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Detection of Eight Different Events of Genetically Modified Maize by Multiplex PCR Method

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Abstract Multiplex PCR was performed to simultaneously detect eight different events of genetically modified (GM) maize. Specific primers were constructed from GA21, T25, TC1507, Mon810, Mon863, Event176, Bt11, and NK603 events of GM maize. Using this PCR method, specific GM maize was monitored in commercialized foods and feed.

Keywords: genetically modified maize, multiplex PCR, primer

Introduction

A number of genetically modified (GM) crops have been developed through the recombinant DNA technology and commercialized by several agricultural biotechnological companies in recent years. The global area for GM crops has continued to grow for the eighth consecutive year, from 1.7 million hectares in 1996 to 67.7 million hectares in 2004 (1). GM maize has been planted on 23% of global area (15.5 million hectares). Korea imported more than 99 % of its maize in 2004. Recently, various events of GM maize are available on the market with different events safety-approved in different countries. In Korea, the safety-assessments of eight different events (GA21, TC1507, Mon810, NK603, Event176, Bt11, T25, and Mon863) among GM maize were recently approved by Korea Food and Drug Administration (KFDA). In response to growing consumer concerns, the genetically modified organism (GMO) labeling system has been enforced in raw materials since March of 2001 and GMO-derived foods since July of 2001 in Korea (2). The labeling system is based on the detection of inserted genes or proteins expressed from inserted genes. Thus, the success of the labeling system is dependent upon the efficiency with which GMO-derived materials can be detected. Consequently, the development of a practical detection method is required to confirm the validity of labeling system and to monitor the status of circulation for GMOs. The method of polymerase chain reaction (PCR) that detects specific genes and immunological method that use specific proteins were used for monitoring of GMOs (3-5). The PCR method has been found to be more sensitive than the protein-based method for the detection of GMOs in raw materials and processed foods (6). In addition, with the increasing number of GMOs developed for food applications, the ability to detect various GMOs with high specificity and low experimental time and cost becomes an important feature of all detection methods. Multiplex PCR has adequate sensitivity to simultaneously detect various GMOs in a single reaction, without loss of specificity. The detection method for GMO using the PCR was reported (7-10); however, multiplex PCR method of eight different events of GM maize has not yet been developed.

In this study, the multiplex PCR was developed to efficiently monitor eight different events of GM maize in a single reaction, and designed primer sets were validated by sequencings of PCR products.

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Materials and Methods

Maize samples Eight events of GM maize (herbicide-tolerant GA21 and NK603 from Monsanto, T25 from AgrEvo; insect-resistant Mon810 and Mon863 from Monsanto; insect and herbicide-resistant TC1507 from Mycogen; insect and herbicide-resistant Bt11 and insect-resistant Event176 from Syngenta) and non-GM maize were provided by Korea Food and Drug Administration. The schematic diagram of eight events of GM maize is shown in Fig. 1. To monitor the events of GM maize, corn processed foods (corn grits, cooking oil, corn for feeding, canned corn, mixed vegetables, corn soup, corn chip, popcorn, and cereal; all imported goods) purchased from Korean markets were used as reference and analysis materials.

DNA extraction Samples were ground in an electric mill. The DNeasy Plant Mini kit (Qiagen, Germany) was used according to the manufacturer's instructions. A ground sample (100 mg) was mixed in a 2-mL Eppendorf tube with 500 μL of buffer AP1, and 5 μL of RNase A (100 mg/mL), and the mixture was incubated for 10 min at 65°C. Subsequently, 130 μL of buffer AP2 was added to the lysate, mix, and incubated for 5 min on ice. The lysate was applied to the QIAshredder mini spin column placed in a 2-mL tube and centrifuged for 2 min at 20,000×g (14,000 rpm). Flow-through fraction was transferred to a new tube and added with 1.5 volumes of buffer AP3. The

tube was mixed, applied to the DNeasy mini spin column, and centrifuged for 1 min at 10,000×g. The column was then placed in a new 2-mL tube and washed in buffer AW. After washing, genomic DNA was eluted with 50 μL of preheated (65°C) sterile distilled water.

Polymerase chain reaction (PCR) PCR was carried out on a PCR thermal cycler (ASTEC, Japan). The reaction mixture in 25 μL volume contained 2.5 μL of 10×buffer (Takara, Japan), 200 nM of dNTP (Takara), and 1 unit of *Taq* DNA polymerase (Takara). The concentrations of template DNA and the optimized primer pairs are shown in Table 1. The conditions for PCR were pre-incubation at 94°C for 5 min and 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, and terminal elongation at 72°C for 10 min.

Oligonucleotide primers Eight sets of primer pairs were designed for the multiplex PCR to detect and distinguish the events of GM maize. The inserted DNAs and amplified regions for GA21, TC1507, Mon810, T25, Bt176, Bt11, NK603, and Mon863 are described in Fig. 1. The primers of cry1F 5', cry1F 3', PEPC 1, and CTP 3' were designed on the basis of delta-endotoxin gene derived from *Bacillus thuringiensis* var. *aizawai* (11), promoter from the maize phosphoenolpyruvate carboxylase gene (12), and chloroplast transit peptide leader sequence from *Arabidopsis thaliana* (13) using the GenBank (Accession No. AF336114, X15642 and X06613). The primers of HS01 and cry-CR01 were designed by referring to other publication (14). The primers of GA21 3-5' and GA21 3-3' were provided by Nippon Gene (15). The primer pair, Zel 1-5' and Zein 3'-1, was used for detection of the intrinsic zein gene (Accession No. M23537) as a

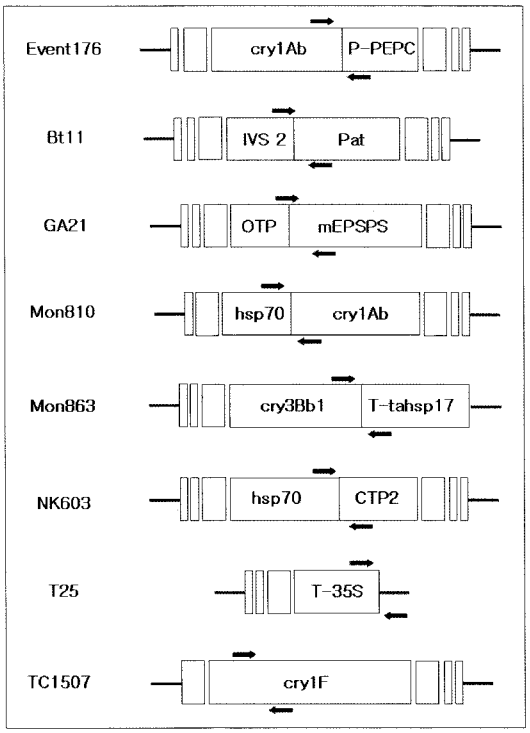

Fig. 1. Schematic diagram of eight events of GM maize.

Table 1. List of PCR primers

Primer name	Specificity			Sequene(5'-3')	Reference
	Target	pM	Size(bp)		
Zein 1-5'	Intrinsic gene(Zein)	3	99	ATCGGCCTCAGTCGCACATA	This study
Zein 1-3'		3		AGCTAGGAGAGCGAACAATG	This study
GA21 3-5'	GA21	0.6	133	GAAGCCTCGGCAACGGCA	Kuribara *et al.* (2002)
GA21 3-5'		0.6		ATCCGGTTGGAAAGCGACTT	Kuribara *et al.* (2002)
pUC 1	T25	15	152	TTGTAAAACGACGGCCAGTG	This study
T35s 1		15		AGGGTTTCGCTCATGTGTTG	This study
C1f03-5'	TC1507	3	172	GTGACTGCAGAGACTGTTAG	This study
C1f03-3'		3		GATCTGACAAGGTCCGATAG	This study
HSO1	MON810	10	193	AGTTTCCTTTTTGTTGCTCTCCT	Matsuoka *et al.* (2000)
cry-CRO1		1		GATGTTTGGGTTGTTGTCCAT	Matsuoka *et al.* (2000)
C3b1-5'	MON863	1.5	223	CGCCTGTTCGTCCAGAACTC	This study
TAP1-3'		1.5		ACAGCTGGACGGGGATGAAC	This study
PEPC1	Event176	1	248	GGTTACCGCCGATCACATGC	Heo *et al.* (2004)
cry-CRO1		10		GATGTTTGGGTTGTTGTCCAT	Matsuoka *et.al.* (2000)
IVS2-S	Bt11	10	265	TTCTTGGCGGCTTATCTGTC	This study
PAT-S		10		AGCAATACCAGCCACAACAC	This study
HSO1	NK603	1	314	AGTTTCCTTTTTGTTGCTCTCCT	Matsuoka *et al.* (2000)
CTP164-3'		10		CGTGGATGCTGCTGCGTCTT	This study

maize internal control (16, 17). The sequences of the oligonucleotide primers are shown in Table 1.

Agarose gel electrophoresis The PCR products were separated by gel electrophoresis, and the inserted DNAs were identified based on the length of the amplified DNA fragments. After PCR, 10 μL of each of products was loaded on a 3.5% agarose gel containing 0.5 μg/mL ethidium bromide. The φX174 RF DNA/*Hae* III (Gibco-BRL) was used as a size standard for amplified DNA fragments.

DNA Sequencings PCR products of the eight GM maize were extracted from agarose gel using gel extraction kit (Qiagen, Germany). The pGEM-T easy vector (Promega) was used to clone the amplified DNA fragments and transformed into *Escherichia coli* strain DH5α. The selected *E. coli* containing the recombinant plasmid was cultivated and purified. The sequencing of the amplified DNA fragment in pGEM-T easy vector was executed twice using the ABIPRISM 3700 DNA analyzer (Perkin Elmer, USA). DNA sequences were analyzed based on the GenBank data.

Results and Discussion

DNA extraction from various maize foods Homogenization of the sample is a critical point in the DNA extraction. The efficiency of DNA is influenced by the size of the ground particles submitted for analysis (17). Thus, all samples were milled to a fine powder. DNAs from maize powders and several commercially available maize foods, such as popcorn, corn snack, and corn bread, were extracted using the DNeasy Plant Mini kit. The purity and quantity of the extracted DNA were evaluated using the 260/280 nm UV absorption ratio between 1.7-2.0. Extracted DNAs showed different patterns according to the conditions used during processing (data not shown).

Sensitivity of primer pairs and specificity for the multiplex PCR To optimize the multiplex PCR, the optimal condition for primer-to-primer ratio was investigated. The presence of more than one primer pair in the multiplex PCR brought about not only unexpected PCR products, but also several difficulties, including poor sensitivity and specificity. All primer pairs in multiplex PCR should be able to give similar amplification efficiencies for their respective targets. Therefore, initially equimolar primer concentration of 0.5 μM each was used in the multiplex PCR. When there was uneven amplification, changing the proportions of various primers in the reaction was required, with an increase in the amount of primers for weak intensity and a decrease for strong intensity (18). This multiplex PCR was optimized by combining each primer at different concentrations (Table 1). Multiplex PCR with non-GM and GM maizes were performed with nine sets of primers in a single tube. The amplified product from a mixture of eight references of GM maizes using multiplex PCR is shown in lane 2 of Fig. 2. The intrinsic zein primer set amplified 99 bp specific DNA fragment. The multiplex PCR product was amplified to the sizes of 133, 152, 172, 193, 223, 248, 265, and 314 bp for the introduced genes in GA21, T25, TC1507, Mon810, Mon863, Event176, Bt11, and NK603, respectively (Fig. 2). No inserted DNAs were observed from non-GM maizes with nine sets of primers except intrinsic zein gene (lane 3 in Fig. 2). The specific bands of each line were clearly distinguished on agarose gel.

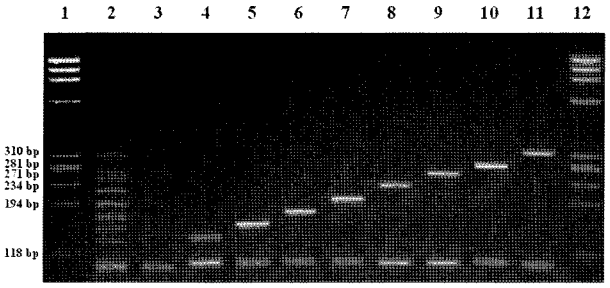

Fig. 2. Multiplex PCR products amplified from 100% GM maize containing zein gene (endogenous gene). Lane 1 and 12: Marker; ØX174 DNA/HaeIII(TaKaRa, Japan), lane 2: Zein (endogenous gene, 99 bp), GA21(133 bp), T25(152 bp), TC1507 (172 bp), Mon810(193 bp), Mon863(223 bp), Event176(248 bp), Bt11 (265 bp) and NK603(314 bp), lane 3: Zein, lane 4: Zein and GA21, lane 5: Zein and T25, lane 6: Zein and TC1507, lane 7: Zein and Mon810, lane 8: Zein and Mon863, lane 9: Zein and Event176, lane 10: Zein and Bt11, lane 11: Zein and NK603.

Multiplex PCR for the detection of GM maize from samples We specifically detected the eight inserted DNAs and intrinsic zein gene in one reaction using multiplex PCR method to monitor GM maizes from various maize samples in Korean markets. The results showed that no PCR amplification related to GM maize was observed in DNAs extracted from corn grits, cooking oil, canned corn, corn soup, popcorn, and cereal (Fig. 3). On the contrary, raw materials for feeding (lane 6 in Fig. 3) showed PCR products with resolved bands of 113, 193, 248, 271, and 328 bp from GA21, Mon810, Event176, Bt11, and NK603, respectively. Mixed vegetables imported from the United States (lane 8 in Fig. 3) showed bands of 173 and 271 bp from TC1507 and Bt11, respectively. Corn chip showed 193 bp from Mon810. These results indicate some processed foods and feed imported from the United States were prepared from one or several events of GM maizes. Although differences were observed in the

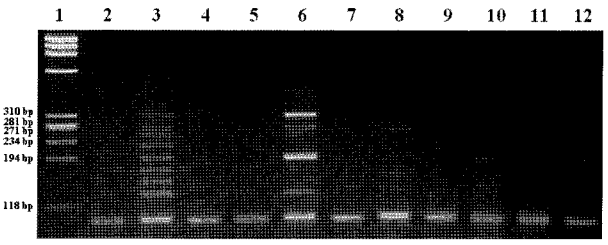

Fig. 3. Multiplex PCR products amplified from various maize foods. Lane 1: Marker; ØX174 DNA/*Hae*III(TaKaRa, Japan), lane 2: Negative control, lane 3: Positive control, lane 4: Corn grits, lane 5: Cooking oil from corn, lane 6: Corn for feeding, lane 7: Canned corn, lane 8: Mixed vegetables, lane 9: Corn soup, lane 10: Corn chip, lane 11: Popcorn, lane 12: Cereal.

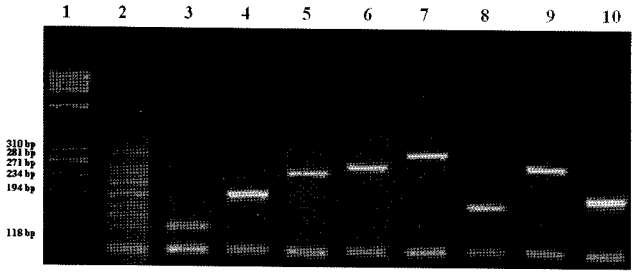

Fig. 4. Results of a single PCR for feed, mixed vegetables and corn chip containing GM maize. Lane 1: Marker; ØX174 DNA/*Hae*III(Takara, Japan), lane 2: Positive control, lane 3-7: T25, Mon810, Event176, Bt11 and NK603 amplified from corn for feeding, lane 8-9: TC1507 and Bt11 amplified from mixed vegetables, lane 10: Mon810 amplified from corn chip.

intensity of PCR products caused by the amount of GM maize in raw materials, they were not quantitated. To quantify the amount of GM maize in foods and feed, real-time PCR should be applied to monitor the GM maize. For the verification of our results, a single PCR was performed on feeding, mixed vegetables and corn chip, samples containing GM maize using specific primer sets of each target (Fig. 4). Each PCR product was sequenced twice, and its specific GM maize was confirmed.

In conclusion, this detection method using multiplex PCR could distinguish the eight different events of GM maize in a single reaction, thereby saving experimental time and cost as compared to using single or duplex PCR. Thus, this method would be helpful to monitor the reliability of the labeling system concerning GM maize in foods and feed market.

Acknowledgments

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