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Development of a Multiplex Polymerase Chain Reaction Method for Simultaneous Detection of Eight Events of Genetically Modified Maize

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In this study, we developed a novel multiplex polymerase chain reaction (PCR) method for simultaneous detection of up to eight events of genetically modified (GM) maize within a single reaction. The eight detection primer pairs designed to be construct specific for eight respective GM events (i.e., Bt11, Event176, GA21, MON810, MON863, NK603, T25, and TC1507) and a primer pair for an endogenous reference gene, *sslIb*, were included in the nonaplex(9plex) PCR system, and its amplified products could be distinguished by agarose gel and capillary electrophoreses based on their different lengths. The optimal condition enabled us to reliably amplify two fragments corresponding to a construct specific sequence and a taxon specific *sslIb* in each of the eight events of GM maize and all of nine fragments in a simulated GM mixture containing as little as 0.25% (w/w) each of eight events of GM maize. These results indicate that this multiplex PCR method could be an effective qualitative detection method for screening GM maize.

KEYWORDS: Multiplex PCR; genetically modified (GM); maize; Bt11; Event176; GA21; MON810; MON863; NK603; T25; TC1507; *sslIb*

INTRODUCTION

Biotechnology has been used in modern farming and is advantageous to the related industries, since the initial commercialization of the genetically modified (GM) tomato in the United States in 1994. In fact, the global area of GM crops increased nearly 50-fold between 1996 and 2004 (from 1.7 million to 81.0 million hectares) (*1*) and is expected to continue to rise. GM crops have been authorized for food and/or feed by many countries based on their own criteria for safety assessment. However, consumers have been demanding appropriate information and labeling for foods derived from GM crops. Thus, labeling systems have been introduced for GM foods in the European Union, Korea, Japan, Australia, and other countries, and each of these systems is distinct. For example, the threshold levels for the unintentional presence of GM materials in non-GM crops have been defined as 0.9% in the European Union (*2*), 3% in Korea (*3*), and 5% in Japan (*4*).

For the monitoring of labeling systems, it has been necessary to develop methods for detecting genetically modified organisms (GMOs) in foods. Two approaches are available as follows: detecting the protein produced by the introduced trait gene through the detection of its specific antibody, such as by enzyme-linked immunosorbent assay (reviewed in *5*, *6*), and detecting a specific DNA sequence used for gene modification by the polymerase chain reaction (PCR) (reviewed in *6*−*9*). For detection of GM maize in foods and food materials, PCR-based detection methods that are able to detect even small amounts of transgenes in raw materials and processed foods have been routinely used (*10*−*19*).

On the other hand, the cost and duration of analyses could constitute one of the major bottlenecks for providing consumers with cost effective labeling and detection of the conventional or non-GM food supply chains. Thus, there is a need to develop time and cost effective detection methods to preserve the freedom of choice of consumers. We have been tackling the research and development of multiplex PCR methods as a solution. Multiplex PCR is a useful method that employs several

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primer pairs in the same amplification reaction for a sample DNA and can detect multiple target DNA sequences by simultaneous amplifications in one tube. Because the number of GM crops is continuously increasing, multiplex PCR would be one useful method for screening GMO content in raw materials and/or processed foods. Several methods using multiplex PCR for the detection of GM maize and/or soy have already been described (*20*−*22*). We also reported a multiplex PCR system for the simultaneous detection of five events of GM maize, i.e., Bt11, Event176, GA21, MON810, and T25, that were authorized for food and feed in Japan and the United States before the year 2000 (*10*, *23*). After publication of the report, another three events of GM maize (MON863, NK603, and TC1507) were authorized in Japan. In this study, we designed construct specific primers for these three GM maize events using recombinant DNA (r-DNA) information in the public domain. Additionally, we redesigned the primer pair for T25 involved in the previous report (*23*), because the target sequence amplified by the primer pair is contained not only in T25 maize but also in TC1507 maize. Then, we developed a method for simultaneously detecting all eight authorized GM maize events in the same tube by multiplex PCR.

MATERIALS AND METHODS

Maize (*Zea mays*) and Other Cereal Materials. Dry seeds of eight events of GM maize, i.e., one progeny each of Bt11 and Event176 developed by Syngenta Seeds AG (Basel, Switzerland), a progeny of TC1507 developed by Dow Agrosciences LLC (Indianapolis, IN), one progeny each of MON810, MON863, GA21, and NK603 developed by Monsanto Co. (St. Louis, MO), and a progeny of T25 developed by Bayer CropScience AG (Monheim am Rhein, Germany), were kindly provided by their developers. Dry seeds of QC9651 maize (Quality Traders, Inc., Huntley, IL) were also provided by the developer and used as a non-GM control. We used several other cereal materials as nontarget controls, such as soy, rice, wheat, and barley. Dry seeds of Roundup Ready (RR) soy (Monsanto Co.) were directly imported from the United States. Dry soybeans harvested in Ohio in 1998 were also directly imported and used as non-GM soy. Seeds of the rice (*Oryza sativa*) variety Kinuhikari, the wheat (*Triticum aestivum*) variety Haruyutaka, and the barley (*Hordeum vulgare*) variety Harrington were obtained in Japan.

Preparation of Test Samples and DNA Extraction. Dry seeds were ground with a P-14 speed rotor mill (Fritsch GmbH, Ibar-Oberstein, Germany). The ground materials were freeze-dried for 24 h in a FDU-540 freeze drier (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and stored at −20 °C until use. The seven levels of simulated GM mixture samples containing 0, 0.05, 0.1, 0.25, 0.5, 1, and 5% (w/w) of each of eight events of GM maize in non-GM maize used for experiments were made from the ground materials. DNA extractions were performed using the DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany) as described in our previous reports (*18*). The DNA concentration of solutions was determined by measuring UV absorbance at 260 nm, and the quality was evaluated by the absorbance ratios at 260/280 and 260/230 nm; in the majority of maize varieties studied, the absorption ratio at 260/230 nm was >1.7 and that at 260/280 was between 1.7 and 2.0. These DNA samples were used for the subsequent experiment.

Oligonucleotide Primers. To design primers, we chose the construct specific regions from the artificial junction contained in r-DNAs and found the most suitable oligonucleotide sequences in these regions using Primer Express software (Applied Biosystems, Foster City, CA). The oligonucleotides were checked using a public database (i.e., DDBJ), and it was confirmed that they did not match unintended DNA. The primer pairs developed herein for use in the sequence analyses of transgenes and multiplex PCR are listed in **Tables 1** and **2**, respectively. These primers were synthesized by Fasmac Co., Ltd. (Kanagawa, Japan) and purified by high-performance liquid chromatography. Each oligonucleotide was diluted to the appropriate concentration to conduct multiplex PCR with the appropriate volume of sterilized water.

DNA Sequencing. The structural information of r-DNA integrated in GM maize was available in safety assessment documents published by the Ministry of Health, Labor and Welfare (MHLW) and the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan. In this study, we analyzed DNA sequence involved in artificial junctions between DNA segments (e.g., the promoter and structural genes) and verified the r-DNA structures (data not shown). The regions sequenced in this study are indicated in **Figure 1**. These regions were amplified under the following conditions: The reaction volume of 25 μL contained 25 ng of genomic DNA, 0.2 μmol/L of each of the primers listed in **Table 1**, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, and 0.625 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). Reactions were buffered by the addition of the PCR buffer II. Amplification was performed in a thermal cycler according to the following PCR step-cycle program: preincubation at 94 °C for 10 min, 40 cycles consisting of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. The amplified products were directly sequenced by Fasmac Co., Ltd.

Simplex PCR Conditions. The reaction volume of 25 μL contained 25 ng of genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 0.2 μmol/L of the 5′- and 3′-primers, and 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). The reactions were buffered with the PCR buffer II (Applied Biosystems) and amplified in a thermal cycler, the Silver 96 well GeneAmp PCR System 9700 (Applied Biosystems) in max mode, according to the following step-cycle program: preincubation at 95 °C for 10 min; 10 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 63 °C for 1 min, and extension at 72 °C for 1 min; 27 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 7 min.

Multiplex PCR Conditions. Two different multiplex PCR reaction mixes were used in this study. For the tetraplex (4-plex) PCR that could detect four events of GM maize, i.e., MON836, NK603, T25, and TC1507, the reaction volume of 25 μL contained 25 ng of genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 0.2 μmol/L each of the M810 1−5′, NK603 1−3′, T25 2−5′, T25 2−3′, M863 1−5′, M863 1−3′, TC1507 1−5′, and TC1507 1−3′ primers. For the nonaplex (9-plex) PCR that could detect eight events of GM maize and a maize endogenous reference gene (*ssIIb*) in one tube, the reaction volume of 25 μL contained 25 ng of genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 1.25 units of AmpliTaq Gold DNA polymerase, and 15 primers at the following concentrations: 0.2 μmol/L for M810 1−5′, NK603 1−3′, M863 1−5′, M863 1−3′, Bt11 1−5′, and cryIA 1−3′; 0.1 μmol/L for T25 2−5′, T25 2−3′, GA21 1−5′, GA21 1−3′, TC1507 1−5′, and TC1507 1−3′; 0.05 μmol/L for Event176 1−5′; and 0.045 μmol/L for SSIIb 1−5′ and SSIIb 1−3′. On both of the multiplex PCRs, the reactions were amplified in a Silver 96 well GeneAmp 9700 (Applied Biosystems) in max mode, according to the following step-cycle program: preincubation at 95 °C for 10 min; 10 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min; 27 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 7 min.

Analysis of PCR Products. The PCR products were analyzed by agarose gel electrophoresis and/or capillary electrophoresis. The agarose gel electrophoresis was performed in a 3% (w/v) LO3 agarose (Takara Bio Inc., Shiga, Japan) gel with 0.5 μg/mL ethidium bromide (Sigma-Aldrich, St. Louis, MO). A 5 μL aliquot of each PCR product was electrophoresed at a constant voltage (100 V) for 30 min in 1 × TAE buffer 40 mmol/L Tris-HCl, 40 mmol/L acetic acid, and 1 mmol/L EDTA (pH 8.0) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After the electrophoresis was finished, the gel was scanned by a Molecular Imager FX system (Bio-Rad Laboratories Inc., Hercules, CA). The capillary electrophoresis analysis was performed using an SV1210 microchip electrophoresis analytical instrument with an IC-4100 short DNA fragment analysis reagents kit (Hitachi High-Technologies, Tokyo, Japan). One microliter aliquots of 10-fold dilutions of PCR products were electrophoresed with the internal control fragments (10 and 500 bp) in a water soluble cellulose derivative at a

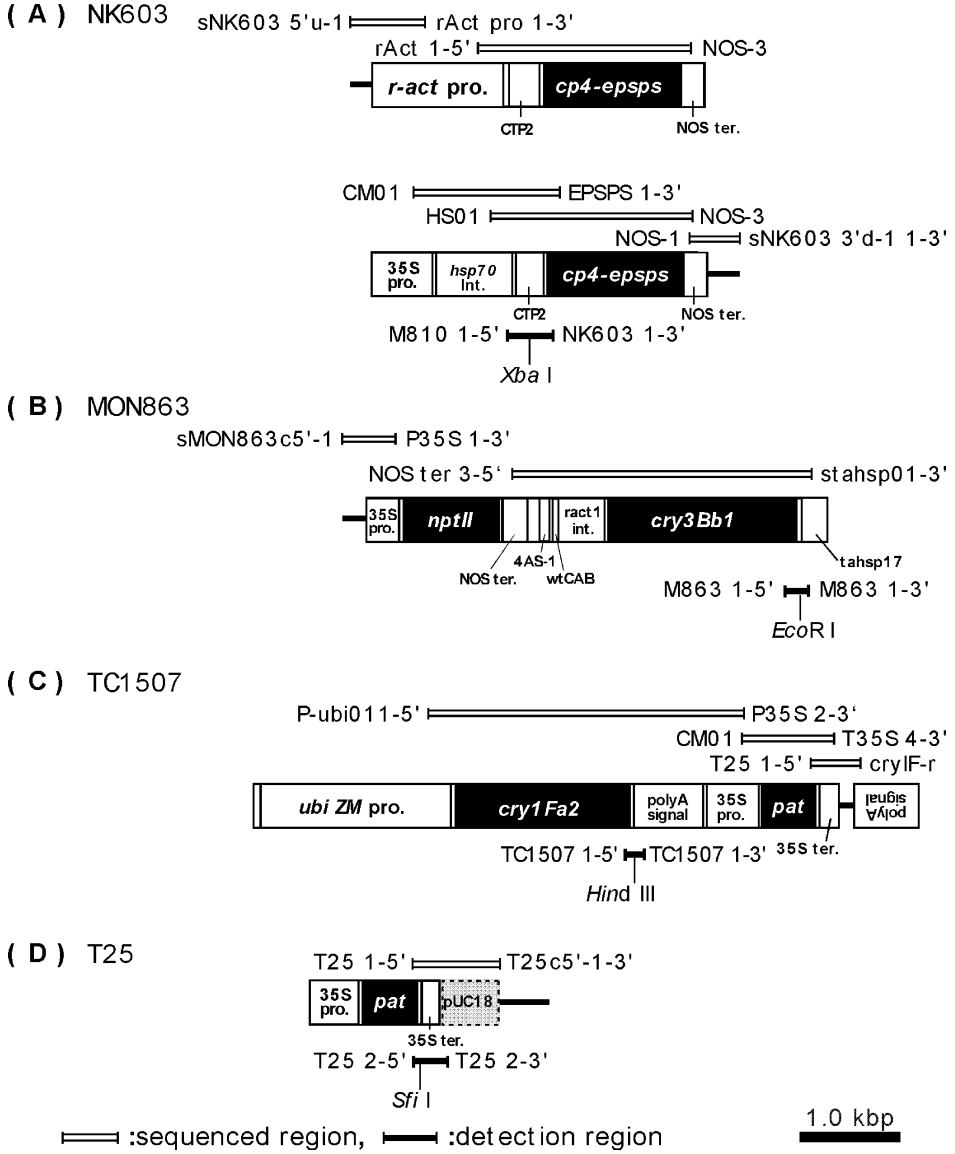

Figure 1. Schematic diagrams of the designed primers in relation to the r-DNAs. The structures of the r-DNAs transformed into NK603, MON863, TC1507, and T25 are shown as boxes in **A**–**D**, respectively. The structural trait genes are shown in filled boxes, and the other elements, including the promoters and terminators, are shown in open boxes. The gray boxes outlined with a dashed line indicate the 610 bp region of the pUC18 vector segment contained in T25 r-DNA. The broad lines attached to the boxes indicate the sequenced border sequences of DNA fragments introduced to each GM event. The amplified regions for sequencing are indicated as double lines with the primers used for the amplification shown at the end. The detection regions of the multiplex PCR method are indicated as broad lines with the primers used for the amplification shown at the end. The restriction enzymes that were used in the restriction analyses are indicated on their digestion sites.

constant voltage (350 V) for 4.5 min and analyzed by the software included in the system.

RESULTS AND DISCUSSION

DNA Sequencing and Primer Design. To design primer pairs suitable for the multiplex PCR, we first examined structures of the r-DNA integrated in three target events of GM maize (i.e., the three newly authorized events: NK603, MON863, and TC1507). Structural information about the r-DNAs of GM maize events authorized in Japan was taken from public domain sources as described in the Materials and Methods section. We were also able to obtain sequence information on each of the DNA segments introduced to GM maize events from the DNA Data Bank of Japan (DDBJ). Then, we analyzed the DNA sequence involved in the artificial junctions between DNA segments (e.g., the promoter and structural gene) and verified the r-DNA structures. The 10 amplified DNA segments used for the sequence analysis in this report are shown in **Figure 1A**−**C**, and the primers are shown in **Table 1**. The sequencing results showed that the r-DNA structures agreed with the information from the public domain, and the sequences of DNA segments involved in the sequenced regions matched the information from DDBJ. The sequencing results are not provided due to a nondisclosure agreement with developers.

On the basis of the sequencing results, we chose the construct specific regions from the artificial junction(s) contained in r-DNAs and found the most suitable oligonucleotide sequences in these regions using a software package (**Table 2**). The oligonucleotides were checked using the public database (i.e., DDBJ), and it was confirmed that they did not match unintended DNA. To ensure the detection specificities, one primer from each pair was designed to anneal to a trait gene of its target GM maize, and the other was designed to anneal to another element that places an artificial junction(s) and adjoins the trait

Table 1. List of Sequencing Primers

targeted GM	name	sequence	specificity	amplicon (bp)	ref
NK603	sNK603 5′u-1	5′-GGAGTCAAGAAGGGGAGGAGG-3′	NK603 plant genome/sense	742	*a*
	rAct pro 1-3′	5′-GGGCTTGCTATGGATCGTG-3′	r-act pro./antisense		*11*
	rAct 1-5′	5′-ATCTTTGGCCTTGGTAGTTTG-3′	*r-act* pro./sense	2163	*23*
	NOS-3	5′-TTATCCTAGTTTGCGCGCTA-3′	NOS ter./antisense		*30*
	CM01	5′-CTCTAGAAATGCCATCATTGCGATA-3′	35S pro./sense	1465	*10*
	EPSPS 1-3′	5′-ATCCTGGCGCCCATGGCCTGCATG-3′	*cp-4 epsps*/antisense		*31*
	HS01	5′-AGTTTCCTTTTTGTTGCTCTCCT-3′	*hsp 70* int./sense	1943	*10*
	NOS-3	5′-TTATCCTAGTTTGCGCGCTA-3′	NOS ter./antisense		*30*
	NOS-1	5′-GAATCCTGTTGCCGGTCTTG-3′	NOS ter./sense	466	*11*
	sNK603 3′d-1	5′-AGAGTGGAAGTGTGTCGCGTG-3′	NK603 plant genome/antisense		*a*
T25	T25 1-5′	5′-GCCAGTTAGGCCAGTTACCAA-3′	*pat*/sense	933	*23*
	T25c5′-1-3′	5′-CTGGCGCCGTCGACTC-3′	T25 plant genome/antisense		*b*
MON863	NOS ter 3-5′	5′-GTCTTGCGATGATTATCATATAATTTCTG-3′	NOS ter./sense	3062	*11*
	atahsp01-3′	5′-TGCACTCAAAATCAGAACAATTTATT-3′	tahsp17/antisense		X13431
	sMON863c5′-1-5′	5′-CCAAGAGCGCTTATGTCATATGG-3′	MON863 plant genome/sense	504	*a*
	P35S 1-3′	5′-CCTCTCCAAATGAAATGAACTTCCT-3′	35S pro./antisense		*23*
TC1507	P-ubi011-5′	5′-CTGCCTTCATACGCTATTTATTTGC-3′	*ubi* pro./sense	3193	S94464, U29159,
	P35S 2-3′	5′-CCTCTCCAAATGAAATGAACTTCCT-3′	35S pro./antisense		US6054574, *32*, *33*
					11
	CM01	5′-CACTACAAATGCCATCATTGCGATA-3′	35S pro./sense	961	*10*
	T35S 4-3′	5′-TTTTAGTACTGGATTTTGGTTTTAGGAATTAG-3′	35S ter./antisense		*11*
	T25 1-5′	5′-GCCAGTTAGGCCAGTTACCCA-3′	*pat*/sense	458	*23*
	Cry1F-r	5′-CGAATAGAAAATACTGCACTGCAA-3′	3′-polyA/antisense		*c*

[a] Design of these primers was based on the information provided from Monsanto with a nondisclosure agreement. [b] Design of this primer was based on the information provided from Bayer CropScience with a nondisclosure agreement. [c] Design of this primer was based on the information provided from Dow AgroScience with a nondisclosure agreement.

Table 2. List of Primers for the Multiplex PCRs

targeted GM	name	sequence	specificity	amplicon (bp)	restriction enzyme (digestion products)[a]	ref
NK603	M810 1–5′	5′-GAGTTTCCTTTTTGTTGCTCTC 3′	*hsp70* int./sense	444	*Xba*I	*23*
	NK603 1–3′	5′-GCTGCTTGCACCGTGAAG-3′	*cp-4 epsps*/antisense		(174 bp/270 bp)	
Event176	E176 1–5′	5′-GTAGCAGACACCCCTCTCCACA-3′	*PEPC* pro./sense	343	*Bst*EII	*23*
	cryIA 1–3′	5′-TCGTTGATGTTKGGGTTGTTGTTC-3′	*cryIA(b)*/antisense		(91 bp/252 bp)	*23*
T25	T25 2–5′	5′-GGCATGATGTTGGTTTTTGGCAAAG-3′	*pat*/sense	311	*Sfi*I	
	T25 2–3′	5′-AATTCGAGCTCGGTACCCCT-3′	pUC18/antisense		(59 bp/252 bp)	
GA21	GA21 1–5′	5′-ACGGTGGAAGAGTTCAATGTATG-3′	OTP/sense	270	*Sfi*I	*23*
	GA21 1–3′	5′-TCTCCTTGATGGGCTGCA-3′	*m-epsps*/antisense		(114 bp/156 bp)	*23*
MON863	M863 1–5′	5′-GATGACCTGACCTACCAGA-3′	*cry3Bb1*/sense	234	*Eco*RI	
	M863 1–3′	5′-GCACACACATCAACCAAATT-3′	tahsp17/antisense		(171 bp/63 bp)	
MON810	M810 1–5′	5′-GAGTTTCCTTTTTGTTGCTCTC-3′	*hsp70* int./sense	199	*Bsa*AI	*23*
	cryIA 1–3′	5′-TCGTTGATGTTKGGGTTGTTGTTC-3′	*cryIA(b)*/antisense		(83 bp/116 bp)	*23*
ssIIb	SSIIb 1–5′	5′-CTCCCAATCCTTTGACATCTGC-3′	*ssIIb*/sense	151		*12*
	SSIIb 1–3′	5′-TCGATTTCTCTCTTGGTGACAGG-3′	*ssIIb*/antisense			*12*
TC1507	TC1507 1–5′	5′-TTGACAGGTTTGAGTTGATTCCAG-3′	*cryIFa2*/sense	131	*Hin*dIII	
	TC1507 1-3′	5′-CCAAGAACTCATGTTAGTCGCAA-3′	3′-polyA/antisense		(65 bp/66 bp)	
Bt11	Bt11 1–5′	5′-CCATTTTTCAGCTAGGAAGTTC-3′	*adh1*-1S IVS6/sense	110	*Xba*I	*23*
	cryIA 1–3′	5′-TCGTTGATGTTKGGGTTGTTGTTC-3′	*cryIA(b)*/antisense		(72 bp/38 bp)	*23*

[a] The lengths of PCR products after restriction digestions shown in **Figure 4B**.

gene. Each primer pair was also designed to distinguish the length of the amplified product from the length of other amplified products and from the four events of GM maize utilized in the previous report (*23*), i.e., MON810, Event176, Bt11, and GA21. The differences in PCR product lengths are shown in **Table 2**. For detection of NK603, we designed a new 3′-primer, NK603 1−3′, on the *cp4-epsps* gene. The MON810 1−5′ primer, which had been previously designed on the intron sequence of the *Arabidopsis thaliana hsp70* gene for the detection of MON810 (*23*), was used as a 5′-primer, as shown in **Figure 1A** and **Table 2**. For detection of MON863, 5′- and 3′-primers were designed on the *cry3Bb1* gene and the terminator of the *Triticum aestivum hsp17* gene, respectively (**Figure 1B** and **Table 2**). For the detection of TC1507, 5′- and 3′-primers were designed on the *cry3Fa2* gene and a polyadenylation signal sequence from *Agrobacterium tumefaciens ORF25*, respectively (**Figure 1C** and **Table 2**).

In addition, we needed to design a new T25 construct specific primer pair that does not cross-react with TC1507, because our previously reported target sequence for T25 construct specific detection (*23*) was also found in the construct of TC1507, as shown in **Figure 1C**,**D**. It was also reported that the r-DNA introduced to T25 included two DNA fragments matching with the pUC18 vector in T25 r-DNA (*19*). We therefore focused on the analysis of DNA sequences in the 3′-flanking regions of the cauliflower mosaic virus (CaMV) 35S terminator, as shown in **Figure 1D**, to detect the construct specific sequence of T25. As a result of the analyses of the downstream regions of *pat*

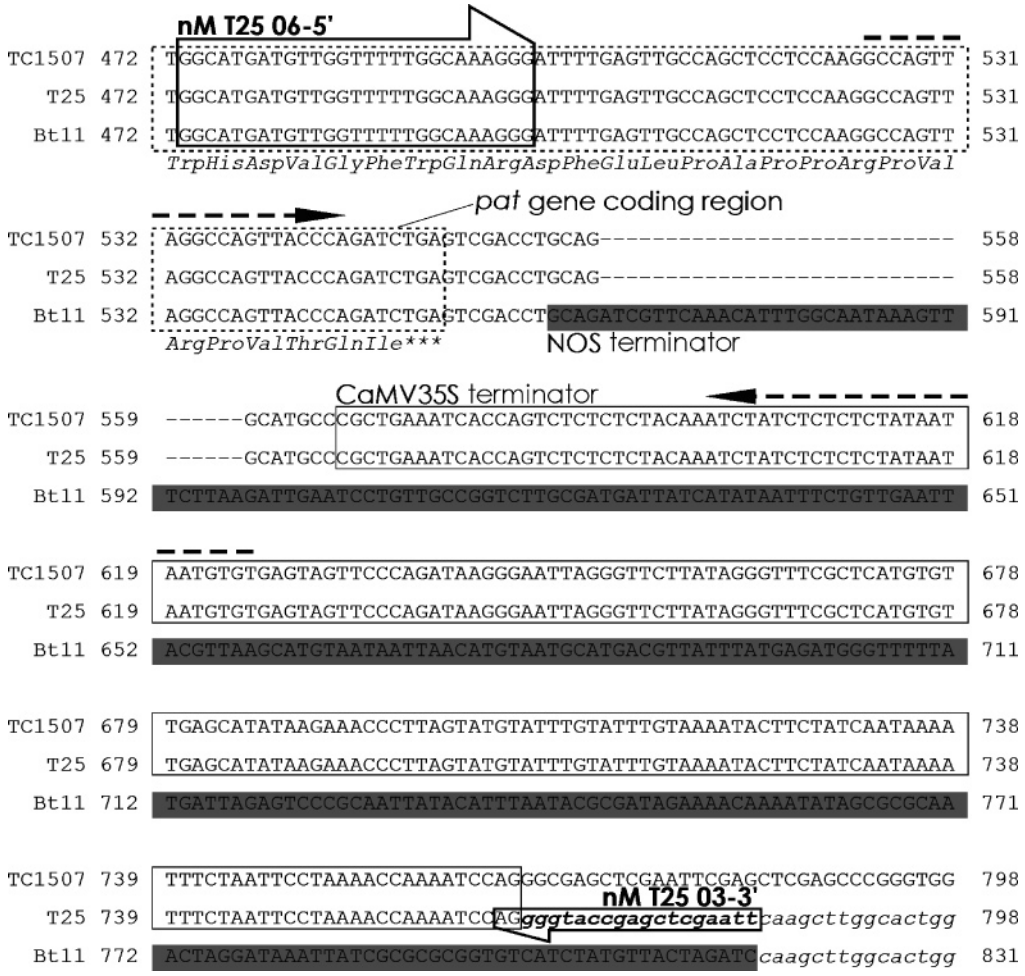

Figure 2. r-DNA sequence comparison of T25, TC1507, and Bt11. All of these shared the same *pat* gene (indicated by dashed boxes). In the region downstream of the *pat* gene, TC1507 and T25 have a CaMV35S terminator (open boxes), and Bt11 has a NOS terminator (gray boxes). The italicized lower case letters indicate the pUC18 vector segments contained in T25 and Bt11, and the 16 bp specific to T25 is shown in bold italics. The new detection primers designed in this study are indicated by boxed arrows. The dashed arrows on the DNA sequences indicate the detection primers previously reported (*15*). The numbers at the end of each line show the positions from the initiation codon of the *pat* gene.

genes in T25, TC1507, and Bt11, we found a unique sequence of 16 bp at the junction region between the CaMV 35S terminator and the pUC18 vector in T25 (**Figure 2**). A new 3′-primer including the 16 bp sequence (T25 2−3′) and a new 5′-primer (T25 2−5′) on the *pat* gene were designed for the detection of T25 (**Figure 2** and **Table 2**).

Because the amplification products for NK603, T25, MON863, and TC1507 have only one digestion site each, *Xba*I, *Sfi*I, *Eco*RI, and *Hin*dIII, respectively (**Figure 1**), it was possible to confirm that each amplification product definitely corresponded to its specific target by means of restricted enzyme digestion (**Table 2**). The restriction enzymes used to confirm the amplification products of the previous multiplex PCR method have already been described (*23*); however, we thought that performing the restriction analyses as an additional confirmation could improve the reliability of this method.

The specificities of the designed primer pairs were individually assessed by simplex PCR tests. The DNAs extracted from the seeds of each of eight events of GM maize and from the other cereal crops were used as the template genomic DNAs. As shown in **Figure 3**, all simplex PCR systems specifically amplified products of the expected lengths from the DNA extracted from each target GM maize sample. In particular, the new T25 primer pair could specifically detect T25 DNA and no amplification products from TC1507 (**Figure 3B**). In contrast, each of these primer pairs amplified no product from DNAs extracted not only from other events of GM maize and non-GM maize but also from other crops, i.e., soy, rice, wheat, and barley. On the other hand, we also performed simplex PCR tests using the four primer pairs to detect Bt11, Event176, GA21, and MON810, which were used in the previous multiplex PCR method (*23*) to reconfirm the specificities of previous PCR systems. The results showed that these primer pairs amplified no products from DNAs extracted from NK603, T25, MON863, and TC1507 (data not shown). Therefore, we concluded that all eight of these primer pairs were specific to their respective target events in maize samples. The specificity for other GM crop events, such as cotton, sugar beet, and rapeseed, should be tested in the future, although the major raw materials for foods are soy and corn.

Tetraplex PCR Method for Detection of Newly Authorized GM Maize. We examined the specificity of these primer pairs under the multiplex PCR conditions. Tetraplex PCR for detection of four events of GM maize, i.e., NK603, MON863, TC1507, and T25, was performed using a reaction mixture including four primer pairs at a concentration of 0.2 μmol/L each. The other experimental conditions were the same as described in the previous report (*23*). The DNAs extracted from eight events of pure GM maize, non-GM maize, soy, rice, wheat, and barley were used as template DNAs. In addition, the DNAs

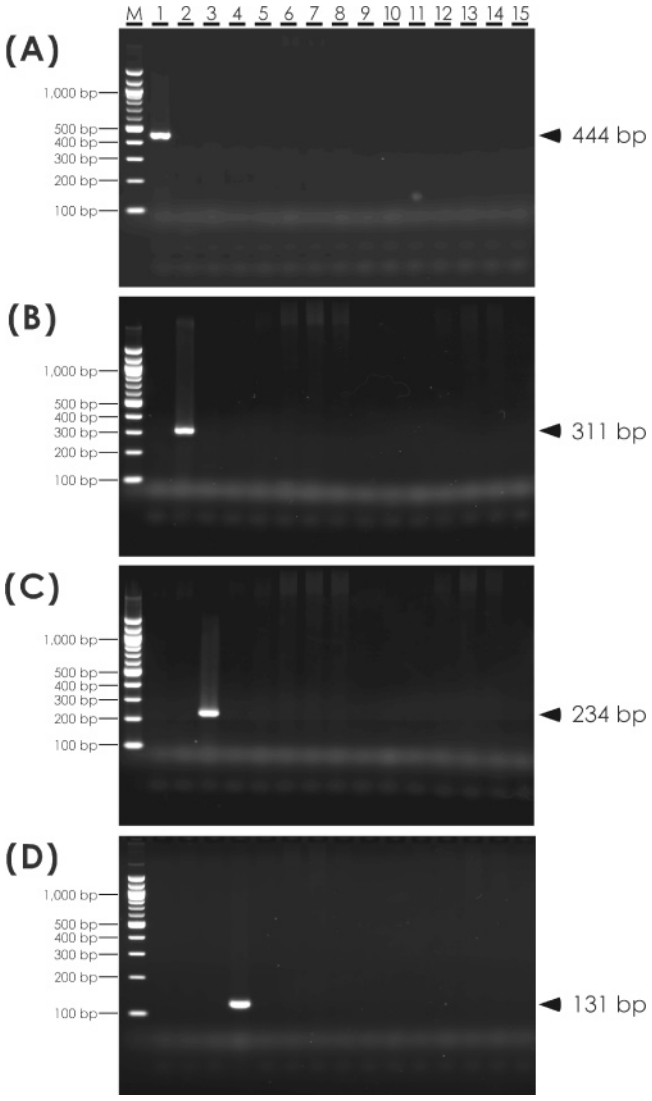

Figure 3. Specificities of detection primer pairs designed for the multiplex PCR. Simplex PCR products amplified with the NK603 primer pair (**A**), T25 primer pair (**B**), MON863 primer pair (**C**), and TC1507 primer pair (**D**) were electrophoresed on 3% agarose gel. Lanes 1–9, amplification of maize DNAs extracted from the seeds of each representative variety of maize of NK603, T25, MON863, TC1507, non-GM variety, Event176, GA21, MON810, and Bt11 maize, respectively; lanes 10–14, amplification of DNAs from non-GM soy, RR soy, rice, wheat, and barley, respectively; lane 15, negative control (no DNA); and M, 100 bp ladder size standard.

extracted from the simulated GM mixture that included equal weights of each of eight events of GM maize [equivalent 1% (w/w) of each of eight events of GM maize] in non-GM maize were used as template DNAs in these specificity tests. As shown in **Figure 4A**, tetraplex PCR amplified only a fragment that corresponded to the expected DNA length in each of the four target events and clearly amplified all four of the PCR products in the simulated GM mixture. Furthermore, the restriction analyses of these amplified products clearly showed that the products corresponded to the target sequences of NK603, T25, MON863, and TC1507 (**Figure 4B**). In contrast, no amplification was observed with the DNA extracted from the nontarget GM maize, non-GM maize, soy, rice, wheat, and barley. These results indicated that these four primer pairs were compatible in the multiplex PCR system.

To evaluate the sensitivity of this tetraplex PCR method, we prepared seven levels of simulated GM mixture samples containing 0, 0.05, 0.1, 0.25, 0.5, 1, and 5% (w/w) of each of eight events of grained GM maize in grained non-GM maize. We made 2−4 independent mixtures for each concentration, and the DNA was extracted from all samples in duplicate and was used to analyze in duplicate ($n = 8-16$). All of the four target specific amplified products were reliably detected with DNAs extracted from the simulated GM mixture containing over 0.1% of each GM event (data not shown). These results show that this tetraplex PCR system could distinguish each of four events of GM maize newly designed from other events of GM maize, soy, rice, wheat, and barley. We have already reported a hexaplex (6-plex) PCR system that could detect and distinguish five events of GM maize (i.e., Bt11, Event 176, GA21, MON810, and T25) and an endogenous reference gene (*zein*) (*23*). In this study, we confirmed that the removal of a primer pair for the detection of T25 from the hexaplex PCR system had no effect on the detection specificities of the other four events and an endogenous reference gene (data not shown). We therefore conclude that it is possible to detect and distinguish these eight events of GM maize by using either of these two multiplex PCR reactions.

Evaluation of the Nonaplex PCR Method for Detecting Eight Events of GM Maize. As described above, the four primer pairs designed in this study showed high detection specificities under the multiplex PCR conditions. Because the amplification products from these two multiplex systems were designed to have mutually different lengths, we could apply these primer pairs to multiplex PCR. In addition, we also changed the control DNA sequence for a taxon specific gene to a *starch synthase IIb* (*ssIIb*) gene from *zein*, which we previously used, because the *zein* gene belongs to a multigene family and shows polymorphism in the maize variety (*24*). The *ssIIb* is a single copy gene in the maize genome (*25*). The taxon specific sequence of the *ssIIb* gene has already been applied in our quantitative PCR method (*12*, *13*), which was adopted as a standard (the official method) in Japan and Korea (*26*−*29*). As the endogenous control primers for the new nonaplex PCR, we used the primers SSIIb 1−5′ and SSIIb 1−3′, which amplify the 150 bp fragment (*12*). Moreover, the copy numbers of taxon specific sequences (*ssIIb*) per genome have also been assessed for 20 representative varieties of maize by the comparison of Ct values in our quantitative PCR system (data not shown). Thus, we attempted to develop a nonaplex PCR system to simultaneously detect all eight events of GM maize and the *ssIIb* gene by multiplex PCR in the same tube.

In attempting to develop the nonaplex PCR, we first performed reactions under the same conditions as for the tetraplex PCR, including the concentration of primers (0.2 μm/L each), but we were not able to simultaneously observe all nine of the amplified bands from the simulated GM mixture containing 1% each of eight GM maize events. We therefore attempted to determine the optimal conditions for the nonaplex PCR, e.g., the concentrations of each primer, thermal cycle program, and concentration of $MgCl_2$. The optimal concentrations of eight GM events and the taxon specific primer pairs containing the primer mixture were examined using DNA extracted from the simulated GM mixture containing 1% of each GM event and were modulated to obtain PCR products having electrophoretic bands with intensities as nearly equivalent as possible. The thermal cycling program was optimized for the Silver 96 well GeneAmp 9700. We examined several cycling programs with different annealing temperatures (i.e., ranging from 60 to 65 °C) and different numbers of cycles. A two-step annealing−extension process was used, in which the first 10

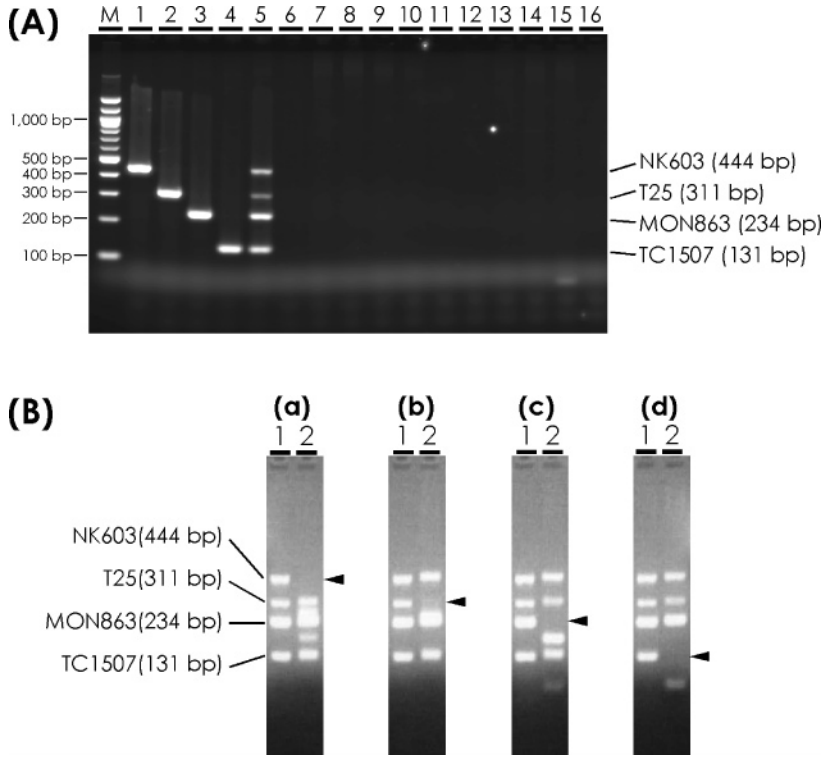

Figure 4. (**A**) Specificities of the multiplex PCR for detection of four events of GM maize: NK603, T25, MON863, and TC1507. The specificities were investigated by multiplex PCR conditions including the four primer pairs of NK603, T25, MON863, and TC1507 for the template DNAs extracted from the seeds of each of the GM events and non-GM maize and other cereal materials. Lanes 1–10, amplification of maize DNAs from NK603, T25, MON863, TC1507, non-GM maize, Event176, GA21, MON810, and Bt11, respectively; lanes 11–15, non-GM soy, RR soy, rice, wheat, and barley, respectively; lane 16, negative control (no DNA); and M, 100 bp ladder size standard. PCR products were electrophoresed on 3% agarose gel. (**B**) Restriction analyses of the amplified products of the multiplex PCR. The products amplified from the simulated GM mixture containing 1% of each of eight events by the multiplex PCR for the detection of four events of GM maize were digested with *Xba*I (a), *Sfi*I (b), *Eco*RI (c), and *Hin*dIII (d). The electrophoregrams of nondigested products and digested products are shown in lanes 1 and 2, respectively, and digested bands are indicated by arrowheads. The lengths of digestions were roughly correspondent to the expected lengths described in **Table 2**.

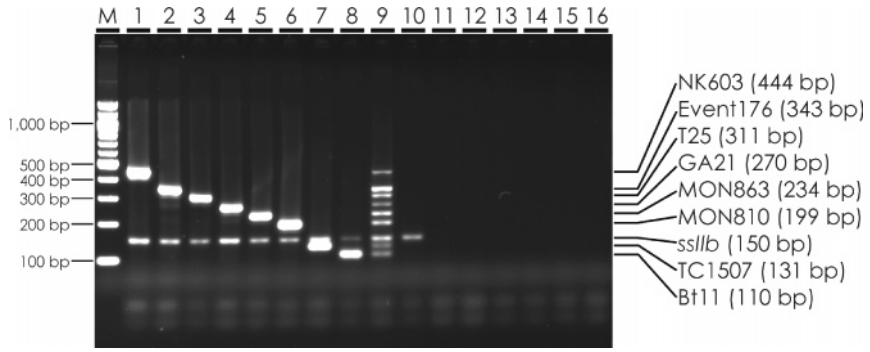

Figure 5. Specificities of the one tube multiplex PCR for eight events of GM maize. Lanes 1–8, amplification of DNAs extracted from the seeds of each event of GM maize, NK603, T25, MON863, TC1507, Event176, GA21, MON810, and Bt11, respectively; lane 9, the simulated GM mixture containing 1% of each event of eight GM maize; lane 10, non-GM maize; lanes 11–15, non-GM soy, RR soy, rice, wheat, and barley, respectively; lane 16, negative control (no DNA); and M, 100 bp ladder size standard. PCR products were electrophoresed on 3% agarose gel.

cycles contained an annealing step with a higher temperature than the subsequent cycles in order to reduce nonspecific primer annealing, and the following 27 cycles were performed to amplify specific products effectively. The other experimental conditions, including the concentration of $MgCl_2$, were the same as described above. Although we examined the use of a higher concentration of $MgCl_2$ in the reaction solution, we found that higher $MgCl_2$ enhanced the amplification of some targets but increased the number of nonspecific amplifications from other crops.

Figure 5 shows a typical result of the multiplex PCR performed under the optimized conditions. The multiplex PCR amplified two fragments corresponding to a construct specific sequence and a taxon specific *ssIIb* in each of the eight events of GM maize and amplified only an *ssIIb* fragment in the non-GM maize (**Figure 5**). Because it was expected that amplifications of *ssIIb* with low concentrations of primers (0.045 mmol/L each) were as sensitive as the GM specific amplifications included in the multiplex PCR to the absence of PCR inhibitors in sample solutions, the amplification of *ssIIb* bands could be used as an internal control to distinguish true negative results from PCR inhibition or failure when the samples included maize genomic DNA. This multiplex PCR causes no amplified products from soy, rice, wheat, and barley. These results

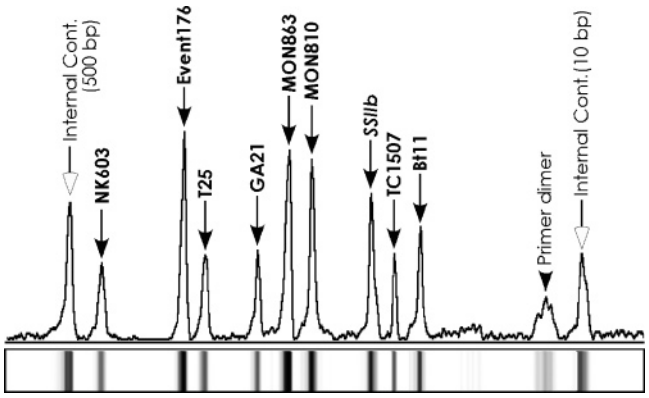

Figure 6. Capillary electrophoresis analysis of the one tube multiplex PCR products. A product amplified from the simulated GM mixture containing 1% of each of eight events was analyzed. The peaks of the amplifications specific to GM maize and the taxon marker are indicated by dark arrows. The lengths of the peaks were estimated by the comparisons of mobility with the internal controls contained in the reagents kit (indicated by the white arrows), and the sizes of the specific peaks corresponded with the expected length. The profile shown under the chromatogram is the simulated gel image generated from the results of the capillary electrophoresis analysis by the software included in the SV1210 microchip system.

indicated that this method is sufficient to distinguish the eight events of GM maize. Furthermore, from the simulated GM mixture containing 1% of each GM event, this multiplex PCR could simultaneously amplify nine different lengths of fragments corresponding to the taxon specific *ssIIb* marker and the eight events of GM maize, respectively, as shown in lane 9 of **Figure 5**, although the intensities of the bands derived from the nine amplified products were not equivalent.

We analyzed the amplified products by electrophoresis on 3% agarose gels and could distinguish all nine products from the one-tube multiplex PCR. Agarose gel electrophoresis is suitable for routine analyses, because it can be performed simply and cheaply. However, we also examined the capillary electrophoresis analysis using an SV1210 microchip electrophoresis analytical system, which electrophoreses PCR products in a capillary filled with a water soluble cellulose derivative. **Figure 6** shows a typical result of the capillary electrophoresis analysis of the amplified products from DNA extracted from the simulated GM mixture containing 1% of each of eight events of GM maize. The capillary electrophoresis could separate fragments more clearly and more rapidly than the agarose gel electrophoresis. The high separation ability of the capillary electrophoresis will enable separation of more amplified products as the number of GM events that the multiplex PCR method can detect continues to grow.

Sensitivity of the Nonaplex PCR Method. The sensitivity of the multiplex PCR system was evaluated by applying simulated GM mixtures described above to agarose gel electrophoresis. We performed a one-tube multiplex PCR with DNAs extracted from the simulated GM mixtures and were able to reliably observe all of the nine amplified bands simultaneously from that the stimulated GM mixtures containing more than 0.25% of each of eight events of GM maize (**Figure 7**). These amplifications, however, could detect the DNAs extracted from the simulated GM mixtures that contained either 0.1 or 0.05% of each of the GM events, but there were few instances in which all of the bands were simultaneously detected, e.g., four out of ten trials for the stimulated GM mixtures containing 0.1% of each of the GM events and one out of eight trials for the

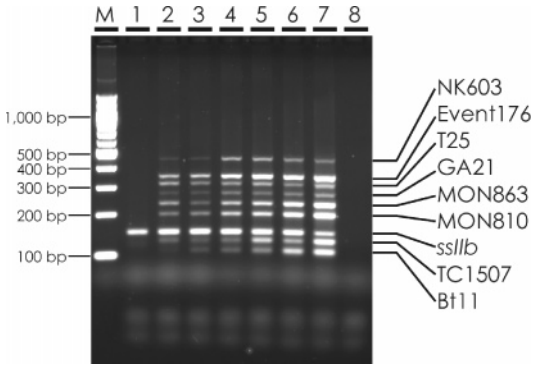

Figure 7. Sensitivity analyses of the one tube multiplex PCR. Amplified products from the simulated GM mixtures containing either 0.05, 0.1, 0.25, 0.5, 1, or 5% (w/w) of each of the eight events of GM maize are shown in lanes 2–7, respectively. Lane 1, non-GM maize; lane 8, negative control (no DNA); and M, 100 bp ladder size standard. PCR products were electrophoresed on 3% agarose gel.

stimulated GM mixtures containing 0.5% of each of the GM events. Therefore, we concluded that this multiplex PCR method has sufficient sensitivity to detect all eight events of GM maize simultaneously from the samples made up of the stimulated GM mixtures containing 0.25% of each of the GM events The sensitivity of multiplex PCR, however, might be affected when the one GM event is more concentrated than the others, because the amplifications from a more concentrated GM event might inhibit the amplification of another less abundant target. These results indicated that the multiplex PCR system has sufficient performance as a method for screening GM maize. As a next step, we plan to conduct interlaboratory collaborative trials to determine the practical limits of detection (LOD) of this method, as well as the applicability of this method to processed food samples.

In this study, we proposed a novel qualitative multiplex PCR method to simultaneously detect eight GM events in maize that were currently being grown for commercial purposes. This method was able to specifically detect all eight events with high sensitivity. These results indicated that this multiplex PCR method is an effective qualitative method to screen for the presence of GM maize in non-GM maize materials. Furthermore, this method could be useful as a monitoring method for hybrid stack progenies between two different events of GM maize, because the acreages of these varieties are quickly increasing in the United States. Plans are underway to confirm the reliability of the one-tube multiplex PCR method through interlaboratory collaborative studies.

ABBREVIATIONS USED

bp, base pair(s); CaMV, cauliflower mosaic virus; GM, genetically modified; GMO, genetically modified organism; LOD, limit of detection; PCR, polymerase chain reaction; r-DNA, recombinant deoxyribonucleic acid; RR, Roundup Ready.

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