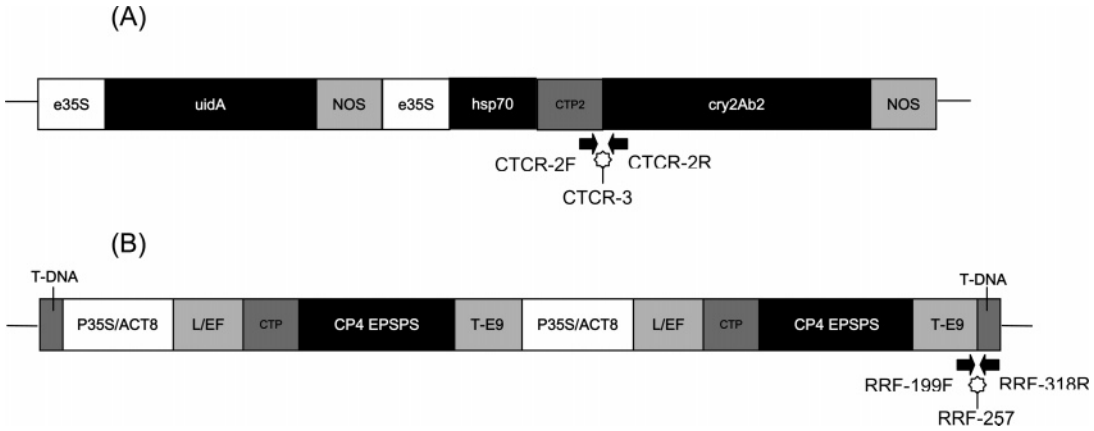

Figure 1. Schematic diagram of PCR strategy to detect biotech cotton MON 15985 and MON 88913. Arrows indicate specific primers and stars specific probes for qualitative or quantitative detection for biotech cotton MON 15985 (**A**) and MON 88913 (**B**).

Cotton (*Gossypium hirsutum* L.) is an important economic crop. Cotton bolls are used as the fiber, cotton seed oil as a cooking oil or snack food, and cotton meal and hulls as important protein concentrates for livestock. Although biotech cotton is not a crop consumed by humans for food, it will be treated as an LMO for feed by the LMO law in South Korea. MON 15985 (BollgardII) expresses both the Cry1Ac and Cry2Ab2 insecticidal proteins, which are highly selective and are active against lepidopteran insects. These proteins bind to the midgut of susceptible insects, leading to gut paralysis and eventual death due to bacterial sepsis (*17*). Additionally, MON 88913 (Roundup Ready Flex) cotton was developed by inserting two CP4-EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) genes to allow the use of glyphosate, which is the active ingredient of the herbicide Roundup as a weed control option in cotton production. Compared with the previously developed MON 1445, MON 88913 tolerates glyphosate applications beyond the fourth leaf stage of MON 1445 (*18*).

In this study, we report the qualitative and quantitative detection methods for biotech cotton products MON 15985 and MON 88913 by using two kinds of specific primer pairs, probes, and one standard plasmid, and confirmed the applicability for practical use by in-house validation experiment.

MATERIALS AND METHODS

Cotton and Other Plant Samples. Ground seeds of biotech cotton, MON 15985 and MON 88913, were provided from Monsanto Co. (St. Louis, MO). Conventional cotton, three biotech cotton products (MON 531, MON 1445, and LLcotton25), and biotech maize T25 were purchased from the American Oil Chemists' Society (AOCS; Champaign, IL). Conventional maize, Roundup Ready soybean, and seven biotech maize products (Bt11, MON810, Event176, GA21, NK603, TC1507, and MON863) were purchased from the Institute for Reference Materials and Measurements (IRMM; Geel, Belgium). In addition, soybean (*Glycine max*) variety 'Taekwang', rice (*Oryza sativa*) variety 'Akibare,' and barley (*Hordeum vulgare*) variety 'Saessal' were used as some conventional plants.

Extraction of Genomic DNA and Quality Check. Cotton and other plant samples were ground by an electric mill (Fritsch Pulverisette 14). According to the modified manufacturer's protocol (*22*), the genomic DNA was extracted from about 1 g of the ground sample by using the DNeasy Plant Maxi kit (Qiagen). The concentration of the extracted DNA was measured by a UV spectrophotometer DU530 (Beckmann Coulter Inc.) and confirmed on agarose gel by electrophoresis. As a good quality of DNA for PCR, the absorption ratios at 260/280 and 260/230 nm wavelengths should be all ≥ 1.7.

The extracted DNA was checked for the absence of PCR inhibitors by quantitative PCR. Two levels of concentration (50 and 10 ng/μL) with MON 15985 or MON 88913 genomic DNAs were prepared and quantitated by fsACP-2 and RRF-199F/318R or CTCR-2F/2R primer/probe sets, respectively. The presence of PCR inhibitor was confirmed by comparison with copy numbers between two levels of concentrations.

Primers and Probes. As a cotton-specific reference system, the fsACP-2 primer pair and fsACP-3 probe were designed to qualitatively or quantitatively detect fiber-specific acyl carrier protein (fsACP) gene (GenBank accession no. U48777), which is used as a taxon-specific reference gene for cotton. On the other hand, as the construct-specific target systems for two kinds of biotech cotton, the CTCR-2F/2R primer pair and a CTCR-3 probe were designed to qualitatively or quantitatively detect MON 15985 on the basis of the junction region between the chloroplast transit peptide2 (CTP2) gene (GenBank accession no. X06613) and Cry2Ab2 gene (GenBank accession no. X55416). The RRF-199F/318R primer pair and an RRF257 probe were also designed to qualitatively or quantitatively detect MON 88913 on the basis of the junction region between the E9 terminator gene (GenBank accession no. X00806) derived from *Pisum sativum* and the T-DNA gene (GenBank accession no. X00493) derived from *Agrobacterium tumefacience* Ti plasmid. The locations of primers and probes are shown in **Figure 1**, and their nucleotide sequences are listed in **Table 1**.

All primers and probes were designed using Primer Express software 3.0 (Applied Biosystems). They were synthesized and purified by Genotech Co. (Daejeon, South Korea). The probes were labeled with 6-carboxy-fluorecein and 6-carboxytetramethyl-rhodamine at the 5′ and 3′ ends, respectively.

Conventional PCR for Qualitative Analysis. Conventional PCR was performed to confirm the specificity of the designed primers for the amplification of each of the targets on the genomic DNAs extracted from conventional cotton, five biotech cotton varieties including MON 15985 and MON 88913, conventional maize, eight biotech maize varieties, conventional soybean, biotech soybean, rice, and barley. To determine the sensitivity of the qualitative PCR method, the DNA mixtures were prepared from two biotech cotton products, MON 15985 and MON 88913, and conventional cotton using the following dilutions: 0.01, 0.05, 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0% (v/v).

The conventional PCR was run with a 25 μL volume of reaction mixture using a thermocycler GeneAmp PCR System 9700 (Applied Biosystems). Twenty-five microliters of reaction mixture contained 50 ng of genomic DNA, 2.5 μL of 10× PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 1.25 units of AmpliTaqGold DNA polymerase (Applied Biosystems). The PCR was performed according to the following program: 1 cycle of 10 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C, and a final extension cycle of 7 min at 72 °C. The PCR product was resolved on 2% (w/v) agarose gel by electrophoresis.

Reference Molecule for Quantitative PCR. As a reference molecule, a standard plasmid was constructed on the basis of a PCR2.1 vector (Invitrogen) integrated with three PCR products, which were amplified from the specific primer pairs for cotton fsACP gene, MON 15985 and MON 88913. The standard plasmid was constructed according to the method of Kuribara et al. (*11*) and is briefly described below.

Table 1. Primers and Probes Used for Qualitative and Quantitative PCRs

target	name	sequence (5′ → 3′)	specificity	length (bp)
		Endogenous		
fsACP	fsACP-2F	CAA ACA AGA GAC CGT GGA TAA GGT A	fsACP	116
	fsACP-2R	CAA GAG AAT CAG CTC CAA GAT CAA G	fsACP	
	fsACP-3 Taq	FAM-TTA GCT TTA GAC AAT GAC AAA CCA ATC ACC GG-TAMRA	fsACP	
		Universal		
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		
MON15985	CTCR-2F	ATT GAA GAA GAG TGG GAT GAC GTT A	CTP2	116
	CTCR-2R	GAC CAG AGT TCA GGA CGG AGT T	Cry2Ab2	
	CTCR-3 Taq	FAM-CGG CGT GCA TGC TTG CCA TG-TAMRA	CTP2/Cry2Ab2	
MON88913	RRF-199F	AAT CTT TAT TTC GAC GTG TCT ACA TTC A	T-DNA	120
	RRF-318R	TGC ATT TTA TGA CTT GCC AAT TG	E9	
	RRF-257 Taq	FAM-ACT CGA GTG GCT GCA GGT CGA TTG A-TAMRA	T-DNA/E9	

Two kinds of PCR reactions were performed for connection and cloning of PCR products. First, to make a blunt-end PCR product for connection, 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.), and 2 ng of plasmid DNA as a reaction template. Second, to connect the first PCR products for cloning, a 25 μL reaction solution contained 2.5 μL of 10× PCR buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.5 μM primer pair, 1.25 units of AmpliTaqGold DNA polymerase, and 2 μL of the two first PCR products as reaction templates. All PCR reactions were performed by a thermocycler GeneAmp PCR system 9700 (Applied Biosystems) according to the following PCR program: 1 cycle of 10 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C, and a final extension cycle of 7 min at 72 °C.

The final connected PCR product was then ligated into the pCR2.1 plasmid vector using the TOPO TA cloning kit (Invitrogen). This recombinant plasmid was used to transform *Escherichia coli* strain TOP10 cell (Invitrogen). The cloned plasmid was selected by *Eco*RI digestion (Roche Applied Science) and confirmed through DNA sequencing analysis by Solgent Co. (Daejeon, South Korea).

The cloned plasmid was extracted by the Qiagen Plasmid Midi kit (Qiagen), and the extracted plasmid DNA was digested with *Hin*dIII restriction endonuclease (Roche). The linearized plasmid DNA was purified from 2% agarose gel by the QIA Quick Gel Extraction kit (Qiagen). The concentration of the plasmid DNA was measured using a UV spectrophotometer DU-530 (Beckmann Coulter Inc.). As a calibrant for quantitation, the standard plasmid was serially diluted to 2×10, 1.25×10^2, 1.5×10^3, 2×10^4, and 2.5×10^5 copies per 2.5 μL with ColE1 DNA (5 ng/μL, Wako).

Real-Time PCR for Quantitative Analysis. For quantitation of biotech cotton DNA, a real-time PCR was performed in three triplicate wells for each DNA sample using the ABI PRISM 7900 (Applied Biosystems). In each well, a 25 μL volume of reaction mixture was composed of 50 ng of sample DNA, 0.5 μM primer pair, 0.1 μM probe, and 12.5 μL of Universal Master Mix (Applied Biosystems). The PCR amplification was run with the following program: uracil-*N*-glycosylate treatment at 50 °C for 2 min, denaturation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 30 s, and annealing or extension at 59 °C for 1 min.

Five concentrations of standard plasmid were used for the standard curve as calibrants, which are reference molecules. ColE1 DNA (5 ng/μL) was used as a no template control (NTC). To calculate the biotech cotton content, the conversion factor (C_f) was required. It defines the ratio of the DNA copy numbers between an introduced gene and an endogenous gene, and it is used to change the experimental value into a real value.

To validate the quantitative method, test samples were prepared by mixing DNAs from conventional cotton, MON 15985, and MON 88913 at six levels: 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0%.

RESULTS AND DISCUSSION

Specific Detection of Qualitative PCR Method. The fsACP-2 primer pair was designed to differentiate cotton from other crops such as maize, soybean, rice, and barley. The fsACP-2 primer pair is specific to a fiber-specific acyl carrier protein (fsACP) gene of cotton. The fsACP gene presents as a single copy gene in the tetraploid cotton genome (*23*) and has been used as an endogenous gene control of cotton in Monsanto's cotton detection methods (http://www.monsanto.com). The tNOS primer pair is a universal primer pair that can distinguish some biotech plants from conventional plants. Although the tNOS primer pair can screen out some biotech cottons except MON 88913, it cannot identify each event from a sample mixed with several events of biotech cotton. However, the newly designed primer pairs were specific to MON 15985 and MON 88913, respectively.

We used the qualified genomic DNAs, which had no PCR inhibitor, extracted from MON 15985 and MON 88913 (data not shown). When the MON 15985 or MON 88913 DNA was used as a template for the CTCR-2F/2R or RRF-199F/318R primer set, an amplified fragment of 116 or 120 bp was observed, respectively. On the other hand, no amplification was observed from the conventional cotton, MON 531, MON 1445, LLcotton25, conventional maize, eight biotech maize products, conventional soybean, biotech soybean, rice, and barley (**Figure 2**).

This specificity is attributable to the specific primer pair being designed to amplify the junction region between two genes of each biotech event. In the case of CTCR-2F/2R primers, there is no CTP2-Cry2Ab2 construct of MON 15985 in other GM cotton varieties. In the case of RRF-199F/318R primers, although the E9-T-DNA construct of MON 88913 is also present in MON 1445, two T-DNAs are different from each other because their plasmids are different (*17*−*21*). Therefore, the CTCR-2F/2R and RRF-199F/318R primer sets are construct-specific. These results showed that the developed qualitative PCR detection methods could be applicable for the identification of MON 15985 and MON 88913.

Limit of Detection (LOD) of Qualitative PCR Method. A sensitivity test was performed three times to determine the LOD of the new qualitative PCR method. As a result, the LODs for MON 15985 and MON 88913 were all 0.05% (**Figure 3**). Considering the lowest threshold value permitted is 0.9% by the EU, this result indicated that the qualitative PCR method developed from this study could be used in monitoring work for the GMO labeling systems worldwide.

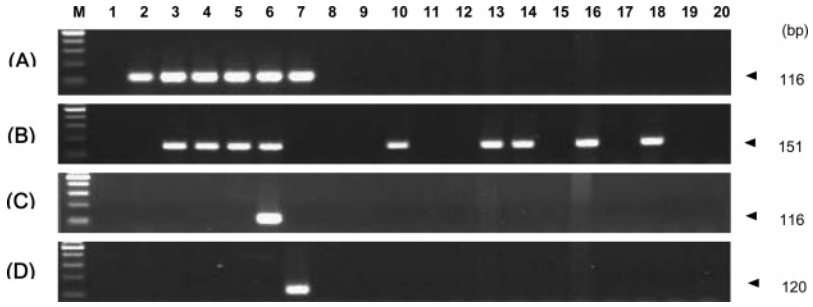

Figure 2. Specificity of the primer pairs designed for biotech cotton MON 15985 and MON 88913. PCR products were electrophoresed on a 2% agarose gel. Arrowheads indicate expected PCR amplification products. The respective primer pairs for detecting fsACP (**A**), tNOS (**B**), MON 15985 (**C**), and MON 88913 (**D**) were used. Template DNAs for each lane were as follows: 1, no template control; 2, conventional cotton; 3, MON 531; 4, MON 1445; 5, LLcotton25; 6, MON 15985; 7, MON 88913; 8, conventional maize; 9, MON 810; 10, Bt11; 11, Event176; 12, T25; 13, GA21; 14, NK603; 15, TC1507; 16, MON 863; 17, conventional soybean; 18, RR soybean; 19, rice; 20, barley; M, 100 bp size ladder.

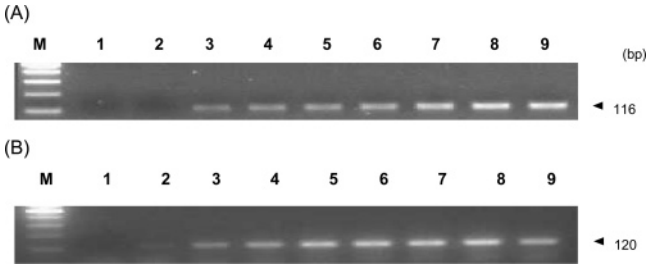

Figure 3. Sensitivity of primer pairs designed for biotech cotton MON 15985 and MON 88913. PCR products were electrophoresed on a 2% agarose gel. The primer pairs for detection of biotech cotton MON 15985 (**A**) and MON 88913 (**B**) were used. Template mixing DNAs for each lane were as follows: 1–9, 0, 0.01, 0.05, 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0%, respectively; M, 100 bp size ladder.

Standard Plasmid as a Reference Molecule Used in Quantitative Method. Plasmid pCTRF was used as a reference molecule. pCTRF was constructed by the integration of three PCR amplicons, which were from one endogenous gene, fsACP, and two introduced genes of MON15985 and MON88913 into a pCR2.1 vector (Invitrogen). The nucleotide sequences of the integrated PCR amplicons of pCTRF are shown in **Figure 4**.

As a reference molecule, five levels of standard plasmid were set to 2×10, 1.25×10^2, 1.5×10^3, 2×10^4, and 2.5×10^5 copies per reaction for the quantitative real-time PCR. This was sufficient to quantitate 0.1−100% of biotech cotton using 50 ng of genomic DNA per reaction ranging from 2×10 to 2.5×10^5 copies. The linearity of the standard curves for MON 15985 or MON 88913 was confirmed in the quantitative PCR using the designed primer pair, probe, and the standard plasmid and resulted in an R^2 value of 0.999 (**Figure 5**).

As for the five levels of the standard plasmid (pCTRF), the repeatability of the copy number at each level of standard plasmid was confirmed from the data of triplicate reactions. The values of relative standard deviation (RSD) of the triplicate reactions ranged from 1.16 to 26.55% (**Table 2**). Although the larger RSD values were observed in the lower levels, most of RSD values were below 20%. The variation within this range was not significant, so the standard plasmid was confirmed to be a stable and reliable reference molecule.

In Europe, the genomic DNAs from the seeds have been used as reference molecules. Compared with the genomic DNA, the standard plasmid can be supplied as unlimited quantities with a consistent quality, and a single plasmid can be used as a single reference molecule for several biotech events. As a result of this study, we propose that this pCTRF plasmid can be used as a reference molecule for quantitating MON 15985 and MON 88913 in biotech cotton.

Figure 4. Standard plasmid pCTRF as a reference molecule: (**A**) schematic diagram of pCTRF; (**B**) sequence of the integrated PCR amplicon of fsACP gene, MON 15985, and MON 88913 in the pCTRF. Lower case letters in bold indicate the sequence of primers with arrows indicating the orientation. Capital letters in italics and bold indicate the sequence of TaqMan probes.

Conversion Factors for Quantitation. Biotech cotton products have different copy numbers of recombinant DNA, so it is necessary to know the original copy numbers of a recombinant DNA of each biotech cotton event for the standard-plasmid quantitative method. The conversion factor (C_f) is a ratio of the copy numbers between a recombinant DNA and a taxon-specific DNA in a biotech cotton event. The amount of biotech cotton in a cotton sample can be calculated from the following formula: (copy number of a construct-specific recombinant DNA sequence/copy number of a taxon-specific DNA sequence)

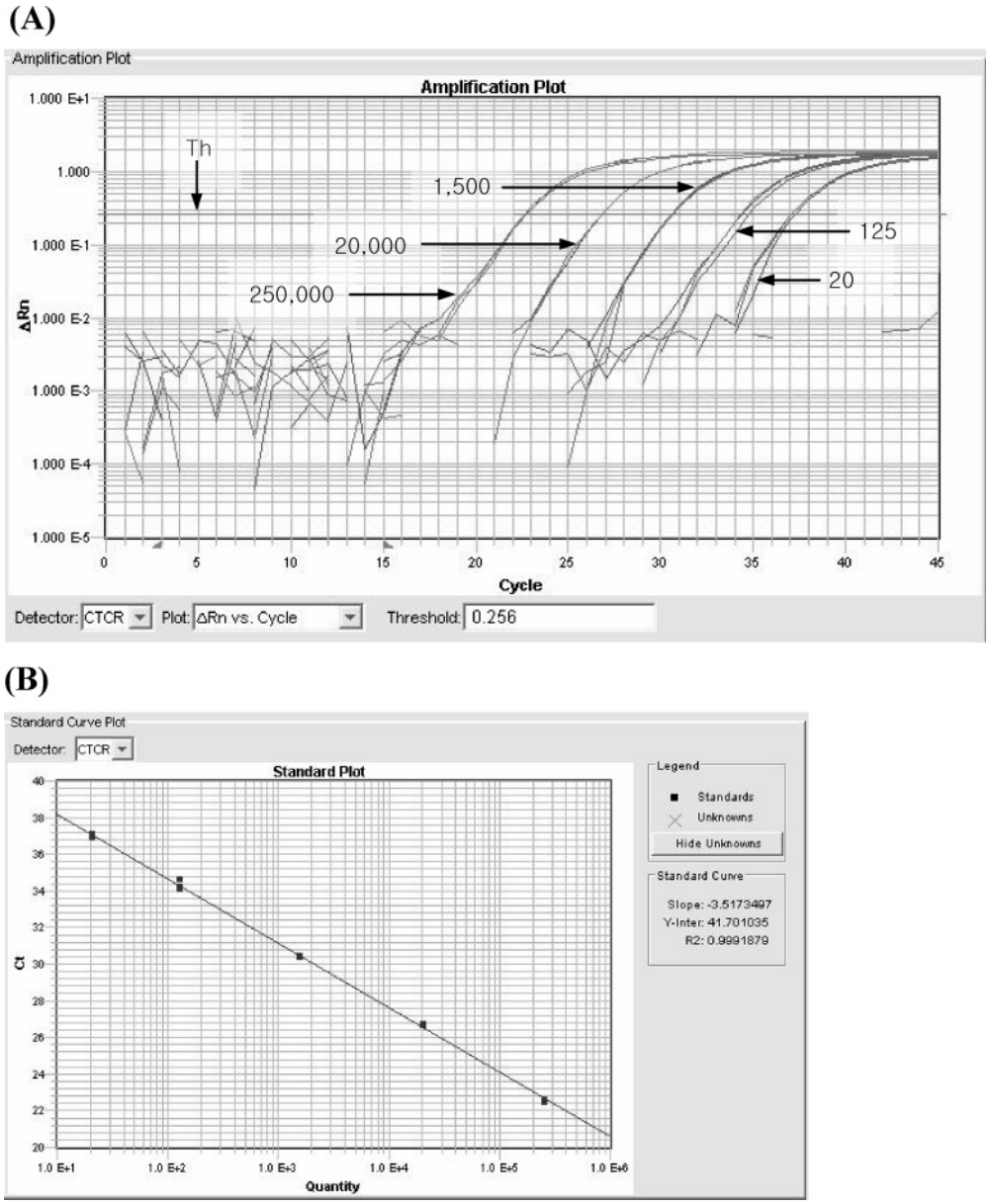

Figure 5. Amplification plots and standard curve of real-time PCR: (**A**) amplification plots generated from PCR of CTCR-2F/R primers and CTCR-3 Taq probe for the detection of biotech cotton MON 15985 by the six levels of pCTRF; (**B**) standard curve from the data of the above amplification plots.

$\times 1/C_f \times 100$ (%). All experiments were repeated three times, and the mean value was determined as the C_f for real content. Cotton (*G. hirsutum* L.) is an allotetraploid species (AADD) consisting of one A-subgenome and one D-subgenome as haploid genome (*24*). Cotton is primarily a self-pollinating plant. Thus, the theoretical C_f of cotton F_1 seed with a single copy transgene haploid genome will be 1.0 because of the endogenous fsACP gene, which is a single copy per haploid genome of cotton. The mean C_f values are 1.16 for MON 15985 and 1.00 for MON 88913 (**Table 3**). The results indicated that the F_1 ground seeds of MON 15985 and MON 88913 provided by Monsanto Co. contain a single copy of the introduced gene, respectively.

Validation of the Quantitative PCR Method. The accuracy of the method was evaluated as the bias (percent) of the experimental mean value from the theoretical value. The precision was evaluated by the relative standard deviation (RSD). To evaluate the accuracy and precision of the method, the test DNA samples were prepared by mixing at six levels: 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0%. According to the GMO labeling systems of some countries, thresholds for the adventitious presence (AP) level of biotech products are 0.9% in the EU, 3.0% in South Korea, and 5.0% in Japan. As a result, at 1.0, 3.0, and 5.0% mixing levels, in the case of MON 15985, the biases (mean vs true value) were 5.0, 3.3, and 0.6%, respectively, and their RSDs were 9.5, 6.5, and 7.6%, respectively. In the case of MON 88913, the biases (mean vs true value) were 4.0, 3.0, and 1.2%, respectively, and their RSDs were 17.7, 5.8, and 10.7%, respectively. Overall, the values of biases and relative standard deviations (RSDs) of the three times repeated tests ranged from 4.7 to 18.2% at six levels (**Table 4**). All variations were within 20%; therefore, the accuracy and precision of this quantitative method were credible for the method's practical application (*25*). On the other hand, the limits of quantitation (LOQs) of the detection methods for biotech cotton MON 15985 and MON 88913 were all 0.1%. Therefore, our method can be applicable for GMO labeling systems worldwide because the lowest AP

Table 2. Repeatability of the Copy Numbers of pCTRF

target	copy no.		
	true value	mean value	RSD[a]
ACP-2	20	22.30	23.11
	125	115.19	7.08
	1500	1469.99	6.60
	20000	19459.86	2.92
	250000	263779.68	9.18
MON15985	20	21.17	6.70
	125	121.77	16.52
	1500	1510.15	3.49
	20000	18052.72	2.25
	250000	271027.26	4.89
MON88913	20	21.32	26.55
	125	125.66	10.80
	1500	1460.84	11.56
	20000	18388.50	7.07
	250000	270290.69	1.16

[a] Average of the relative standard deviations of the triplicate reactions in a single experiment for MON15985 or MON88913.

Table 3. Conversion Factors of Quantitative PCR for MON15985 and MON88913

target	mean	SD[a]	RSD[b]
MON15985	1.16	0.081	6.98
MON88913	1.00	0.006	0.60

[a] Standard deviation. [b] 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		
		mean	bias			
biotech event	true value (%)	exptl value (%)	true value (%)	SD[a]	RSD[b]	below 20 copies[c]
MON15985	0.1	0.11	10.0	0.02	18.2	0/3
	0.5	0.54	8.0	0.03	5.6	0/3
	1.0	0.95	5.0	0.09	9.5	0/3
	3.0	3.10	3.3	0.20	6.5	0/3
	5.0	5.03	0.6	0.38	7.6	0/3
	10.0	9.95	0.5	0.47	4.7	0/3
MON88913	0.1	0.11	10.0	0.02	18.2	0/3
	0.5	0.52	4.0	0.07	13.5	0/3
	1.0	0.96	4.0	0.17	17.7	0/3
	3.0	3.09	3.0	0.18	5.8	0/3
	5.0	4.94	1.2	0.53	10.7	0/3
	10.0	8.79	12.1	0.67	7.6	0/3

[a] Standard deviation. [b] Relative standard deviation. Experiments were repeated three times. [c] Below 20 copies = no. of experiments below 20 copies/total no. of experiments.

threshold is 0.9% required by the EU. In conclusion, we report that the qualitative and quantitative PCR methods for biotech cotton MON 15985 and MON 88913 were developed and validated in house. As a result, we confirmed these methods could be used as a practical monitoring method for biotech cotton.

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