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Simultaneous Detection of Recombinant DNA Segments Introduced into Genetically Modified Crops with Multiplex Ligase Chain Reaction Coupled with Multiplex Polymerase Chain Reaction

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We developed a multiplex polymerase chain reaction (PCR)−multiplex ligase chain reaction (LCR) (MPCR−MLCR) technique as a novel approach for the simultaneous detection of recombinant DNA segments (e.g., promoters, trait genes, and terminators) of genetically modified (GM) crops. With this technique, target DNA regions were amplified by multiplex PCR, the PCR products were then subjected to multiplex LCR as template DNAs, and the LCR products were then analyzed by polyacrylamide gel electrophoresis and subsequent fluorescent scanning. Seven recombinant DNA segments commonly introduced into some GM crop lines were selected as target DNA regions. In addition, another MPCR−MLCR system for the simultaneous detection of three endogenous DNA segments was designed as a positive control test. The specificity and sensitivity of the method were examined. The method allowed us to detect GM crops comprehensively and is expected to be utilized for efficient screening of GM crops into which any one of the seven recombinant DNA segments have been introduced, and for profiling the segments.

KEYWORDS: Multiplex PCR; genetically modified (GM); ligase chain reaction (LCR)

INTRODUCTION

The acreage of commercial cultivation of genetically modified (GM) crops has been continuously increasing, and the number of countries engaging in GM cultivation grew to 23 in 2007 (*1*). Despite some opposition, GM crops are generally considered as important sources of food and feed. Additionally, the production of biofuels such as ethanol and diesel oil is ongoing, and GM crops are expected to be a source of such fuels (*2*). Under these circumstances, novel types of GM crops are being developed all over the world, and the number of GM events obtaining regulatory approval in certain countries is increasing. Until now, GM event-specific detection methods have been developed and widely used for regulatory purposes. For example, many event-specific quantitative methods have been authorized and published under the European Commission (*3*). Such circumstances have made the monitoring and control of GM crops more difficult, because the enforcement of event-specific detection for all approved events would not be realistic. Thus, testing methods which are capable of providing a great deal of information on GM crops and applicable for many types of GM crops are in demand.

Multiplex polymerase chain reaction (PCR) methods (*4*−*7*) have been developed and reported as simultaneous detection tools for GM crops, as have DNA chip analyses (*8*−*16*) and membrane-hybridization methods (*17*). Although gel-based multiplex PCR assay is one of the most efficient techniques, such a method is difficult to develop for practical use because false-positive amplifications tend to occur more often than in simplex reactions (*16*, *18*, *19*). Ligase chain reaction (LCR) has been studied as a specific DNA detection technique and applied for clinical inspections such as the detection of pathogenic microorganisms and the discrimination of point mutations in human genomic DNA (*20*−*28*). LCR is a DNA amplification method that depends on the activity of thermostable ligase, and the amplification mechanism requires four LCR probes designed in adjacent and complementary positions in one target DNA region. In the thermal cycling of LCR, the four LCR probes anneal at the target sequence on the denatured DNA strands and two adjacent probes are linked by a thermostable DNA ligase (*28*). The ligated probes serve as targets for the subsequent cycles, leading to exponential amplification. LCR amplification has been reported to demand complete complementarity at the junction of the adjacent probes (*20*). Therefore, detection methods using the LCR technique are expected to have higher specificity for target nucleotide sequences than PCR amplification. In addition, for the simultaneous detection of tandemly

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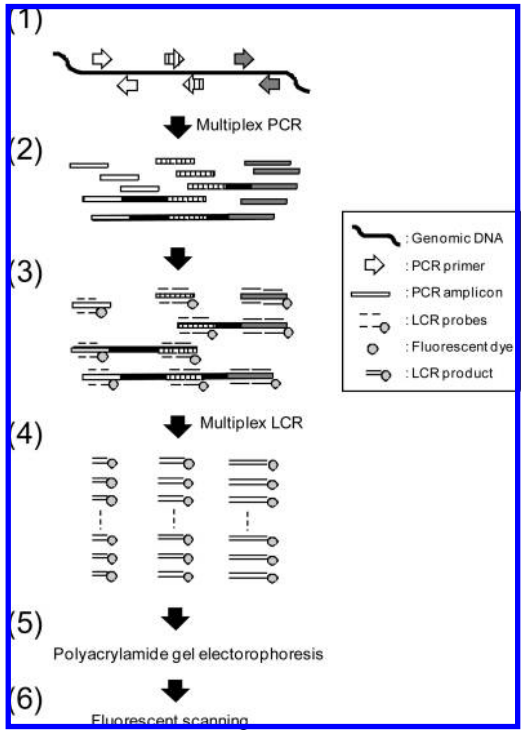

Figure 1. Schematic representation of MPCR−MLCR. (1) PCR primers for target DNA regions in a multiplex detection are designed and PCR mixture is prepared. (2) The target DNAs are exponentially amplified by multiplex PCR. In case that the target regions are closely located in a nucleotide chain, intervening DNA regions may also be amplified. (3) LCR probes are designed in the respective target regions of multiplex PCR, and LCR mixture is prepared with the LCR probes labeled with fluorescent dyes. (4) LCR products with different lengths for the respective targets are exponentially amplified by multiplex LCR. (5) Polyacrylamide gel electrophoresis is performed, and LCR products are separated by the lengths of nucleotide chains. (6) The separated LCR products are detected by fluorescent scanning.

arranged recombinant DNA regions such as the multiplex detection of a promoter, a trait gene and a terminator in one cassette, multiplex LCR may provide further advantages over multiplex PCR. This is because multiplex LCR produces the amplified products with the specific lengths even for the adjacent targets, while multiplex PCR may produce unexpected amplified products containing multiple target regions.

In this study, we applied multiplex LCR for the simultaneous detection of target DNA regions and adopted multiplex PCR as an efficient preamplification method in order to obtain higher sensitivity in this novel system. The newly designed multiplex PCR−multiplex LCR (MPCR−MLCR) systems were intended for the simultaneous detection of recombinant DNA segments commonly introduced into some GM crop lines and that of endogenous DNA segments as a positive control test. The systems would be useful for the screening detection of GM crops. An overview of the MPCR−MLCR technique is shown in **Figure 1**.

MATERIALS AND METHODS

Cereal Materials. We used the following as representative GM maize lines: Bt11, Event176, GA21, MON810, MON863, NK603, T25, and TC1507. The representative GM soy line was 40-3-2 (Roundup Ready soybean, RRS). F1 generation seeds of Bt11, Event176 and ground F1 generation seeds of GA21 were kindly provided by Syngenta Seeds AG (Basel, Switzerland). F1 generation seeds of MON810, MON863, and NK603 were kindly provided by MONSANTO Co. (St. Louis, MO). F1 generation seeds of TC1507 were kindly provided by Dow AgroSciences LLC (Indianapolis, IN). F1 generation seeds of T25 and progeny seeds of RRS were directly imported from the USA. Dry maize seeds (Quality Technology International, Inc., Huntley, IL) and dry soybeans harvested in Ohio in 1998 were directly imported and used as non-GM maize and non-GM soy, respectively. Seeds of the conventional rice variety Kinuhikari (*Oryza sativa*), the conventional wheat variety Haruyutaka (*Triticum aestivum*), and the conventional barley variety Harrington (*Hordeum vulgare*) 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 stored at −20 °C until DNA extraction. DNA extraction was performed with the DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany) as described in our previous report (*29*). The DNA concentration was determined by measuring ultraviolet (UV) absorbance, and quality was evaluated by the absorbance ratios at 260/280 nm and 260/230 nm. The absorbance ratios at 260/230 nm and 260/280 nm were above 1.7 and between 1.7 and 2.0, respectively. All extracted DNAs were diluted to 20 ng/μL. As a template DNA, simulated maize DNA mixtures and simulated soy DNA mixtures were prepared by mixing genomic DNA (20 ng/μL) from GM flour with genomic DNAs (20 ng/μL) from non-GM flour at the volume ratio as follows. Simulated maize DNA mixtures, each containing one of the GM maize lines, i.e., Bt11, Event176, GA21, MON810, MON863, NK603, T25, or TC1507, at the concentration of 1% (v/v) or 0.2% (v/v), were prepared. Simulated soy DNA mixtures containing a GM soy line, RRS, at the concentration of 1% (v/v) were prepared. Simulated maize DNA mixtures containing all eight GM maize lines, i.e., Bt11, Event176, GA21, MON810, MON863, NK603, T25, and TC1507, at the concentrations of 1% (v/v) each, 0.5% (v/v) each, 0.25% (v/v) each, 0.1% (v/v) each, 0.05% (v/v) each, and 0.025% (v/v) each were prepared. Simulated maize DNA mixtures at the concentration of 99.9% (v/v) MON810 and 0.1% (v/v) GA21, 99.5% (v/v) MON810 and 0.5% (v/v) GA21, 99% (v/v) MON810 and 1% (v/v) GA21, 1% (v/v) MON810 and 99% (v/v) GA21, 0.5% (v/v) MON810 and 99.5% (v/v) GA21, and 0.1% (v/v) MON810 and 99.9% (v/v) GA21 were also prepared.

PCR Primers and LCR Probes. Target regions were selected as follows: a region of phosphinotricin-*N*-acetyltransferase gene derived from *Streptomyces viridochromogenes* (PAT), a region of 5-enolpyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium* sp. CP-4 (CP4-EPSPS), a region of phosphinotricin-*N*-acetyltransferase gene from *Streptomyces hygroscopicus* (BAR), a region of neomycin phosphotransferase II gene (NPTII), the terminator region of nopaline synthase derived from *Rhizobium radiobacter* (TNOS), the 35S promoter region derived from Cauliflower mosaic virus (P35S) and the promoter region of rice Actin gene (PACT). In addition, another MPCR−MLCR system for three endogenous DNA segments was designed as a positive control test. Target regions for this assay were selected as follows: a region of lectin 1 gene of *Glycine max* (Le1), a region of starch synthase IIb gene of *Zea mays* (SSIIb), and the consensus region of 18S rRNA gene in plant genomes (18SrRNA).

The sequences of PCR primers and their references have been previously reported as listed in **Table 1** (*29*−*31*) except for 18SrRNA. The oligonucleotide DNAs for PCR primers were synthesized by Fasmac Co., Ltd. (Atsugi, Japan). The primers, 18SrRNA 3-5′ and 18SrRNA 3-3′, were designed for the amplification of 18S rRNA genes as highly conserved nucleotide sequences in crops. For the design of this primer pair, 18S rRNA gene sequences of some crops, such as *Zea mays* (Genbank Accession No. AF168884), *Hordeum vulgare* (Genbank Accession No. AY552749), *Gossypium hirsutum* (Genbank Accession No. L24145), *Solanum tuberosum* (Genbank Accession No. X67238), *Oryza sativa* (Genbank Accession No. AF069218), *Glycine max* (Genbank Accession No. X02623), *Nicotiana tabacum* (Genbank Accession No. AJ236016), and *Triticum aestivum* (Genbank Accession No. AJ272181), were aligned. The primer pair was designed in a DNA region completely matching in these crops.

For LCR amplification of a target DNA segment, four LCR probes were designed in a region between the forward primer and the reverse primer of each corresponding PCR amplicon. The designed probes and

Table 1. List of PCR Primer Sequences

target name	primer name	sequence	amplicon	ref
PAT	pat 1-5′	5′-AAGAGTGGATTGATGATCTAGAGAGGT-3′	161 bp	*30*
	pat 1-3′	5′- ATGCCTATGTGACACGTAAACAGTACT-3′		
CP4-EPSPS	epsps 1-5′	5′- GCCTCGTGTCGGAAAACCCT-3′	118 bp	*30*
	epsps 3-3′	5′- TTCGTATCGGAGAGTTCGATCTTC-3′		
BAR	bar 2-5′	5′- ACTGGGCTCCACGCTCTACA-3′	186 bp	*30*
	bar 2-3′	5′- AAACCCACGTCATGCCAGTTC-3′		
NPTII	npt 1-5′	5′- GACAGGTCGGTCTTGACAAAAAG-3′	155 bp	*30*
	npt 1-3′	5′- GAACAAGATGGATTGCACGC-3′		
TNOS	NOS ter 2-5′	5′-GTCTTGCGATGATTATCATATAATTTCTG-3′	151 bp	*29*
	NOS ter 2-3′	5′-CGCTATATTTTGTTTTCTATCGCGT-3′		
P35S	P35S 1-5′	5′-ATTGATGTGATATCTCCACTGACGT-3′	101 bp	*29*
	P35S 1-3′	5′-CCTCTCCAAATGAAATGAACTTCCT-3′		
PACT	rAct pro 2-5′	5′- CGTTGCAGCGATGGGTAT-3′	121 bp	*30*
	rAct pro 1-3′	5′- GGGCTTGCTATGGATCGTG-3′		
Le1	Le1n02-5′	5′- GCCCTCTACTCCACCCCCA-3′	118 bp	*29*
	Le1n02-3′	5′- GCCCATCTGCAAGCCTTTTT-3′		
SSIIb	SSIIb 3-5′	5′- CCAATCCTTTGACATCTGCTCC-3′	114 bp	*31*
	SSIIb 3-3′	5′- GATCAGCTTTGGGTCCGGA-3′		
18SrRNA	18SrRNA 3-5′	5′- CCGTTAACGAACGAGACCTCAGCC-3′	238 bp	this study
	18SrRNA 3-3′	5′- AATGATCTATCCCCATCACGATGAAAT-3′		

Table 2. List of LCR Probe Sequences

target name	probe name	sequence	amplicon	reference
PAT	PAT-1-A	5′-GATAGATACCCTTGGTTGGTTGCTGAGGTTGAGGGTGT-3′	76 bp	Genbank
	Phos-PAT-1-B	5′-phosphate-TGTGGCTGGTATTGCTTACGCTGGGCCCTGGAAGGCTA-3′		DQ156557
	FAM-PAT-1-C	5′-FAM-TAGCCTTCCAGGGCCCAGCGTAAGCAATACCAGCCACA-3′		
	Phos-PAT-1-D	5′-phosphate-ACACCCTCAACCTCAGCAACCAACCAAGGGTATCTATC-3′		
CP4-EPSPS	EPSPS-1-A	5′-AAACCCTGTCACGGTGGACGATGCCACGATGAT-3′	71 bp	US patent
	Phos-EPSPS-1-B	5′- phosphate-CGCCACGAGCTTCCCGGAGTTCATGGACCTGATGGCCG-3′		No. 5,804,425
	FAM-EPSPS-1-C	5′-FAM-CGGCCATCAGGTCCATGAACTCCGGGAAGCTCGTGGCG-3′		
	Phos-EPSPS-1-D	5′-phosphate-ATCATCGTGGCATCGTCCACCGTGACAGGGTTT-3′		
BAR	BAR-1-A	5′-GGTCGCTGTCATCGGGCTGCCCAACGACCCGAG-3′	66 bp	Genbank
	Phos-BAR-1-B	5′-phosphate-CGTGCGCATGCACGAGGCGCTCGGATATGCCCC-3′		X05822
	FAM-BAR-1-C	5′-FAM-GGGGCATATCCGAGCGCCTCGTGCATGCGCACG-3′		
	Phos-BAR-1-D	5′-phosphate-CTCGGGTCGTTGGGCAGCCCGATGACAGCGACC-3′		
NPTII	NPT-2-A	5′-TCTTGACAAAAAGAACCGGGCGCCCCTGCGCTG-3′	61 bp	Genbank
	Phos-NPT-2-B	5′-phosphate-ACAGCCGGAACACGGCGGCATCAGAGCA-3′		U00004
	FAM-NPT-2-C	5′-FAM-TGCTCTGATGCCGCCGTGTTCCGGCTGT-3′		
	Phos-NPT-2-D	5′-phosphate-CAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGA-3′		
TNOS	TNOS-1-A	5′-GTAATGCATGACGTTATTTATGAGATGG-3′	56 bp	*29*
	Phos-TNOS-1-B	5′-phosphate-GTTTTTATGATTAGAGTCCCGCAA-3′		
	FAM-TNOS-1-C	5′-FAM-ATAATTGCGGGACTCTAATCATAAAAAC-3′		
	Phos-TNOS-1-D	5′-phosphate-CCATCTCATAAATAACGTCATGCATTAC-3′		
P35S	P35S-1-A	5′-GACGCACAATCCCACTATCCTTC-3′	51 bp	*29*
	Phos-P35S-1-B	5′-phosphate-GCAAGACCCTTCCTCTATATAAGGAAGT-3′		
	FAM-P35S-1-C	5′-FAM-ACTTCCTTATATAGAGGAAGGGTCTTGC-3′		
	Phos-P35S-1-D	5′-phosphate-GAAGGATAGTGGGATTGTGCGTC-3′		
PACT	PACT-2-A	5′-AGACTCAAAACATTTACAAAAAC-3′	46 bp	*32*
	Phos-PACT-2-B	5′-phosphate-AACCCCTAAAGTTCCTAAAGCCC-3′		
	FAM-PACT-2-C	5′-FAM-GGGCTTTAGGAACTTTAGGGGTT-3′		
	Phos-PACT-2-D	5′-phosphate-GTTTTTGTAAATGTTTTGAGTCT-3′		
Le1	Le1-1-A	5′-GGTGAAGTTGAAGGAAGCGGCGA-3′	51 bp	*29*
	Phos-Le1-1-B	5′-phosphate-AGCTGGCAACGCTACCGGTTTCTTTGTC-3′		
	FAM-Le1-1-C	5′-FAM-GACAAAGAAACCGGTAGCGTTGCCAGCT-3′		
	Phos-Le1-1-D	5′-phosphate-TCGCCGCTTCCTTCAACTTCAC-3′		
SSIIb	SSIIb-1-A	5′-AGCAAAGTCAGAGCGCTGCAATG-3′	46 bp	*31*
	Phos-SSIIb-1-B	5′-phosphate-CAAAACGGAACGAGTGGGGGCAG-3′		
	FAM-SSIIb-1-C	5′-FAM-CTGCCCCCACTCGTTCCGTTTTG-3′		
	Phos-SSIIb-1-D	5′-phosphate-CATTGCAGCGCTCTGACTTTGCT-3′		
18SrRNA	18SrRNA-3-A	5′-CGTGCGGCCCAGAACATC-3′	41 bp	this study
	Phos-18SrRNA-3-B	5′-phosphate-TAAGGGCATCACAGACCTGTTAT-3′		
	FAM-18SrRNA-3-C	5′-FAM-ATAACAGGTCTGTGATGCCCTTA-3′		
	Phos-18SrRNA-3-D	5′-phosphate-GATGTTCTGGGCCGCACG-3′		

their references are listed in **Table 2** (*29*, *31*, *32*). Among the four LCR probes, one probe was synthesized with a fluorescent dye, 6-carboxyfluorescein (FAM) at the 5′ end, and two probes were synthesized with phosphate at the 5′ ends. The length of each LCR product was designed to be different from the others as described in **Table 2**. The oligonucleotide DNAs for LCR probes were synthesized by Fasmac Co., Ltd. The LCR probes for the amplification of PAT, CP4-EPSPS, BAR, and NPTII were designed by referring to the publicly available nucleotide sequences (**Table 2**). The LCR probes for amplification of TNOS, P35S, PACT, Le1, and SSIIb were designed by referring to previous reports (*29*, *31*, *32*), whereas those of 18SrRNA were designed by referring to the nucleotide sequence obtained in the alignment of the nucleotide sequences of 18S rRNA genes as described above.

Table 3. DNA Segments in GM Lines of Maize and Soy

		recombinant DNA segment							endogenous DNA segment		
crop name	event name	PACT	P35S	TNOS	NPTII	BAR	CP4-EPSPS	PAT	Le1	SSIIb	18SrRNA
maize	Bt11	−	+	+	−	−	−	+	−	+	+
maize	Event176	−	+	−	−	+	−	−	−	+	+
maize	GA21	+	−	+	−	−	−	−	−	+	+
maize	MON810	−	+	−	−	−	−	−	−	+	+
maize	MON863	−	+	+	+	−	−	−	−	+	+
maize	NK603	+	+	+	−	−	+	−	−	+	+
maize	T25	−	+	−	−	−	−	+	−	+	+
maize	TC1507	−	+	−	−	−	−	+	−	+	+
soy	RRS	−	+	+	−	−	+	−	+	−	+

The structural information of recombinant DNA integrated in GM maize and GM soy is compiled in **Table 3**, as taken from safety assessment documents published by the Ministry of Health, Labour and Welfare and the Ministry of Agriculture, Forestry and Fisheries of Japan or documents downloadable from the Web site of the Japanese Biosafety Clearing-House (http://www.bch.biodic.go.jp/) managed by the Ministry of the Environment of Japan.

Multiplex PCR. For heptaplex (7-plex) PCR designed to amplify seven recombinant DNA segments, 25 μL of a reaction mixture was assembled containing 50 ng of genomic DNA, 200 μM dNTPs, 0.625 units of AmpliTaq Gold Polymerase (Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$, 2.5 μL of 10× PCR buffer II (Applied Biosystems) and seven primer pairs at the following concentrations: 0.1 μM for PAT, CP4-EPSPS, TNOS, and P35S; 0.14 μM for NPTII; 0.2 μM for PACT; and 0.4 μM for BAR. The concentrations of primer pairs were experimentally adjusted. For triplex (3-plex) PCR designed to amplify three endogenous DNA segments, 25 μL of a reaction mixture was assembled containing the same components as the heptaplex PCR reaction mixture with the exception of three primer pairs at the following concentrations: 0.1 μM for Le1 and SSIIb, and 0.02 μM for 18SrRNA. The PCR amplification was carried out on the ABI PRISM 9700 (Applied Biosystems) with thermal cycles consisting of 95 °C for 10 min for preincubation, 45 cycles of 95 °C for 30 s for denaturation, 55 °C for 30 s for annealing and 72 °C for 30 s for extension, and 72 °C for 7 min for final extension. For the analysis of PCR products, agarose gel electrophoresis was carried out with 3% (w/v) LO3 agarose (Takara Bio Inc., Otsu, Japan) gel in TAE buffer (Wako Pure Chemical Industries, Ltd., Osaka, Japan) with 0.2 μg/mL of ethidium bromide (Sigma Aldrich, St. Louis, MO). Eight microliters of each reaction mixture underwent electrophoresis at a constant voltage (100 V) for 30 min in the TAE buffer. After the electrophoresis, the gel was photographed under UV radiation by using the Densitograph system (ATTO, Tokyo, Japan).

LCR Conditions. For simplex LCR, a 10 μL reaction mixture consisting of 1 μL of multiplex PCR mixture as template DNA, 0.1 unit of Ampligase (EPICENTRE Biotechnologies, Madison, WI), 1 μL of the 10× reaction buffer [200 mM Tris/HCl (pH 8.3), 250 mM KCl, 100 mM $MgCl_2$, 5 mM NAD^+, 0.1% of Triton-X], 1.5 μg of salmon testes DNA (Sigma Aldrich), and a set of 4 LCR probes for one target DNA region with a concentration of 12.5 nM each was used. For the heptaplex LCR which is capable of amplifying seven recombinant DNA segments, a 10 μL volume of reaction mixture was prepared that was the same as the simplex LCR reaction mixture except that seven sets of the LCR probes listed in **Table 2** were used for PAT, CP4-EPSPS, BAR, NPT, TNOS, P35S, and PACT as target DNA with a concentration of 12.5 nM each. For the triplex LCR that can amplify three endogenous DNA segments, a reaction mixture was prepared that was the same as the simplex LCR reaction mixture except that three sets of LCR probes (**Table 2**) were used for Le1, SSIIb, and 18SrRNA with a concentration of 12.5 nM each. The LCR amplification was carried out on the ABI PRISM 9700 with thermal cycles consisting of 2 cycles of 94 °C for 1.5 min and 55 °C for 6 min, and 15 cycles of 91 °C for 30 s and 55 °C for 6 min.

Analysis of LCR Products. For analysis of LCR products, polyacrylamide gel electrophoresis was carried out on polyacrylamide gel (10% T and 2.6% C) (Bio-Rad Laboratories, Inc., Hercules, CA) in TBE buffer. Six microliters of LCR reaction solution mixed with 1.5 μL of a sample loading buffer (6× loading buffer triple dye, NIPPON GENE CO., Ltd., Tokyo, Japan) was applied along with a DNA size marker, Fluorescein MapMarker 1000 (BioVentures, Inc., Murfreesboro, TN), and electrophoresed at a constant voltage (300 V) for 60 min in TBE buffer. After the electrophoresis, the gel was scanned with the Molecular Imager FX (Bio-Rad Laboratories) connected to 488 nm external laser in the FAM detection mode (medium range). The image data were analyzed with the analytical software Quantity One Version 4.5.2 (Bio-Rad Laboratories).

RESULTS AND DISCUSSION

Simplex and Multiplex PCR. The specificity of simplex PCR amplification with the PCR primers was confirmed previously (*29*−*31*) except for 18SrRNA. Simplex PCR with the primer pair for 18SrRNA at the concentration of 0.5 μM was performed with genomic DNAs from maize, soy, wheat, barley, and rice as template DNA. DNA amplification was observed on the conventional crop samples of these 5 crops (data not shown). Heptaplex PCR for the seven recombinant DNA segments (i.e., PAT, CP4-EPSPS, BAR, NPTII, TNOS, P35S, and PACT) and triplex PCR for the three endogenous DNA segments (i.e., Le1, SSIIb, and 18SrRNA) were performed. Agarose gel electrophoreses of heptaplex PCR products and triplex PCR products are shown in **Figure 2** (**A**) and (**B**), respectively. Some amplification products with the expected lengths (**Table 1**) were observed corresponding to the DNA segments listed in **Table 3**. At the same time, many amplification products with unexpected lengths were observed, and they were possibly attributed to non-specific amplification with unpaired primers. Based on the results of the multiplex PCRs, we could not determine whether GM crops were included or what kind of DNA segments were contained in each template DNA. This result showed the necessity of the following multiplex LCR.

Multiplex PCR−Simplex LCR. Simplex LCR amplification following multiplex PCR was performed as shown in **Figure 3**. On the odd-numbered lanes of both (**A**) and (**B**), LCR amplification was not observed because of a lack of template DNA, and only unreacted LCR probes were found as bands. On the even-numbered lanes, LCR-amplified products with the expected lengths were observed along with concomitantly decreasing LCR probes. It was confirmed that LCR amplification proceeded successively with PCR products as template DNA. In this investigation, multiplex PCR products without purification were added into LCR reaction mixtures as template DNA. Any adverse effects caused by the addition of PCR reaction mixture were not observed in the results of LCR, suggesting that the purification of PCR products was not necessary in our method.

MPCR–MLCR. MPCR−MLCR assays for the various DNA samples were performed, and the specificity was examined as shown in **Figure 4**. The LCR amplification observed in each

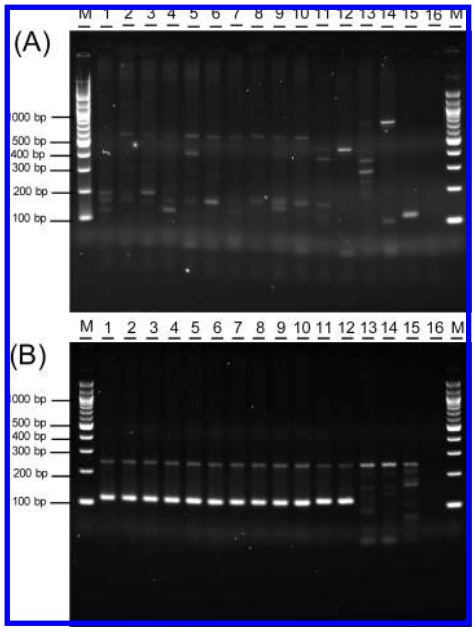

Figure 2. Results of agarose gel electrophoresis of multiplex PCR products. Heptaplex PCR with various kinds of template DNA was performed and electrophoresed (**A**). Triplex PCR with various template DNA was performed and electrophoresed (**B**). The template DNA in the multiplex PCR reaction mixture for each lane was as follows: lane 1, the simulated maize DNA mixture containing all eight GM maize lines at the concentration of 1% (v/v) each; lanes 2−9, the simulated maize DNA containing each of the GM maize lines (Bt11, Event176, GA21, MON810, MON863, NK603, T25, or TC1507, respectively) at the concentration of 1% (v/v); lane 10, non-GM maize genome; lane 11, the simulated soy DNA containing RRS at the concentration of 1% (v/v); lane 12, non-GM soy genome; lanes 13−15, non-GM wheat, non-GM barley, and non-GM rice genome, respectively; lane 16, sterilized distilled water (negative control); lane M, 100 bp ladder size standard.

lane corresponded to the information of the DNA segments listed in **Table 3**. Regarding non-GM maize, soy, wheat, and barley, no amplification of recombinant DNA segments was observed as expected. In the recombinant DNA segment assay with template DNA from non-GM rice (**Figure 4** (**A**), lane 15), only one band indicating the PACT segment was observed. This amplification was also expected because PACT is a DNA segment originally derived from the conventional rice. The results of endogenous DNA segment detection were shown to be specific for the relevant crops (**Figure 4** (**B**)). 18SrRNA was detected for the various crop samples. Thus, the 18SrRNA detection test as a positive control may expand the applicability of this proposal detection method for a broader range of plant species.

The sensitivity of the assay for recombinant DNA segments was tested with a simulated maize DNA mixture with each GM line at the concentration of 0.2% (v/v). The expected amplification was clearly observed in the same way as **Figure 4** (**A**) (data not shown). In addition, the assay with simulated maize DNA mixtures containing all eight GM maize lines with several concentrations was carried out for the simultaneous amplification of seven segments (**Figure 5** (**A**) and (**B**)). Even when a simulated maize DNA mixture with eight GM lines at the concentration of 0.05% (v/v) each was used as template DNA, seven LCR products were clearly observed as expected. The results indicated that the amplification in the multiplex system was well-performed, even with the low concentration of target DNA segments. In this experiment, several segments were included redundantly in the reaction mixture as template DNA.

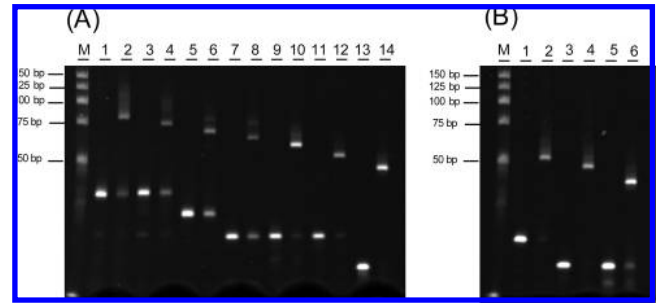

Figure 3. DNA amplification in multiplex PCR−simplex LCR. Simplex LCR was performed with heptaplex PCR products as template DNA, and polyacrylamide gel electrophoresis was carried out (**A**). LCR for amplification of the following segments was used: lanes 1 and 2, PAT; lanes 3 and 4, CP4-EPSPS; lanes 5 and 6, BAR; lanes 7 and 8, NPTII; lanes 9 and 10, TNOS; lanes 11 and 12, P35S; lanes 13 and 14, PACT. In odd-numbered lanes, LCR was carried out with PCR reaction products with no template DNA. In even-numbered lanes, LCR was performed with PCR products amplified from the simulated maize genomic DNA containing all eight GM maize lines at the concentration of 1% (v/v) each. Polyacrylamide gel electrophoresis was carried out with simplex LCR products amplified with triplex PCR products as template DNA (**B**). LCR for amplification of the following segments was used: lanes 1 and 2, Le1; lanes 3 and 4, SSIIb; lanes 5 and 6, 18SrRNA. Triplex PCR products amplified from no template DNA were used as template DNA in LCR in odd-numbered lanes, triplex PCR products amplified from non-GM soy genome were used as template DNA for LCR in lane 2, and triplex PCR products amplified from non-GM maize genome were used as template DNA for LCR in lanes 4 and 6. In Lane M, the DNA size marker was electrophoresed.

For example, P35S was found in genomic DNA of seven GM lines and TNOS was also found in four GM lines (**Table 3**). Therefore, we additionally noted that the copy numbers of each DNA segment were not equal in the simulated maize DNA mixtures which consisted of all eight GM maize lines at the same concentration for each line. Furthermore, we evaluated the detection sensitivity for a recombinant DNA segment when another target segment dominantly coexisted in a sample (**Figure 5** (**C**) and (**D**)). P35S derived from MON810 and TNOS and PACT derived from GA21 were selected as representative target segments. In lane 10−12 of **Figure 5** (**C**), the detection of TNOS and PACT under the dominant P35S amplification was evaluated. When the sample contained 0.5% (v/v) GA21 in MON810 genome, all the expected amplification was observed, including weak signal of the PACT amplification. Meanwhile, in the sample containing 0.1% (v/v) GA21 in MON810 genome, the PACT amplification was not observed. For the detection of P35S with the dominant TNOS and PACT amplification, all the expected amplification was detected even when the sample contained 0.1% (v/v) MON810 in GA21 as shown in **Figure 5** (**C**). In **Figure 5** (**D**), the endogenous DNA segments were detected as expected. From these results, we supposed that the sensitive detection of underrepresented target segments under the coexistence of other dominant target would be achievable by our method. However, the evaluation of method performance with simulated DNA mixtures including various GM lines at diversified concentrations would be demanded, for determining the detection limits of the developed method.

In this report, we proposed the MPCR−MLCR technique as a new approach to the simultaneous detection of recombinant DNA segments in GM crops. For the design of this new technique, we emphasized the high sensitivity and specificity achieved by multiplex PCR and multiplex LCR, respectively. The results of our investigation proved that MPCR−MLCR was

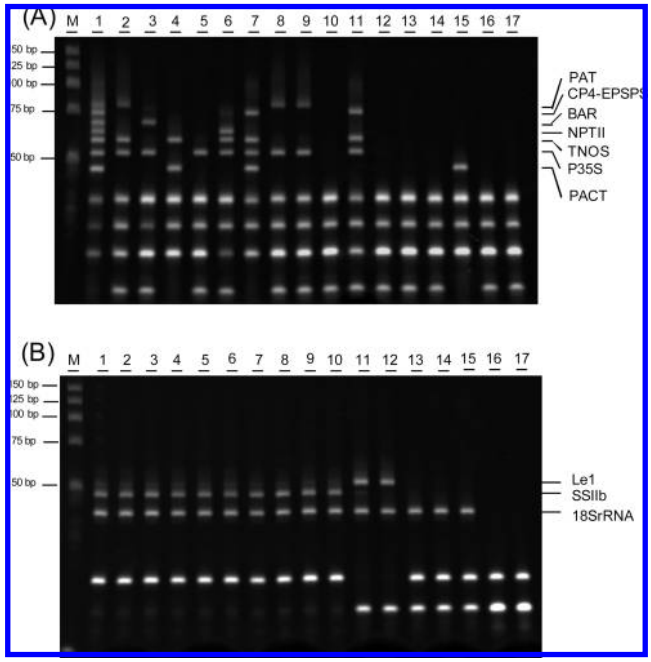

Figure 4. Specificity of MPCR−MLCR detection. The results of polyacrylamide gel electrophoresis with multiplex LCR products for recombinant DNA segment detection are shown in (**A**), and those for endogenous DNA segment detection are shown in (**B**). The MPCR−MLCR products with the following template DNA in multiplex PCR were electrophoresed: lane 1, the simulated maize DNA containing all eight GM maize lines at the concentration of 1% (v/v) each; lanes 2−9, the simulated maize DNA containing each of the GM maize lines (Bt11, E176, GA21, M810, M863, NK603, T25, or TC1507) at the concentration of 1% (v/v); lane 10, non-GM maize genome; lane 11, the simulated soy DNA containing RRS at the concentration of 1% (v/v); lane 12, non-GM soy genome; lane 13, non-GM wheat genome; lane 14 non-GM barley genome; lane 15, non-GM rice genome; lane 16, sterilized distilled water (negative control in multiplex PCR). In lane 17, multiplex LCR products with sterilized distilled water as template DNA for LCR were electrophoresed as negative controls in multiplex LCR. In lane M, the DNA size marker was electrophoresed.

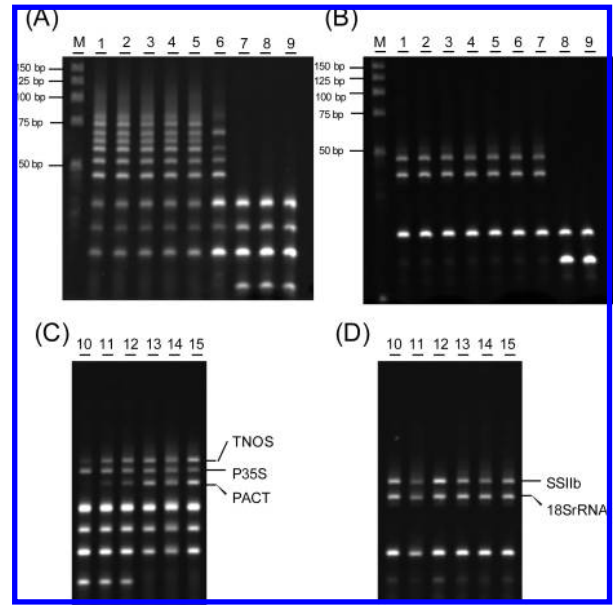

Figure 5. Sensitivity of MPCR−MLCR detection. The results of polyacrylamide gel electrophoresis with reaction mixtures for recombinant segment detection are shown in (**A**) and (**C**), and those with reaction mixtures for endogenous segment detection are shown in (**B**) and (**D**). The MPCR−MLCR products with the following genomic DNAs as template DNA in multiplex PCR were electrophoresed: lanes 1−6, the simulated maize DNA mixture containing all eight GM maize lines at the concentrations of 1% (v/v) each, 0.5% (v/v) each, 0.25% (v/v) each, 0.1% (v/v) each, 0.05% (v/v) each, and 0.025% (v/v) each, respectively; lane 7, non-GM maize genome; lane 8, sterilized distilled water (negative control); lanes 10−15, the simulated maize DNA mixtures at the concentration of 99.9% (v/v) MON810 and 0.1% (v/v) GA21, 99.5% (v/v) MON810 and 0.5% (v/v) GA21, 99% (v/v) MON810 and 1% (v/v) GA21, 1% (v/v) MON810 and 99% (v/v) GA21, 0.5% (v/v) MON810 and 99.5% (v/v) GA21, and 0.1% (v/v) MON810 and 99.9% (v/v) GA21, respectively. In lane 9, multiplex LCR products with sterilized distilled water as template DNA were electrophoresed as negative controls. In lane M, the DNA size marker was electrophoresed.

efficient, and the availability of multiplex PCR products without purification as template DNA in multiplex LCR made the method simple and less contaminative. Combined PCR−ligase detection reaction−universal array (PCR−LDR−UA) methods have been reported as the first report on the GM crop-detection method with using ligase reaction (*9*−*11*). The PCR−LDR−UA aimed at GM line-specific detection with microarray technique, while our investigation aimed at recombinant DNA segment-detection with electrophoresis-based assay. In the PCR−LDR−UA strategy, the target DNA regions were exponentially amplified only in the PCR step, and a successful multiplex PCR was supposed to be essential for the sensitive and robust detection. Meanwhile, the MPCR−MLCR strategy is mainly based on the multiplex LCR technique and it permits the easy design of multiplex PCR without considering the lengths of the multiple amplification products, because multiplex PCR functions just as preamplification. Additionally, the strategy featuring multiplex LCR may render our developed methods more reliable and potentially applicable even to the adjacently located targets. In conclusion, we expected that our novel systems for recombinant DNA segments and endogenous DNA segments would be utilized for the efficient screening of GM crops, including unknown ones into which any one of the seven recombinant DNA segments has been introduced, and for the profiling of the segments.

ABBREVIATIONS USED

CP4-EPSPS, a region of 5-enolpyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium* sp. CP-4; Le1, a region of lectin 1 gene of *Glycine max*; NPTII, a region of neomycin phosphotransferase II gene; PAT, a region of phosphinotricin-*N*-acetyltransferase gene derived from *Streptomyces viridochromogenes*; BAR, a region of phosphinotricin-*N*-acetyltransferase gene from *Streptomyces hygroscopicus*; SSIIb, a region of starch synthase IIb gene of *Zea mays*; FAM, 6-carboxyfluorescein; GM, genetically modified; LCR, ligase chain reaction; MPCR−MLCR, multiplex polymerase chain reaction−multiplex ligase chain reaction; PCR, polymerase chain reaction; RRS, Roundup Ready soybean; 18SrRNA, the consensus region of 18S rRNA gene in plant genomes; PACT, the promoter region of rice actin; P35S, the 35S promoter region derived from Cauliflower mosaic virus; TNOS, the terminator region of nopaline synthase derived from *Rhizobium radiobacter*; UV, ultraviolet.

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