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Multiplex PCR Detection of 4 Events of Genetically Modified Soybeans (RRS, A2704-12, DP356043-5, and MON89788)

Jae-Hwan Kim, Young-Ju Seo, Seol-Hee Sun, and Hae-Yeong Kim*

Institute of Life Sciences and Resources and Department of Food Science & Biotechnology, Kyung Hee University, Yongin, Gyeonggi 446-701, Korea

Abstract A multiplex polymerase chain reaction (PCR) method was developed for the detection of 4 events of genetically modified (GM) soybean. The event-specific primers were designed from 4 events of GM soybean (RRS, A2704-12, DP356043-5, and MON89788). The *lectin* was used as an endogenous reference gene of soybean in the PCR detection. The primer pair YjLec-4-F/R producing 100 bp amplicon was used to amplify the *lectin* gene and no amplified product was observed in any of the 9 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 soybean mixture containing RRS, A2704-12, DP356043-5, and MON89788. In this study, 20 soybean products obtained from commercial food markets were analyzed by the multiplex PCR. As a result, 6 samples contained RRS. These results indicate that this multiplex PCR method could be a useful tool for monitoring GM soybean.

Keywords: multiplex polymerase chain reaction, genetically modified soybean, RRS, A2704-12, DP356043-5, MON89788

Introduction

Since 1996, when the first genetically modified organism (GMO) was commercially grown, consumers have become increasingly concerned about the safety of GMO as foods and food ingredients. The first commercial GM soybean is Roundup Ready soybean (RRS) event GTS 40-3-2 from Monsanto Company. RRS has received regulatory approval in most countries including Korea. In 2007, GM soybean occupied 51% in global biotech area (58.6 million ha) (1).

A number of countries have introduced GM labeling rules. In Korea, GMO labeling system has been enforced in raw materials since March 2001 and GMO-derived foods since July 2001 (2-4). Commodities subject to labeling include soybean, soybean sprout, corn, potato, canola, cotton, and sugar beet. Potatoes were added in March 2002 and canola, cotton, and sugar beet were added in June 2007. GM food labeling policies differ from country to country. The labeling threshold was defined as 0.9% in the European Union (5,6), 5% in Japan (7), and 3% in Korea. In the case of GM soybean, RRS has only been approved in Korea so far.

These GM soybean events (A2704-12, MON89788, and DP356043-5) have not yet been approved to the human safety assessment in Korea Food & Drug Administration (KFDA). Therefore, in respect to Food Sanitation law in Korea, the inflow of non-approved GM soybeans should be thoroughly managed and monitored.

The advantage of multiplex polymerase chain reaction (PCR)-based methods is that several target DNA sequences can be identified in a single reaction. Due to this merit, many researchers have developed various multiplex PCR systems for simultaneous detection of GMOs in commercialized GM crops. Kim *et al*. (8), Matsuoka *et al*. (9), Ahn *et al*. (10), Hernandez *et al*. (11), and Onishi *et al*. (12) reported the detection of GM maize using multiplex PCR. Yang *et al.* (13) reported a multiplex PCR detection method of 3 event of GM cotton (MON531, GK19, and SGK321). Kim *et al*. (14) reported a multiplex PCR method of 4 event of GM cotton (MON1445, MON15985, MON88913, and LLcotton25). Demeke *et al*. (15) and Kim *et al*. (16) reported the multiplex PCR method for detection of GM canola. James *et al*. (17) reported a multiplex PCR method to detect multiple target sequences in different GM crops (soybean, maize, and canola). Germini *et al*. (18) and Forte *et al*. (19) also reported multiplex PCR methods for the simultaneous detection of transgenic soybean and maize.

In this study, we reported multiplex PCR method for 4 GM soybean events (RRS, A2704-12, DP356043-5, and MON89788) using event-specific primer pairs designed based on the junction sequences between the transgenic insert and the host genome. The GM soybean RRS and MON89788 contain a 5-enolpyruvylshikimate-3-phosphate synthase (cp4 *epsps*) gene derived from *Agrobacterium tumefaciens* strain CP4 for herbicide tolerance (20). The GM soybean A2704-12 contains a phosphinothricin *N*-acetyltransferase (*pat*) gene derived from *S. viridochromogenes* for herbicide tolerance (20). The GM soybean DP356043-5 contains a glyphosate *N*-acetyltransferase (*gat4601*) gene derived from *Bacillus licheniformis* and an acetolactate synthase (*gm-hra*) gene which is a modified version of the endogenous soybean acetolactate synthase (*gm-als*) gene for herbicide tolerance (20).

A detection method for non-approved GM soybean was firstly presented in this study. This method would be available for monitoring the presence of non-approved and/or approved GM soybean in food and feed.

*Corresponding author: Tel: +82-31-201-2660; Fax: +82-31-205-6091
E-mail: hykim@khu.ac.kr
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Materials and Methods

Soybean samples Four GM soybean seeds were used in this study. RRS and MON89788 were developed by Monsanto (St. Louis, MO, USA). A2704-12 was developed by Bayer Crop Science (Monheim am Rhein, Germany). DP356043-5 was developed by Pioneer Hi-Bred International Inc (Johnston, IA, USA). GM and non-GM soybeans were provided from the Korea Food & Drug Administration (KFDA). Nine different plant samples, wheat (*Triticum aestivum*), barley (*Hordeum vulgare* var. *hexastichon*), rice (*Oryza sativa*), cotton (*Gossypium hirsutum*), canola (*Brassica napus*), maize (*Zea mays*), buckwheat (*Fagopyrum esculentum Moench*), radish (*Raphanus sativus*), and red bean (*Phaseolus angularis*) were provided from the Rural Development Administration in Korea.

To monitor GM soybean, soybean processed foods (*doenjang*, soymilk, sausage, *tofu*, *cheonggukjang*, and powdered milk) were purchased at commercial food markets in Korea. A schematic diagram of the 4 varieties of GM soybean is shown in Fig. 1.

DNA extraction Samples were ground after freeze-drying in liquid nitrogen using mortars and pestles. Genomic DNAs of soybeans 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 modified manufacturer's manual. DNA isolation of proceed foods was carried out according to Wizard magnetic DNA purification system for food (Promega, Madison, WI, USA) or with CTAB method.

The concentration of the extracted DNA was measured by a ultraviolet (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.

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

PCR condition PCR was carried out on a thermal cycler (ASTEC PC808, Fukuoka, Japan). The reaction mixture in 25 μL volumes contained 100 ng of genomic DNA, 2.5 μL of 10x 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 shown in Table 1. The PCR condition was 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 final extension at 72°C for 7 min.

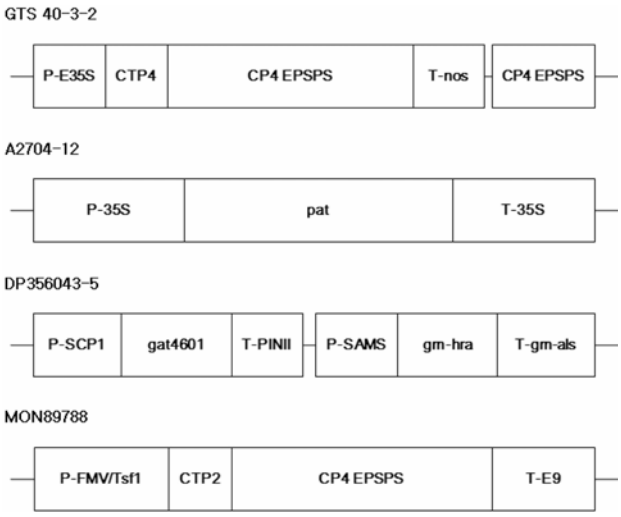

Fig. 1. Schematic diagram of 4 events of GM soybean. P-35S, 35S promoter; P-E35S, enhanced 35S promoter; P-SCP1, SCP1-constitutive synthetic core promoter; P-SAMS, *S*-adenosyl-L-methionine synthetase promoter; P-FMV/Tsf1, a chimeric promoter combining the enhancer sequences from the 35S promoter of the Figwort Mosaic Virus and the promoter from the *Tsf1* gene from *Arabidopsis thaliana*; CTP, chloroplast transit peptide; T-nos, nopaline synthase terminator; T-35S, 35S terminator; T-PINII, *Solanum tuberosum* proteinase inhibitor II terminator; T-gm-als, native soybean acetolactate synthase terminator; T-E9, *Pisum sativum* T-E9 terminator; CP4 EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; pat, phosphinothricin *N*-acetyltransferase; gat4601, glyphosate *N*-acetyltransferase; gm-hra, acetolactate synthase.

Table 1. Primer pairs for multiplex PCR

Primer name	Sequences (5'-3')	Targets	Amplicon size (bp)	Concentration (μM)
YjLec-F	GGG CTT GCC TTC TTT CTC GC	*lectin*	100	3
YjLec-R	CGACGA CTT GAT CAC CAG AC	*lectin*		
YjRRS-F	CTC GAT TTC GGC AAT GCC GC	CP4 EPSPS	127	4
YjRRS-R	CAG CAG AGA TCC CCA GGA AG	Genome		
YjA2704-F	GGG CGT TCG TAG TGA CTG AG	Genome	153	3
YjA2704-R	GCG TTA CCC AAC TTA ATC GC	T-DNA		
YjDP-5-F	GTT AGG TCG AAT AGG CTA GG	Genome	176	10
YjDP-5-R	TCC GAG GAG GTT TCC CGA TA	T-DNA		
YjMON5-F	TCC CGC TCT AGC GCT TCA AT	Genome	198	5
YjMON5-R	GGT TTG GAG ACT CTG TAC CC	P-FMV/Tsf1		

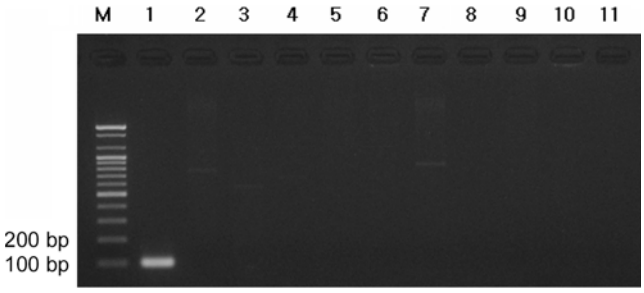

Fig. 2. Specificity analysis of the primer pair YjLec-F/R. Lane M, marker (100 bp DNA ladder); lane 1, soybean; lane 2, wheat; lane 3, barley; lane 4, rice; lane 5, cotton; lane 6, canola; lane 7, maize; lane 8, buckwheat; lane 9, radish; lane 10, red bean; lane 11, no template.

Agarose gel electrophoresis The PCR products were separated by gel electrophoresis and identified based on the length of the amplified DNA fragments. After PCR, 6 μL of each of product was loaded on a 4% agarose gel containing 0.5 μg/mL ethidium bromide. The 100 bp DNA ladder (Takara, Kyoto, Japan) was used as a size standard for amplified DNA fragments.

DNA sequencing PCR products of the non-GM soybean and 4 GM soybean were purified by QIAquick PCR purification kit (Qiagen). The pGEM-T easy vector (Promega) was used to clone the amplified DNA fragment and transformed into *Escherichia coli* strain DH5α. The selected *E. coli* containing the recombinant plasmid was grown and the plasmid was purified. DNA sequencing was performed twice using the ABI PRISM 3700 DNA analyzer (Perkin Elmer, Boston, MA, USA).

Results and Discussion

Specificity and sensitivity of the designed primer pairs The primer pair YjLec-F/R was used to amplify an endogenous soybean gene (*lectin*). For the specificity of the designed intrinsic primer pairs, we performed conventional PCR using 50 ng of genomic DNA from 10 different plant species. As shown in Fig. 2, no amplicon was observed in any of the species tested except soybean.

Specificity of the event-specific primer pairs was also confirmed using conventional PCR. Genomic DNAs extracted from the seeds of 4 varieties of GM soybean were used as the templates. As shown in Fig. 3, the expected amplicons were specifically amplified from each target GM soybean (GTS 40-3-2, A2704-12, DP356043-5, and MON89788). The YjRRS-F/R primer amplified the 3'-flanking region of RRS to a size of 127 bp, the YjA2704-F/R primer amplified the 5'-flanking region of A2704-12 to a size of 153 bp, the YjDP-5-F/R primer amplified the 5'-flanking region of DP356043-5 to a size of 176 bp, and the YjMON5-F/R primer amplified the 5'-flanking region of MON89788 to a size of 198 bp.

For GMO detection, it is important to know information about the gene cassette consisting of promoter, terminator, and a structural gene (encoding region) inserted in GMOs. This information was provided by Agriculture and Biotechnology Strategies Inc. (AGBIOS) (20). In the case of multiplex PCR, the design of primers is especially an important part, because primer specificity and melting temperature (Tm value) are more critical than conventional PCR. Although the specific primers amplify target DNA sequences, they do so with the differential amplification rates. In order to amplify of the specific PCR products with equal efficiency, the concentrations of individual primer pairs were optimized.

Figure 4 shows the result of a multiplex PCR under the optimized conditions. The multiplex PCR amplified 2 fragments corresponding to the chosen target sequences for each GM event and for *lectin* as an internal control in soybean. As shown in lane 6 of Fig. 4, the intensities of amplicon bands on agarose gel were almost 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 four varieties of GM soybean.

Limit of detection (LOD) of the multiplex PCR The

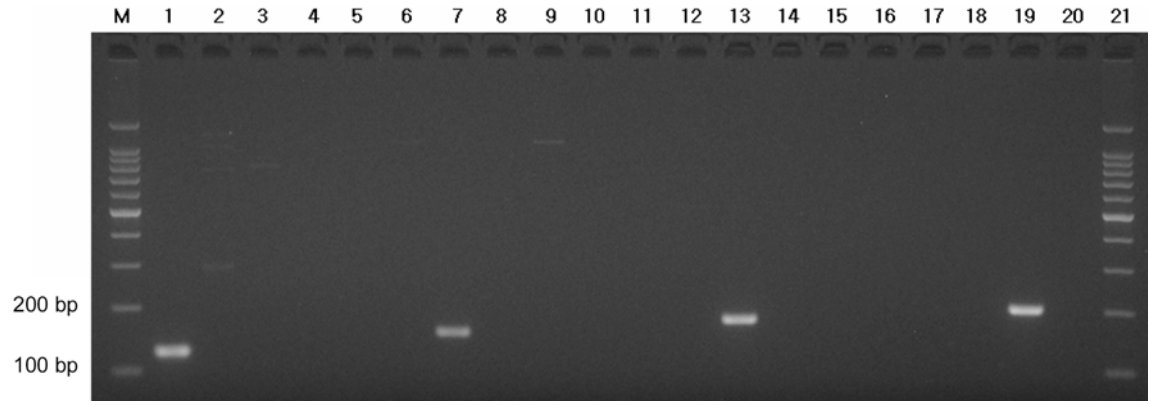

Fig. 3. Specificity of primer pairs designed for multiplex PCR. Lane M: (100 bp DNA ladder); lane 1, 6, 11, 15: GTS40-3-2; lane 2, 7, 12, 16: A2704-12; lane 3, 8, 13, 17: DP356043-5; lane 4, 9, 14, 18: MON89788; lane 5 ,10, 15, 20: no template; lane 1-5: PCR results using GTS40-3-2 event-specific primer pairs (127 bp); lane 6-10: PCR results using A2704-12 event-specific primer pairs (153 bp); lane 11-15: PCR results using DP356043-5 event-specific primer pairs (176 bp); lane 16-20: PCR results using MON89788 event-specific primer pairs (198 bp).

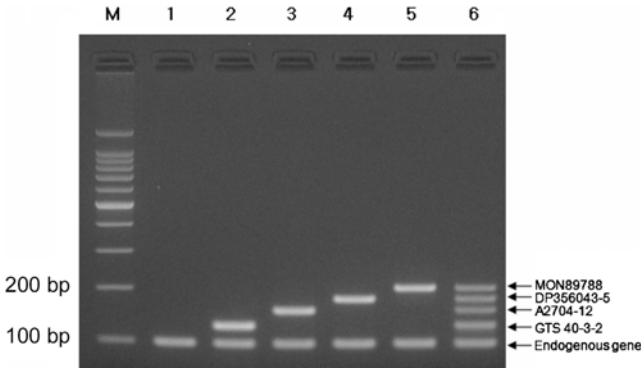

Fig. 4. Multiplex PCR products amplified from GM soybean containing the *SSIIb* gene (an endogenous gene). Lane M, marker (100 bp DNA ladder); lane 1, endogenous gene; lane 2, GTS40-3-2 and endogenous gene; lane 3, A2704-12 and endogenous gene; lane 4, DP356043-5 and endogenous gene; lane 5, MON89788 and endogenous gene; lane 6, GTS40-3-2, A2704-12, DP356043-5, MON89788, and endogenous gene.

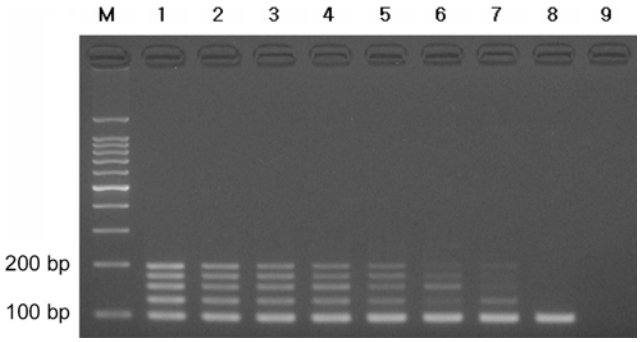

Fig. 6. Sensitivity analysis of the multiplex PCR. Lane M: marker (100 bp DNA Ladder); lane 1-8: 100, 10, 5, 3, 1, 0.5, 0.1, and 0.01% of a mixture of GM soybeans (RRS, A2704-12, DP356043-5 and MON89788); lane 9: no template.

reference materials containing 100, 10, 5, 3, 1, 0.5, 0.1, and 0.01% of the GM soybean mixture were prepared by mixing GM DNA from each variety of GM soybean (RRS, A2704-12, DP356043-5, and MON89788) with non-GM DNA from the control soybean 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 soybean 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.

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 soybean using multiplex PCR in this study.

Monitoring of GM soybean in processed foods using multiplex PCR To monitor GM soybean in markets, 20 soy food samples (2 *doenjang*, 2 *cheonggukjang*, 2 *tofu*, 1 sausage, 4 infant formula, 3 powdered soybean, 4 soymilk, and 2 snack) were purchased from Korean food markets and genomic DNAs extracted from them were used as templates in multiplex PCR assay.

As shown in Fig. 7A, the result of multiplex PCR indicates that 6 samples (*doenjang* B, infant formula A-D, and snack B) contain RRS. To observe false positive or negative results of multiplex PCR, single PCR was performed for RRS, A2704-12, DP356043-5, and MON89788 using designed primers. As a result, there were no differences in the multiplex PCR and single PCR results (Fig. 7). These

***Lectin* (Soybean endogenous gene)**

GGGCTTGCCTTCTTTCTCGCACCAATTGACACTAAGCCACAAACACATGCAGGTTATCTTGGTCTTTTCA
ACGAAAACGAGTCTGGTGATCAAGTCGTCG

GTS 40-3-2

CTCGATTTCGGCAATGCCGCCACGGGCTGCCGCCTGACCATGGGCCTCGTCGGGGTCTACGATTTCAA
GCGCATCATGCTGGGAAATTTTAGCGAGATTATAAGTATCTTCCTGGGGATCTCTGCTG

A2704-12

GGGCGTTCGTAGTGACTGAGGGGGTCAAAGACCAAGAAGTGAGTTATTTATCAGCCAAGCATTCTATTCT
TCTTATGTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATT
AAGTTGGGTAACGC

DP356043-5

GTTAGGTCGAATAGGCTAGGTTTACGAAAAAGAGACTAAGGCCGCTCTAGAGATCCGTCAACATGGTGG
AGCACGACACTCTCGTCTACTCCAAGAATATCAAAGATACAGTCTCAGAAGACCAAAGGGCTATTGAGAC
TTTTCAACAAAGGGTAATATCGGGAAACCTCCTCGGA

MON89788

TCCCGCTCTAGCGCTTCAATCGTGGTTATCAAGCTCCAAACACTGATAGTTTAAACTGAAGGCGGGAAAC
GACAATCTGATCCCCATCAAGCTCTAGCTAGAGCGGCCGCGTTATCAAGCTTCTGCAGGTCCTGCTCGA
GTGGAAGCTAATTCTCAGTCCAAAGCCTCAACAAGGTCAGGGTACAGAGTCTCCAAACC

Fig. 5. Sequencing results of the multiplex PCR products.

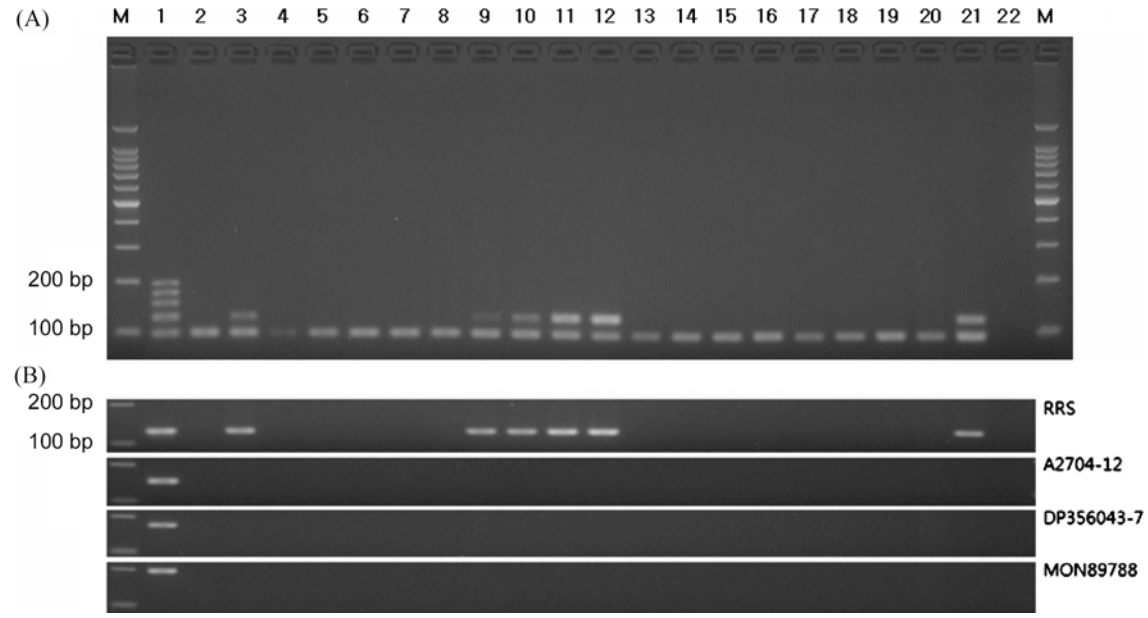

Fig. 7. Detection of GM soybean in processed foods using multiplex PCR. Lane M, marker (100 bp DNA ladder); lane 1, positive control; lane 2-3, *doenjang* A-B; lane 4-5, *cheonggukjang* A-B; lane 6-7, *tofu* A-B; lane 8, sausage A; lane 9-12, infant formula A-D; lane 13-15, powdered soybean A-C; lane 16-19, soymilk A-D; lane 20-21, snack A-B; lane 22, no template. (A) Multiplex PCR to soy processed foods. (B) Single PCR to soy processed foods.

results demonstrate that this multiplex PCR method is suitable for the detection of GM soybeans (RRS, A2704-12, DP356043-5, and MON89788) in processed foods.

The results of multiplex PCR method including non-approved GM soybean have not yet been reported. This method may be useful for identification and monitoring of four GM soybean events (RRS, A2704-12, DP356043-5, and MON89788).

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