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Multiplex PCR Detection of the MON1445, MON15985, MON88913, and LLcotton25 Varieties of GM Cotton

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Abstract A multiplex polymerase chain reaction (PCR) method was developed to simultaneously detect 4 varieties of genetically modified (GM) cotton. The event-specific primers were used to distinguish the 4 varieties of GM cotton (MON1445, MON15985, MON88913, and LLcotton25) using multiplex PCR. The acyl carrier protein 1 (Acp1) gene was used as an endogenous reference gene of cotton in the PCR detection. The primer pair Acp1-AF/AR containing a 99 bp amplicon was used to amplify the Acp1 gene and no amplified product was observed in any of the 13 different plants used as templates. This multiplex PCR method allowed for the detection of event-specific targets in a genomic DNA mixture of up to 1% GM cotton containing MON1445, MON15985, MON88913, and LLcotton25.

Keywords: multiplex polymerase chain reaction (PCR), genetically modified cotton, MON1445, MON15985, MON88913, LLcotton25

Introduction

Cotton (*Gossypium hirsutum* L.) is grown worldwide primarily as a source of fiber in textile manufacturing and cottonseed (a by-product of fiber production) is routinely used in human foods (1). Genetically modified (GM) cotton has been grown in the United States since 1996. More than half of the GM cotton produced in the world is grown in the United States. Farmers in many countries have also grown GM cotton due to the benefits of biotechnology in agriculture. In 2007, GM cotton occupied 15 million hectares at 13% of global biotech crop area (1, 2). GM cotton comes in 3 varieties: herbicide tolerant cotton, insect resistant cotton with a gene of the bacteria *Bacillus thuringiensis* (Bt), and a combination of Bt/herbicide tolerant cotton.

According to the genetically modified organism (GMO) database of Agbios (Agriculture and Biotechnology Strategies Inc., Merrickville, ON, Canada), 18 varieties of GM cotton were developed and approved in several countries (1,2). Recently, the Korea Food & Drug Administration (KFDA) has approved 4 new events of GM cotton (MON1445, MON15985, MON88913, and LLcotton25). The GM cotton MON1445 has been genetically engineered to be tolerant to the herbicide glyphosate. CP4 *5-enolpyruvylshikimate-3-phosphate synthase* (EPSPS) from *Agrobacterium tumefaciens* strain CP4 confer this herbicide tolerance in MON1445. The GM cotton MON15985 contains *cry*1Ac and *cry*2Ab, coding for insecticidal proteins, derived from *B. thuringiensis* subsp. *kurstaki*. The GM cotton MON88913 contains CP4 EPSPS derived from *A. tumefaciens* strain CP4. The GM cotton LLcotton25 has been genetically engineered for tolerance to the herbicide glufosinate-ammonium. The herbicide tolerance is conferred by insertion of *bar* gene. Cottonseed oil derived from cotton lines containing GM cotton events (MON1445, MON15985, MON88913, and LLcotton25) is expected to be used by the food industry. Cottonseed oil is used in salads, margarine, fried snacks, peanut butter, and many other food ingredients. Korea has implemented mandatory labeling requirements for GM foods that contain more than 3% GM crops; therefore, the development of methods for GMO detection are continuously required to monitor various GM crops.

Polymerase chain reaction (PCR) has been successfully employed to screen for the presence of GMOs (3-8). A PCR method that can distinguish one particular variety from all other varieties using primers is described as being 'gene-specific', 'construct-specific', or 'event-specific'. An event-specific PCR system was designed based on the junction between the transgenic insert and the host DNA and was regarded as the most specific approach (10). The simultaneous amplification of multiple targets has routinely been performed using multiplex PCR and a useful strategy involving the optimization of multiplex PCR primer mixtures has been developed in GM maize (9-11), GM canola (12,13), and mixed GM crops (14,15). Qualitative or quantitative PCR methods for GM cotton MON1445, MON531, MON15985, MON88913, and 281-24-236/3006-210-23 have also been previously reported (16-19). However, multiplex PCR results of these GM cottons have not yet been reported. In this study, event-specific primers were developed to simultaneously distinguish the 4 varieties of MON1445, MON15985, MON88913, and LLcotton25.

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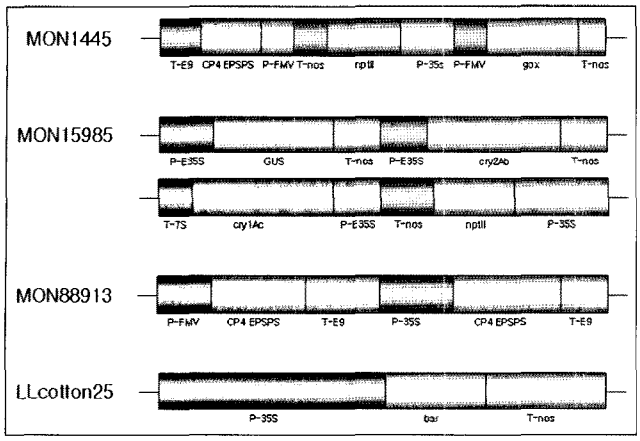

Fig. 1. Schematic diagram of 4 varieties of GM cotton.

Materials and Methods

Cotton samples Four varieties of GM cotton; MON1445, MON15985, and MON88913 from Monsanto (St. Louis, MO, USA), LLcotton25 from Bayer Crop Science (Monheim am Rhein, Germany), as well as non-GM cotton were provided by the KFDA. Thirteen different plants [soybean (*Glycine max*), corn (*Zea mays*), canola (*Brassica napus*), rice (*Oryza sativa*), potato (*Solanum tuberosum*), barley (*Hordeum vulgare*), buck wheat (*Fagopyrum esculentum*), wheat (*Triticum aestivum*), pepper (*Capsicum annuum*), red bean (*Phaseolus angularis*), radish (*Raphanus sativus*), Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), and perilla leaf (*Perilla frutescens*)] were collected from Rural Development Association in Korea and Department of Food Biotechnology in Kyung Hee University. A schematic diagram of the 4 varieties of GM cotton is shown in Fig. 1.

DNA extraction Samples were finely ground in liquid nitrogen using a mortar and pestle. The DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany) was used according to the manufacturer's instructions. A ground sample (1 g) was mixed in a 15-mL tube with 5 mL of buffer AP1 and 10 μL of RNase A (100 mg/mL). The mixture was then incubated for 15 min at 65°C. After incubation, 1.8 mL of buffer AP2 was added to the lysate, which was then mixed and incubated on ice for 10 min. The lysate was applied to a QIAshredder mini spin column, placed in a 50-mL tube, and centrifuged for 5 min at 4,500×g. The flow-through fraction was transferred to a new tube and 1.5 volumes of buffer AP3 were added to the tube. The tube was then mixed, applied to the DNeasy maxi spin column and centrifuged for 5 min at 4,500×g. The column was then placed in a new 50-mL tube and washed with buffer AW. After the column was washed, the genomic DNA was eluted with 500 μL of preheated (65°C) sterile distilled water.

Polymerase chain reaction PCR was carried out on a Mastercycler (Eppendorf, Hamburg, Germany). The reaction mixture in 25 μL volumes contained 2.5 μL of 10× buffer (Applied Biosystems, Foster City, CA, USA), 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 the appropriate concentrations of the template DNA. The optimized primer pairs are shown in Table 1. The PCR conditions were pre-incubation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec, extension at 72°C for 30 sec, and terminal elongation at 72°C for 8 min.

Oligonucleotide primers Five sets of primer pairs were designed for multiplex PCR to detect and distinguish 4 varieties of GM cotton. The sequences of the oligonucleotide primers are shown in Table 1.

Agarose gel electrophoresis The PCR products were separated by gel electrophoresis and the sizes of the inserted DNA fragments were compared to the sizes of the amplified DNA fragments. After PCR, 7 μL of each product was loaded on a 3.5% agarose gel containing 0.5 μg/mL ethidium bromide. A 100 bp DNA ladder (Takara, Kyoto, Japan) was used as the size standard for the amplified DNA fragments.

DNA sequencing The PCR products of MON1445, MON15985, MON88913, and LLcotton25 were extracted from the agarose gel using a gel extraction kit (Qiagen). The pGEM-T easy vector (Promega, Madison, WI, USA) was used to clone the amplified DNA fragments and was transformed into *Escherichia coli* strain DH5α. The selected *E. coli* containing the recombinant plasmid was grown and the plasmid was purified. The sequencing of the

Table 1. Primer pairs for multiplex PCR

Primer name	Sequences (5'→3')	Targets	Amplicon size (bp)
Acp1-AF	CGTGGATAAGGTATGTGAAG	Acp1	99
Acp1-AR	GAATCAGCTCCAAGATCAAG	Acp1	
M1445-AF	GTGAGTGAGATGATCCCAGT	Genome	125
M1445-AR	TCGTTTCCCGCCTTCAGTTT	T-E9	
M88913-AF	GCTGATCCATGTAGATTTCCCG	Vector	141
M88913-AR	AGAAGCGAGACCTACAAGCC	Genome	
LL25-AF	ACATCATCCGTTTCTTGGAC	Genome	169
LL25-AR	GCAACTGTGCTGTTAAGCTC	P-35S	
M15985-AF	GTTGGCACATTTGATGCACT	Genome	197
M15985-AR	GACAGATAGCTGGGCAATGG	P-E35S	

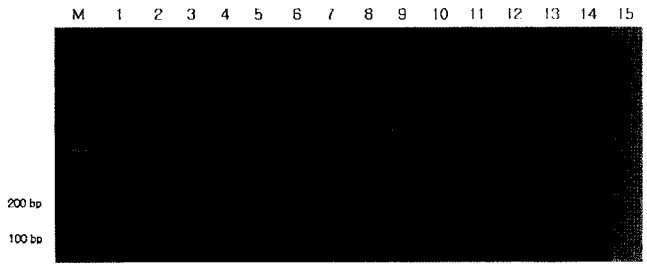

Fig. 2. PCR products amplified from the genomic DNAs of 14 different plants. Lane M, marker (100 bp DNA Ladder); lanes 1-15, soybean (Lectin), maize (SSIIb), canola (FatA), cotton (Acp1), rice (SPS), potato, barley, buck wheat, wheat, pepper, red bean, radish, Chinese cabbage, perilla leaf, and non-template; SSIIb, *starch synthase* IIb; FatA, *Fatty acyl-ACP thioesterase*; Acp1, acyl carrier protein; SPS, *sucrose phosphate synthase*.

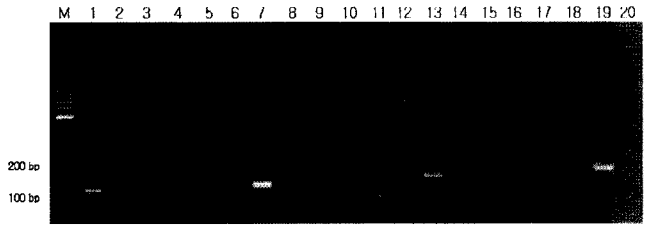

Fig. 3. Specificity of primer pairs designed for multiplex PCR. Lane M: (100 bp DNA ladder); lanes 1, 6, 11, 15: MON1445; lanes 2, 7, 12, 16: MON88913; lanes 3, 8, 13, 17: LLcotton25; lanes 4, 9, 14, 18: MON15985; lanes 5, 10, 15, 20: no template; lanes 1-5: PCR results using MON1445 event-specific primer pairs; lanes 6-10, PCR results using MON88913 event-specific primer pairs; lanes 11-15, PCR results using LLcotton25 event-specific primer pairs; lanes 16-20, PCR results using MON15985 event-specific primer pairs.

amplified DNA fragment in the pGEM-T easy vector was executed twice using an ABIPRISM 3700 DNA analyzer (Perkin Elmer, Boston, MA, USA).

Results and Discussion

Specificity and sensitivity of primer pairs for the multiplex PCR The primer pair Acp1-AF/AR was used to amplify an endogenous cotton gene (Acp1). The primer pair gave rise to a 99 bp amplicon via PCR. We performed a qualitative PCR using 50 ng of genomic DNA from 14 different plant species in order to investigate the specificity of the intrinsic primer pair. As shown in Fig. 2, no amplicon was observed in any of the species tested except for a cotton. Single PCR was used to individually assess the specificity of the designed primer pairs. The genomic DNA fragments extracted from the seeds of 4 varieties of GM cotton were used as the templates. As shown in Fig. 3, the expected amplicons were specifically amplified from each target GM cotton; MON1445, MON15985, MON88913, and LLcotton25. The M1445-AF/AR primer amplified the 5'-flanking region of MON1445 to a size of 125 bp, the M15985 AF/AR primer amplified the 5'-flanking region of MON15985 to a size of 197 bp, the MON88913 AF/AR primer amplified the 3'-flanking region of MON88913 to a size of 141 bp and the LL25 AF/AR primer amplified the 3'-flanking region of LLcotton25 to a size of 141 bp.

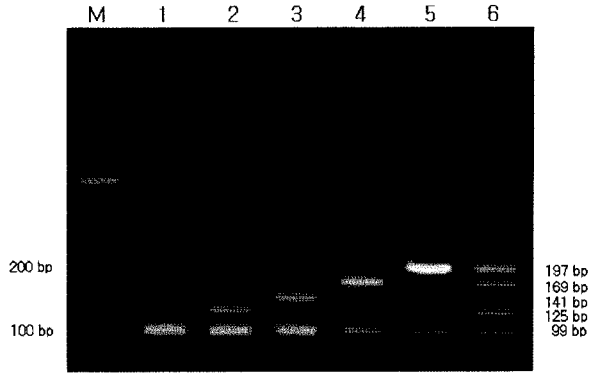

Fig. 4. Multiplex PCR products amplified from GM cotton containing the Acp1 gene (an endogenous gene). Lane M, marker (100 bp DNA ladder); lane 1, endogenous gene; lane 2, MON1445 and endogenous gene; lane 3, MON88913 and endogenous gene; lane 4, LLcotton25 and endogenous gene; lane 5, MON15985 and endogenous gene; lane 6, MON1445, MON88913, LLcotton25, MON15985, and endogenous gene.

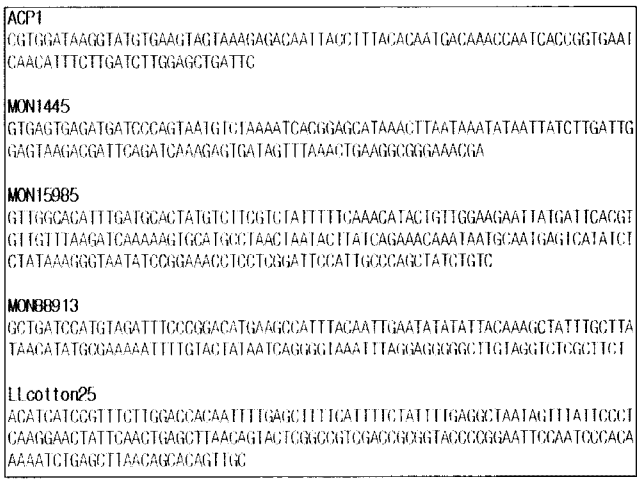

Fig. 5. Sequencing results of the multiplex PCR products. The 5'-flanking region of MON1445 to a size of 125 bp, the 5'-flanking region of MON15985 to a size of 197 bp, the 3'-flanking region of MON88913 to a size of 141 bp, and the 3'-flanking region of LLcotton25 to a size of 169 bp.

Figure 4 shows the result of a multiplex PCR performed under the optimized conditions. The multiplex PCR amplified 2 fragments corresponding to the chosen target sequences for each GM event and for Acp1 as an internal control in the non-GM cotton. As shown in lane 6 of Fig. 4, the intensities of the bands derived from the 4 amplicons were equivalent. The sequences of these amplicons were determined and are shown in Fig. 5. This PCR result showed that this method is sufficient to distinguish the 4 varieties of GM cotton.

Limit of detection (LOD) of the multiplex PCR The LOD is defined as the lowest amount or concentration of analyte that can be reliably detected in a sample. The reference materials containing 10, 5, 3, 1, 0.5, and 0.1% of the GM cotton mixture were prepared by mixing GM DNA from each variety of GM cotton (MON1445, MON15985, MON88913, and LLcotton25) with non-GM

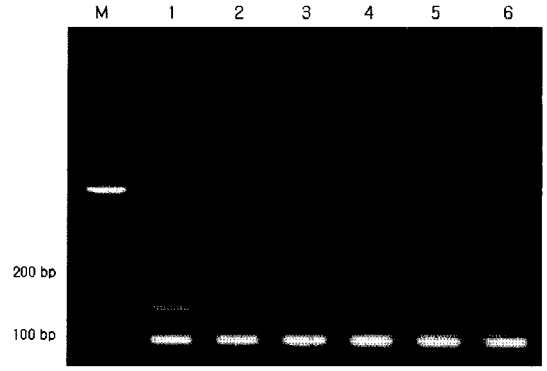

Fig. 6. Sensitivity analysis of the multiplex PCR. Lane M, marker (100 bp DNA ladder); lanes 1-6: 10, 5, 3, 1, 0.5, and 0.1% of a mixture of GM cotton (MON1445, MON88913, LLcotton25, and MON15985).

DNA from the control cotton in order to determine the LOD value of the multiplex PCR. In the multiplex PCR assay, the LOD value was determined to be 1% of the GM cotton mixture in 100 ng of genomic DNA (Fig. 6). This LOD is of significant value for the PCR method used to monitor the labeling system for GM foods.

The advantage of multiplex PCR-based methods is that several target DNA sequences can be identified in a single reaction. In recent year, there are many multiplex PCR systems for simultaneous detection of GMOs in commercialized GM crops. Most of GMOs contain the same or a similar promoter, terminator, foreign gene, or a combination of two of these elements; therefore, event-specific primer pairs were used for the effective and reliable identification of GM cotton using multiplex PCR in this study.

In conclusion, we developed the multiplex PCR method for 4 varieties of GM cotton (MON1445, MON15985, MON88913, and LLcotton25) safety-approved in Korea. This method may offer a useful tool to detect GM cotton in the markets and to monitor a labeling system with 3% threshold of in Korea.

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