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Multiplex PCR Detection for 3 Events of Genetically Modified Maize, DAS-59122-7, TC6275, and MIR604

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Abstract A multiplex polymerase chain reaction (PCR) method was developed to simultaneously detect 3 events of genetically modified (GM) maize. The event-specific primers were used to discriminate the following 3 events of GM maize (DAS-59122-7, TC6275, and MIR604) using multiplex PCR method. The *zein* gene was used as an endogenous maize reference gene in the multiplex PCR detection. The primer pair Zein-F/R producing a 99 bp amplicon was used to amplify the *zein* gene. The primer JI-Das-F1/R1 for DAS-59122-7, JI-TC6275-F3/R3 for TC6275, and JI-MIR F1/R1 for MIR604 yielded an amplicon of 130, 162, and 197 bp, respectively. The detection limit of multiplex PCR was 1% for DAS-59122-7, TC6275, and MIR604 for one reaction.

Keywords: DAS-59122-7, TC6275, MIR604, maize, multiplex polymerase chain reaction (PCR)

Introduction

Genetically modified (GM) crops were grown by approximately 12 million farmers from 23 countries in 2007 (1). GM soybean was grown on 58.6 million hectares (51% of global biotech area), followed by GM maize (35.2 million hectares at 31%), GM cotton (15.0 million hectares at 13%), and GM canola (5.5 million hectares at 5% of global biotech crop area) (1). Fifty-two countries in the world have granted approvals for (GM) crops for food and feed use and for release into the environment since 1996 (1). Among the GM crops approved in these countries, maize has the most events followed by cotton, canola, and soybean. Korea has implemented mandatory labeling requirements for GM foods that contain more than 3% generally modified organism (GMO); therefore, the development of methods for GMO detection has been continuously required to monitor various GM crops (2). As of March, 2007, food safety approvals have been given to 29 events of GM maize containing stacked events by Korea Food & Drug Administration (KFDA).

Various features of GM maize are being continuously developed. Among of them, three GM events have been recently developed and commercialized. The GM maize DAS-59122-7 contains 3 genes, *cry*34Ab1 and *cry*35Ab1 for insect resistance, and *pat* for herbicide tolerance. The GM maize TC 6275 contains 2 genes, *cry*1F and *bar*, for insect resistance and herbicide tolerance, respectively. The GM maize MIR604 contains 2 genes, *mcry*3A conferring insect resistance and containing a cathepsin-G serine protease recognition site within the expressed protein for increased resistance to target pests, and *Escherichia coli manA* gene (*pmi*) encoding phosphomannose isomerase (3).

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Detection methods for GMOs have been previously developed (4-13). The polymerase chain reaction (PCR) is one of the most commonly used methods for identifying the presence of GMOs (6-8). Especially, multiplex PCR method is necessary for simultaneous detection of various GM events (9-12). Event-specific PCR systems have been promoted for many years because of its high specificity based on the flanking sequence of the exogenous integrant (5,13).

In this study, multiplex PCR detection method for GM maize DAS-59122-7, TC6275, and MIR604 using event-specific primer pairs designed based on the junction sequences between the transgenic insert and the host DNA is firstly reported.

Materials and Methods

Maize samples Three events of GM maize; DAS-59122-7 and TC6275 maize from Dow AgroSciences LLC (Indianapolis, IN, USA), and MIR604 maize from Syngenta seeds AG (Basel, Switzerland) as well as non-GM maize were provided by the KFDA. The schematic diagram of 3 events of GM maize is shown in Fig. 1.

DNA extraction Samples were ground in liquid nitrogen using mortars and pestles. The DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany) was used according to the manufacturer's instructions. A ground sample (0.5 g) was mixed in a 15-mL tube with 5 mL of buffer AP1, and 10 μL of RNase A (100 mg/mL) and then the mixture was incubated for 10 min at 65°C. After incubation, 1.8 mL of buffer AP2 was added to the lysate, mixed, and incubated for 10 min on ice. The lysate was applied to the QIAshredder maxi spin column placed in a 50-mL tube and centrifuged for 5 min at 5,000×g. The flow-through fraction was transferred to a new tube and 1.5 volumes of buffer AP3 were added. After the tube was mixed, it was applied to the

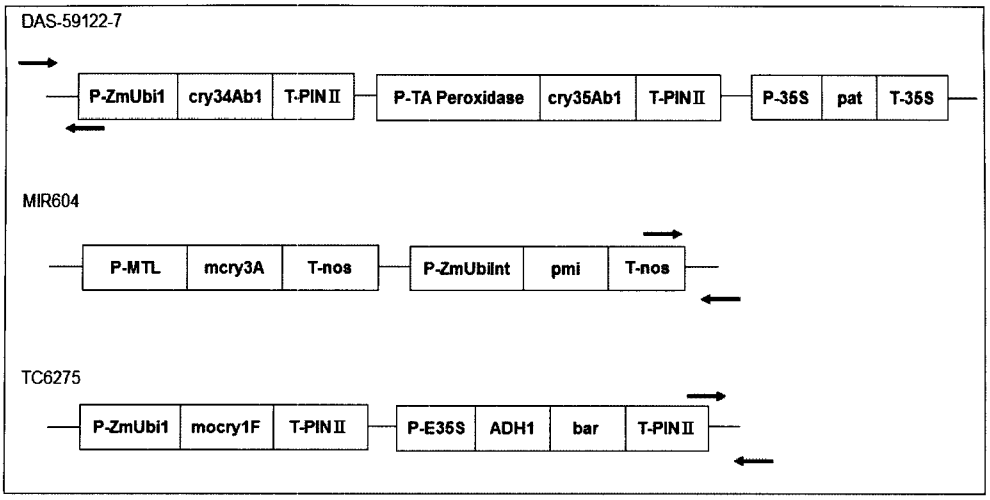

Fig. 1. Schematic diagram of 3 events of GM maize. The primers used for the amplification are indicated by arrows.

DNeasy maxi spin column and centrifuged for 5 min at 5,000×g, after which this column was placed in a new 50-mL tube and washed in buffer AW. After the column was washed, genomic DNA was eluted with 500 μL of preheated (65°C) sterile distilled water.

Oligonucleotide primers Oligonucleotide primer pairs were synthesized by Bionix Inc. (Seoul, Korea). Four sets of primer pairs were designed for multiplex PCR to detect and distinguish 3 events of GM maize. The sequences of the oligonucleotide primers are shown in Table 1.

Multiplex PCR conditions The reaction mixture in 25 μL volumes included 100 ng of genomic DNA, 2.5 μL of 10×buffer (Applied Biosystems, Foster City, CA, USA), 200 μM of each dNTP (Applied Biosystems), 1.5 mM of $MgCl_2$ and 0.8 units of Ampli Gold *Taq* DNA polymerase (Applied Biosystems). The optimized primer pairs are listed in Table 1. PCR was performed in a thermal cycler (ASTEC PC818; Fukuoka, Japan) with the conditions as follows: 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 at 1 min, and final extension at 72°C for 8 min. The PCR product was resolved on 4%(w/v) agarose gel by electrophoresis.

DNA sequencing PCR products of the three GM maize were purified using PCR purification kit (Qiagen). The pGEM-T easy vector (Promega, Madison, WI, USA) was used to clone the amplified DNA fragments and transformed into *E. coli* strain DH5α. The selected *E. coli* containing the recombinant plasmid was cultured and the plasmid was purified. The sequencing of the DNA fragment cloned in pGEM-T easy vector was performed twice using an ABIPRISM 3700 DNA analyzer (Perkin Elmer, Boston, MA, USA).

Results and Discussion

Specificity of primer pairs for a multiplex PCR The primer pair Zein-1-5'/1-3' reported previously (12) was used to amplify an endogenous maize gene (*zein* gene). A single PCR was performed containing 50 ng of genomic DNA from 12 different events of GM maize to confirm the specificity of each primer pair. As shown in Fig. 2, the expected amplicons were specifically amplified from each target. Most of GM crops include the same or similar promoter, terminator, inserted gene, or a combination of two of these elements (14). In this study, event-specific primers were designed to minimize the risk of false positive results. The primer pairs JI-DAS F1/R1 amplified the 5'-flanking region of DAS59122-7 to a size of 130 bp (Fig. 2A). For MIR604 maize, a size of 162 bp was obtained by the use of JI-MIR F1/R1 primer pairs (Fig. 2B). For

Table 1. Sequence of primer pairs used for multiplex PCR

GM crops	Primer name	Sequence (5'-3')	Target	Primer concentration (μM)	Amplicon size (bp)
Zein	Zein-5'	ATC GGC CTC AGT CGC ACA TA	*zein*	2	99
	Zein-3'	AGC TAG GAG AGC GAA CAA TG			
DAS-59122-7	JI-DAS-F1	GCA CCT GTG ATT GGC TCA TA	genome	3.9	130
	JI-DAS-R1	CTC CGC TCA TGA TCA GAT TG	T-DNA		
MIR604	JI-MIR-F1	CGC GGT GTC ATC TAT GTT AC	T-nos	3.9	162
	JI-MIR-R1	AGG CTA CAT CCG TGC AGG AG	genome		
TC6275	JI-TC6275-F3	GGG AGC TCG AAT TCA GTA CA	T-DNA	5	197
	JI-TC6275-R3	TCA CGC AAA GGA CTC TGG AT	genome		

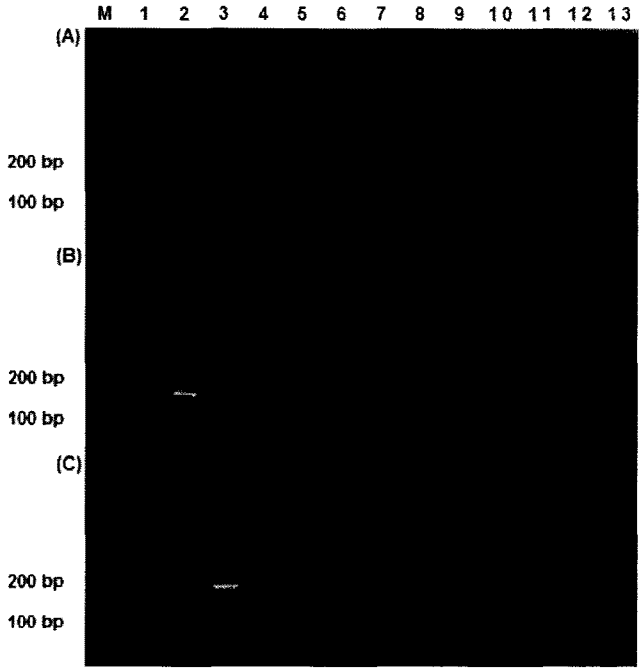

Fig. 2. Specificity of the detection primer pairs designed for the multiplex PCR. Lane M, marker (100 bp DNA ladder); lane 1, DAS-59122-7; lane 2, MIR604; lane 3, TC6275; lane 4, Bt176; lane 5, Bt11; lane 6, Bt10; lane 7, GA21; lane 8, Mon810; lane 9, Mon863; lane 10, NK603; lane 11, TC1507; lane 12, T25; lane 13, no template. The result obtained by using the JI-DAS F1/R1 primer pairs (A); JI-MIR F1/R1(B); JI-TC6275 F3/R3 (C).

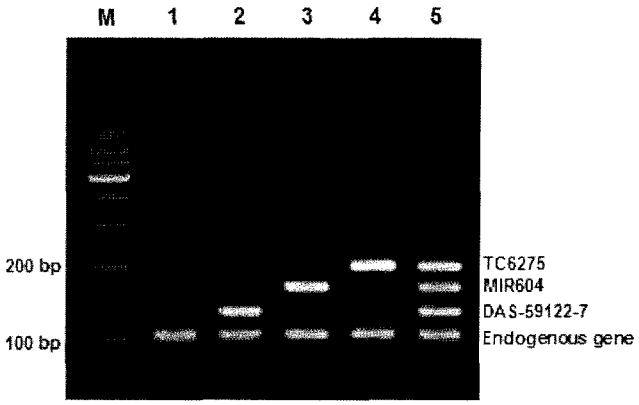

Fig. 3. Multiplex PCR products amplified from GM maize. Lane M, marker (100 bp DNA ladder); lane 1, endogenous gene; lane 2, DAS-59122-7 and endogenous gene; lane 3, MIR604 and endogenous gene; lane 4, TC6275 and endogenous gene; lane 5, DAS-59122-7, MIR64, TC6275, and endogenous gene.

TC6275, an amplified fragment of 197 bp was produced by the JI-TC6275 F3/R3 primer pairs (Fig. 2C).

All primer pairs in a multiplex PCR should be similar amplification efficiencies for their respective targets. In this study, all primer pairs were used to similar amplification at different concentration and the optimized concentrations were determined (Table 1).

Figure 3 shows the result of a multiplex PCR performed under the optimized conditions. Two fragments corresponding

Fig. 4. Sequencing results of the multiplex PCR products. *Zein* (maize endogenous gene); 5'-flanking region of DAS-59122-7; 3'-flanking region of MIR604; 3'-flanking region of TC6275.

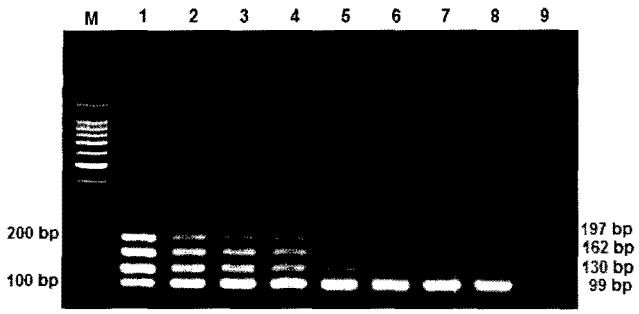

Fig. 5. Sensitivity of the multiplex PCR. Lane M, marker (100 bp DNA ladder); lane 1-7, 100, 10, 5, 3, 1, 0.5, and 0.1% of a mixture of GM maizes (DAS-59122-7, MIR64, and TC6275); lane 8, non-GM maize; lane 9, no template.

to the chosen target sequences for GM maize and *zein* gene as an internal control in the non-GM maize were amplified from the multiplex PCR (Fig. 3). As shown in lane 5 of Fig. 3, the intensities of the bands derived from the 4 amplicons were equivalent. The sequences of these amplicons were determined and are shown in Fig. 4. This PCR results show that this method is sufficient to distinguish the three events of GM maize.

Limit of detection (LOD) of the multiplex PCR The LOD is described as the lowest amount or concentration of sample that can be detected (13). The reference materials including 10, 5, 3, 1, 0.5, 0.1, and 0% of the GM maize mixture were prepared from 3 events of GM maize (DAS-59122-7, TC6275, and MIR604) with the non-GM maize 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 maize mixture (Fig. 5). This LOD is of significant value for the PCR method used for labeling threshold as 0.9% in the European Union (15), 3% in Korea (16), and 5% in Japan (17).

In this study, a multiplex PCR method was developed to detect 3 events of GM maize, which have been recently 'safety-approved' in the world. In fact, these results demonstrate that this multiplex PCR method is suitable for the detection of 3 events of GM maize in food ingredients and feeds.

In this report, we firstly presented the results of the multiplex PCR for 3 events of GM maize (DAS-59122-7, TC6275, and MIR604).

Acknowledgments

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