Food Sci. Biotechnol. Vol. 18, No. 5, pp. 1118 ~ 1123 (2009)

Food Science and Biotechnology

Event-specific Detection Methods for Genetically Modified Maize MIR604 Using Real-time PCR

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Abstract Event-specific real-time polymerase chain reaction (PCR) detection method for genetically modified (GM) maize MIR604 was developed based on integration junction sequences between the host plant genome and the integrated transgene. In this study, 2 primer pairs and probes were designed for specific amplification of 100 and 111 bp DNA fragments from the *zSSIIb* gene (the maize endogenous reference gene) and MIR604. The quantitative method was validated using 3 certified reference materials (CRMs) with levels of 0.1, 1, and 10% MIR604. The method was also assayed with 14 different plants and other GM maize. No amplification signal was observed in real-time PCR assays with any of the species tested other than MIR604 maize. As a result, the bias from the true value and the relative deviation for MIR604 was within the range from 0 to 9%. Precision, expressed as relative standard deviation (RSD), varied from 2.7 to 10% for MIR604. Limits of detections (LODs) of qualitative and quantitative methods were all 0.1%. These results indicated that the event-specific quantitative PCR detection system for MIR604 is accurate and useful.

Keywords: MIR604, genetically modified maize, real-time polymerase chain reaction (PCR)

Introduction

Genetically modified (GM) maize was grown on 35.2 million hectares (13% of global biotech area) in 2007 (1). Since the first commercial genetically modified organism (GMO) in 1996, many countries have approved commercial releases of various events of GM maize. GM maize is used in animal feed and in starch production in many countries. Most countries have recognized current customer concerns in relation to GMO products and the need to decide threshold levels of GMOs. GM food labeling policies differ from country to country. For instance, the labeling threshold was defined as 0.9% in the European Union (2, 3), 3% in Korea (4), and 5% in Japan (5). Therefore, there is a strong need for quantitative methods for determining threshold levels of GMOs.

To confirm the level of GM content in a food matrix, real-time polymerase chain reaction (PCR) is considered as highly specific quantitative assay and has been also adopted as the Official Method in Korea. Until now, some quantitative methods have been published for GM maize T25 (6), Bt11 (7,8), Bt176 and GA21 (8), MON810 and NK603 (9), CBH-351 (10), and MON863 (11) using real-time PCR. Yang *et al*. (12) recently reported event-specific quantitative detection of 9 genetically modified maizes (Bt11, Bt176, GA21, MON810, MON863, NK603, T25, TC1507, and CBH351) using 1 novel standard reference molecule (12).

Recently, GM maize MIR604 was approved from safety-assessment by the Korea Food & Drug Administration (KFDA). MIR604 was developed to control yield losses from rootworm larvae damage by Syngenta Seeds Inc. (13). MIR604 contains a modified *cry*3A gene conferring insect resistance and a *pmi* gene encoding the enzyme phosphomannose isomerase (PMI) as a selectable marker gene (13). A PCR method that can distinguish a particular variety from all other varieties using primers was described as being gene-, construct-, and event-specific detection. Especially, the event-specific detection method was regarded as the most specific approach since its detection target is a unique junction spanning the transgenic insert and the host plant DNA.

In this study, the novel plasmid-based quantitative PCR method to detect MIR604 event-specifically was developed by using the TaqMan-based real-time PCR system and ABI Prism 7500 as a thermal cycler. The method was used a standard plasmid as a reference molecule. The standard plasmid contains *zSSIIb* as a maize endogenous gene and event-specific sequences of MIR604. Standard curves were calibrated using the 5 concentrations (20, 200, 2,000, 20,000, and 200,000 copies/reaction) of reference molecules. This real-time PCR system also validated using MIR604 certified reference materials (CRMs).

Materials and Methods

Plant materials MON810, MON863, NK603, GA21, and LY038 maize were provided by Monsanto (St. Louis, MO, USA). T25 maize was provided by Bayer CropScience (Monheim am Rhein, Germany). Bt176, Bt11, and MIR604 maize were provided by Syngenta Seeds (Basel, Switzerland). TC1507, TC6275, and DAS-59122-7 maize was provided by DOW AgroSciences LLC (Indianapolis, IN, USA). Fourteen different plants [soybean (*Glycine max*), maize (*Zea mays*), canola (*Brassica napus*), cotton (*Gossypium hirsutum*), rice (*Oryza sativa*), potato (*Solanum tuberosum*), barley (*Hordeum vulgare*), buck wheat (*Fagopyrum esculentum*), wheat (*Triticum aestivum*), pepper (*Capsicum annuum*), red-bean (*Phaseolus angularis*),

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Received February 10, 2009; Revised June 8, 2009;
Accepted June 22, 2009

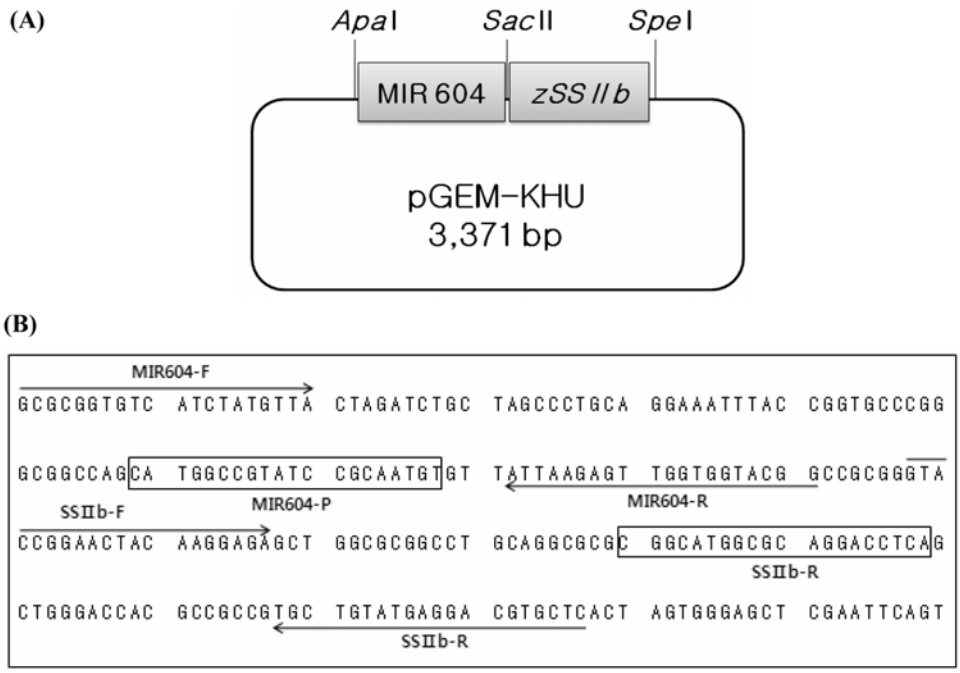

Fig. 1. Schematic diagrams and nucleotide sequences of standard plasmid (pGEM-KHU) as a reference molecule. (A) Schematic diagram of pGEM-KHU; Event-specific fragment from 3'-flanking region of MIR604; *zSSIIb*, maize endogenous gene. (B) Nucleotide sequences of pGEM-KHU; Arrows locate primers with direction and squared boxes indicate TaqMan probes.

Table 1. List of primers and probes for quantitative PCR

Target	Primer name	Sequence (5'-3')	Specificity	Amplicon (bp)
Endogenous gene	SSIIb-F	GTACCGGAACTACAAGGAGA	*zSSIIb*[1)]	100
	SSIIb-R	GAGCACGTCCTCATACAGCA		
	SSIIb-P	FAM CGGCATGGCGCAGGACCTCA TAMRA		
MIR604	MIR604-F	GCGCGGTGTCATCTATGTT	3'-flanking region	111
	MIR604-R	CCGTACCACCAACTCTTAAT		
	MIR604-P	FAM CATGGCCGTATCCGCAATGT TAMRA		

[1)] *Starch synthase* IIb, maize endogenous gene.

radish (*Raphanus sativus*), Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), and perilla leaf (*Perilla frutescens*)] were provided by the Rural Development Administration in Korea.

DNA extraction Genomic DNAs of maize and other plant samples were extracted from the ground sample (about 1 g) using the DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's manual. Concentration of the extracted DNA was measured by a UV spectrophotometer UV-1700 (Shimadzu, Kyoto, Japan) and analyzed by agarose gel electrophoresis. The quality of extracted DNAs was evaluated from the ratio of UV absorptions at 260/280 and 260/230 nm wavelengths.

Primers and probes All primers and probes in this study were designed using the Primer Designer program (version 3.0, Scientific and Educational Software). Primers were synthesized and purified on PAGE columns by Bionics Company (Seoul, Korea). The TaqMan fluorescent dye-labeled probes were synthesized by Applied Biosystems (Foster City, CA, USA). Probes were labeled with 6-carboxyfluorescein (FAM) and tetramethyl-6-carboxy-rhodamine (TAMRA) at the 5' and 3' ends, respectively. Locations of primers and probes are shown in Fig. 1 and their nucleotide sequences are listed in Table 1. SSIIb-F/R primers and the SSIIb-P probe were designed to detect maize starch synthase IIb (*zSSIIb*) gene as an endogenous reference control of maize. MIR604-F/R primers and the MIR604-P probe were designed to detect the 3'-flanking sequence of MIR604 which spans the junction between the transgenic insert and the host genome.

Conventional PCR for qualitative analysis Conventional PCR was carried out to confirm the specificity of the designed primers. To confirm the specificity of the primer pair SSIIb-F/R, genomic DNAs extracted from maize as well as 13 different plant samples were used as templates for PCR. Genomic DNAs extracted from 12 GM varieties of maize were used as templates to evaluate the specificity of the event-specific primers for MIR604. Conventional PCR was carried out on a thermocycler PC808 (ASTEC, Fukuoka, Japan). Reaction mixtures in 25 μL volumes contained 2.5 μL of 10× buffer (Applied Biosystems),

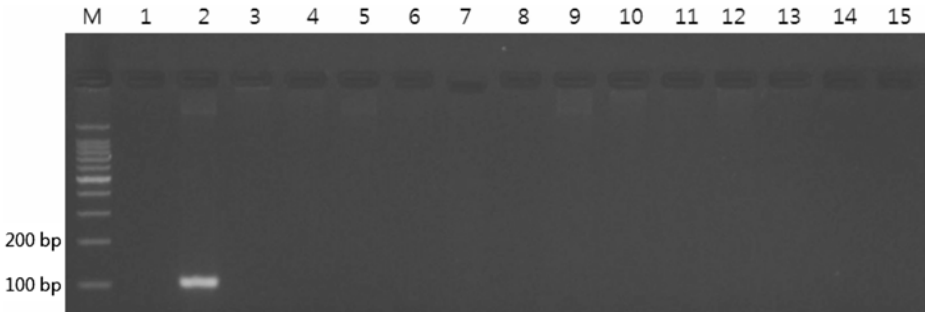

Fig. 2. Analysis of species specificity of maize *zSSIIb* gene using primer pairs, SSIIb-F/R. Lane 1, soybean; lane 2, maize; lane 3, canola; lane 4, cotton; lane 5, rice; lane 6, potato; lane 7, barley; lane 8, buck wheat; lane 9, wheat; lane 10, pepper; lane 11, red-bean; lane 12, radish; lane 13, Chinese cabbage; lane 14, perilla; lane 15, non-template; and lane M, 100 bp DNA ladder.

200 μM of each dNTP (Applied Biosystems), 1.5 mM of $MgCl_2$, 0.8 unit of Ampli Gold *Taq* DNA polymerase (Applied Biosystems), and 50 ng of genomic DNA. PCR conditions were pre-incubation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec, and terminal elongation at 72°C for 8 min.

Standard plasmid as a reference molecule A standard plasmid, a reference molecule with an endogenous *zSSIIb* gene and the event-specific sequences in MIR604, was constructed based on a pGEM Vector (Promega, Madison, WI, USA) integrated with 2 PCR products. To make blunt-end PCR products, 2 kinds of primers were designed, including SSIIb-SacII (F): GTCCCCGCGGGTACCGGAA CTACAAGGAGAGCTGGCGCGC, SSIIb-SpeI (R): GG ACTAGTGAGCACGTCCTCATACAGCACGGCGGCGT GGT, MIR604-ApaI (F): GCGCGCGGGCCCGCGCGGT GTCATCTATGTTACTAGATCT, MIR604-SacII (R): CGC GCCGCGGCCGTACCACCAACTCTTAATAACACATT.

The underlined letters indicate the restriction enzyme sites. PCR was carried out in a thermocycler PC808 (ASTEC). Reaction mixtures in 50 μL volumes contained 5 μL of 10× buffer (Applied Biosystems), 200 μM of each dNTP (Applied Biosystems), 1.5 mM of $MgCl_2$, 0.8 units of Ampli Gold *Taq* DNA polymerase (Applied Biosystems), 0.5 μM of each primer and 100 ng of genomic DNA. PCR conditions were pre-incubation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec, and terminal elongation at 72°C for 8 min.

After PCR, 2 PCR products were digested with a restriction enzyme, purified with a gel extraction kit (Qiagen), and finally ligated into pGEM Vector (Promega). The recombinant plasmid was transformed into *Escherichia coli* strain DH5α (Novagen, Madison, WI, USA). Sequencing analysis of the cloned DNA was performed using an ABI Prism 3700 DNA analyzer (Perkin Elmer, Boston, MA, USA).

On the basis of the maize genome size of 2,504 Mb/haploid genome (14) and the molecular weight of double-stranded DNA (965 Mb weigh 1 pg), one haploid maize genome molecule corresponds to 2.6 pg of DNA. Thus, 1 plasmid molecule (3,371 bp) contains 3.49 ag of plasmid DNA. For example, the mass of plasmid DNA containing 200,000 copies of inserted sequence is:

$$[3.49\times10^{-18}\ \text{g/copy}][200{,}000\ \text{copies}]=6.98\times10^{-13}\ \text{g}$$

Quantitative PCR Real-time PCR assays with a TaqMan probe were performed with an ABI Prism 7500 (Applied Biosystems). PCR reaction mixtures contained the following ingredients in final volumes of 25 μL: 50 ng samples of DNA, 0.5 μM primer pair, 0.2 μM probe, and 12.5 μL Universal Master Mix (Applied Biosystems). Real-time PCR was performed according to the following procedure: 1 cycle of 2 min at 50°C, 10 min at 95°C, 50 cycles of 30 sec at 95°C, 1 min at 60°C. Each sample was quantified in triplicate. Results were analyzed using a sequence detection system provided by the software for ABI 7500. Standard curves were calibrated using 5 concentrations of reference molecules from 20 to 200,000 copies/reaction. A no-template control (NTC) was prepared as a negative control for the analysis.

According to the principle of a standard curve's optimization, the best standard curves were obtained for the real-time PCR assays. The Ct value was used to determine the amount of total DNA using the endogenous *zSSIIb* gene PCR system and the amount of transgenic DNA using the event-specific PCR system based on the standard curves. To evaluate the accuracy and precision of the quantitative PCR method in this study, 50 ng DNAs extracted from each of the CRMs MIR604 (10, 1, and 0.1%) were used as templates. Estimation of precision was shown as relative standard deviation (RSD, the standard deviation of the ΔCt) and standard deviation (SD) (15). RSD value was calculated by dividing the SD by mean value.

Results and Discussion

Specificity of the designed primer pairs for the maize endogenous gene (*zSSIIb*) and MIR604 An endogenous reference gene should be species-specific with a single copy number in the genome and exhibit low heterogeneity among cultivars (16). The *starch synthase IIb* (*zSSIIb*) gene (GenBank No. AF019297) was selected as a maize endogenous gene for quantitative PCR detection. The primers and probes set specific to this DNA sequence was designed for real-time quantitative PCR assays. For a specificity test, Qualitative PCR was performed on 50 ng of genomic DNA from 14 different plant species containing maize. No amplification was observed when

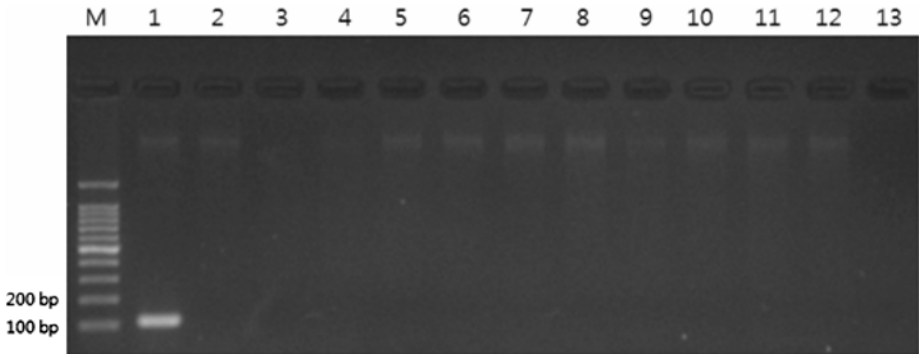

Fig. 3. Specificity of the event-specific primer pairs for MIR604. The respective event-specific primer pair for detection of MIR604 was used. Lane 1, MIR604; lane 2, DAS-59122-7; lane 3, Bt176; lane 4, Bt11; lane 5, MON810; lane 6, MON863; lane 7, NK603; lane 8, GA21; lane 9, T25; lane 10, TC1507; lane 11, TC6275; lane 12, LY038; lane 13, no template; and lane M, 100 bp DNA ladder.

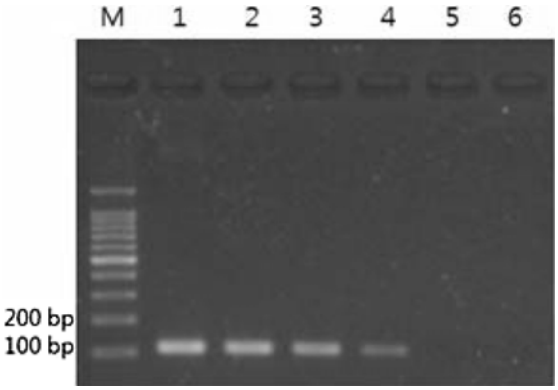

Fig. 4. Sensitivity of event-specific primer pairs designed for MIR604. The respective event-specific primer pair for detection of MIR604 was used. Lane 1-5, 100, 10, 1, 0.1, and 0.01 % of MIR604; lane 6, non-template; and lane M, 100 bp DNA ladder.

genomic DNAs extracted from other species such as soybean, canola, cotton, rice, potato, barley, buck wheat, wheat, pepper, red-bean, radish, Chinese cabbage, and perilla were used as templates (Fig. 2). Specificity of the designed event-specific primers for MIR604 was also confirmed by testing genomic DNA extracted from 12 GM maize varieties, Bt176, Bt11, MON810, MON863, NK603, T25, TC1507, GA21, TC6275, LY038, MIR604, and DAS-59122-7, using real-time PCR. The designed event-specific primer was specific to MIR604 (Fig. 3).

To check the PCR inhibitions, 2 concentration levels (10 and 50 ng/μL) with MIR604 genomic DNA were quantitated by each primer and probe. No PCR inhibitions were observed when copy numbers between the 2 levels of concentrations were compared (data not shown). The presence of PCR inhibitions should be investigated because quantitative PCR can result in false-negative results.

Limit of detection (LOD) of qualitative PCR The LOD was defined as the lowest amount or concentration of analyte that can be reliably detected in a sample (17). To determine the LOD value of the qualitative PCR, certified reference materials (CRMs) MIR604, containing 0, 0.1, 1, 10, and 100% GM materials, were used. Additionally, 0.01% GM material for MIR604 was prepared by mixing with non-GM maize.

Standard plasmid for real-time PCR To test the range of quantification, 5 levels of standard plasmid concentrations were set at 2×10, 2×10^2, 2×10^3, 2×10^4, and 2×10^5 copies per reaction. The plasmid copy number was sufficient to quantify MIR604 from 0.1 to 100% in the 50 ng of genomic DNAs for 1 reaction. Linearity of the standard curve for MIR604 was confirmed in the quantitative PCR using the designed primer pairs, probes, and the standard plasmid. The standard curve had a slope of −3.46 and a square regression coefficient (R^2) of 0.998 (Fig. 5).

According to a recommendation by the European Network of GMO Laboratories (ENGL), minimum acceptance criteria for real-time PCR detection methods require a slope of the standard curve in the range of $-3.1<\chi<-3.6$ and a regression coefficient higher than 0.98 (18). The fine linearity between DNA quantities and fluorescence values (C_t) indicated that these assays were appropriate for quantitative measurements. Reproducibility of the C_t values was determined using the standard plasmid DNA dilution (Table 2). This experiment was performed in triplicate and repeated 3 times.

Measurement of coefficient values using genuine seeds of MIR604 According to previous reports, the ratios of introduced DNA and endogenous sequences in each genuine seed were calculated and defined as the coefficient value (C_v) or conversion factor (C_f) (19-20). The C_v was used for GMO amount (percent) calculations of unknown samples. The amount of GM maize in a maize sample can be calculated from the following formula: (copy number of r-DNA sequence in the DNA extracted from GM seeds × 100)/(copy number of endogenous sequence in the DNA extracted from GM seeds ×C_v).

To determine the C_v of MIR604, each sample was performed 3 times in triplicate wells, and then the mean value was deduced as the C_v. Table 3 shows the C_vs of the tested GM maizes. The value was 0.57 for MIR604. The ideal C_v of GM maize should be 1.0 because the event-specific fragment of the GM maize was unique in the haploid maize genome. The discrepancy between the experimental and theoretical value was be due to differences of PCR efficiencies that resulted from the

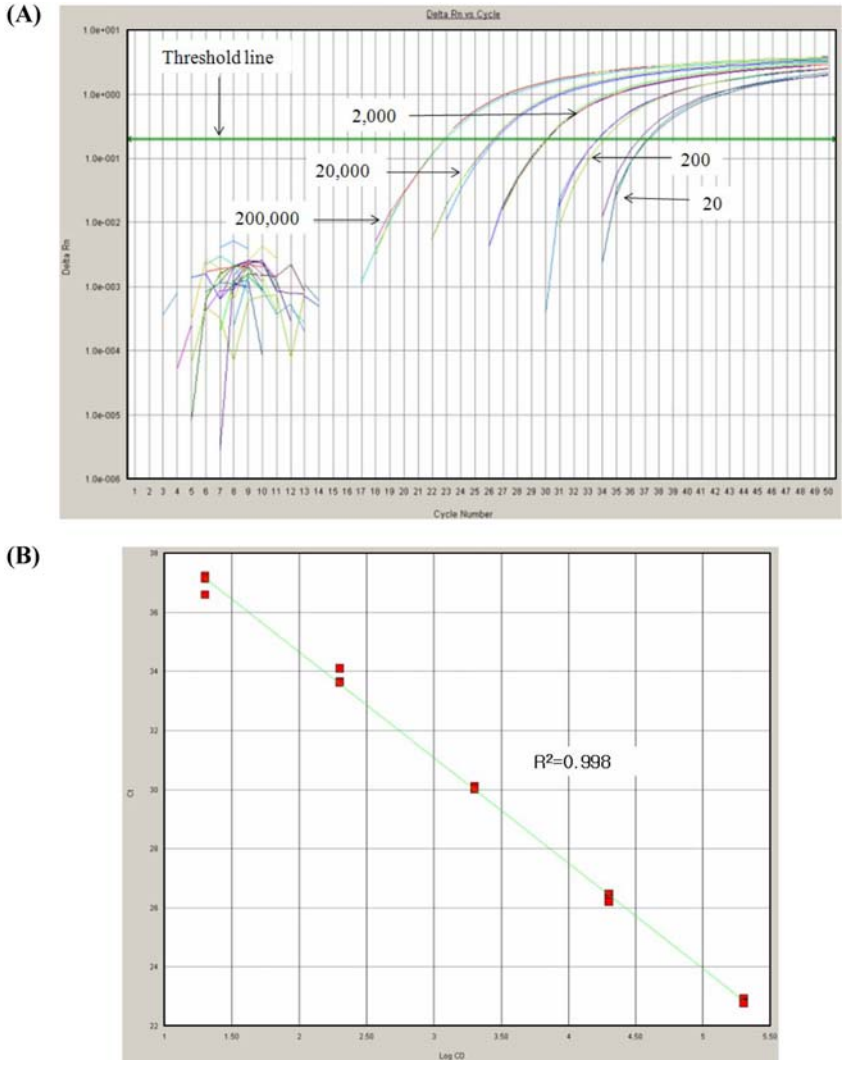

Fig. 5. Amplification plots and standard curve of real-time PCR. (A) Amplification plots generated from PCR of MIR604-F/R primers and MIR604-P probe for detection of MIR604 by 5 levels of pGEM-KHU; (B) parameters of the regression line through data points are indicated within the plot.

Table 2. Repeatability of pGEM-KHU

Target copies	Ct values			Mean Ct	SD[1)]
	Mean 1	Mean 2	Mean 3		
			zSSIIb		
200,000	22.39	22.47	22.51	22.46	0.06
20,000	26.00	26.09	26.07	26.05	0.05
2,000	29.47	29.43	29.52	29.47	0.04
200	33.11	33.07	33.07	33.08	0.02
20	36.77	36.23	36.34	36.45	0.29
			MIR604		
200,000	22.83	22.73	22.77	22.78	0.05
20,000	26.33	26.39	26.43	26.38	0.05
2,000	30.06	29.97	30.09	30.04	0.06
200	33.79	33.64	33.68	33.71	0.08
20	36.99	36.88	36.88	36.92	0.07

[1)]Standard deviation.

Table 3. Conversion factor of quantitative PCR for MIR604

Target	Mean	SD[1)]	RSD[2)]
MIR604	0.57	0.02	3.50

[1)]Standard deviation.
[2)]Relative standard deviation was calculated by dividing the SD by the mean value and given in percent. Each experiment was performed 3 times.

amounts of non-targeted sequences in the plasmid and genomic DNA (9,12). These results indicated that the pGEM-KHU was used effectively as a standard plasmid for quantification of MIR604.

Accuracy and precision of the quantitative PCR method Accuracy of the quantitative PCR method was measured as bias (%) which was calculated as the following equation: |1-calculated GM contents/known GM contents| (12). The calculated bias, SD, and RSD at each

Table 4. Accuracy and precision of the real-time PCR methods

GM event	True value (%)	Coefficient value (CV)	Accuracy[1]		Precision[2]		Below 20 copies[3]
			Mean (%)	Bias (%)	SD	RSD	
MIR604	10.0	0.57	10.11	1.1	0.32	3.17	0/3
	1.0		1.09	9.0	0.03	2.75	0/3
	0.1		0.10	0.0	0.01	10.00	0/3

[1]Bias=(mean value-true value)/true value×100.
[2]SD, standard deviation; RSD, relative standard deviation values were calculated by dividing the SD by the mean value and given in percent.
[3]The number of experiments below 20 copies/total number of experiments.

level are shown in Table 4. For MIR604, biases were 1.1, 9.0, and 0% for the 10, 1, and 0.1% templates, respectively. Their RSDs were 3.17, 2.75, and 10.00, respectively. C_vs and biases in similar ranges were published earlier in connection with other quantitative GMO detection systems (12,15). Therefore, our results fit with the ENGL minimum acceptance criteria for real-time PCR detection methods.

In conclusion, we designed an event-specific primer and a probe to detect MIR604 and also constructed pGEM-KHU as a reference material. Results indicated that our methods are suitable for qualitative and quantitative analyses for MIR604. Especially, the established quantitative PCR system could be used to monitor labeling systems within the EU (0.9% threshold), Korea (3% threshold), and Japan (5% threshold) GMO labeling regulations. However, this method should be validated in ring-trials with several independent laboratories and authorized by the KFDA for practical application to GMO monitoring.

Acknowledgment

This work was supported by a grant (Code #20080401034032) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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