Multiplex PCR法を用いた組換えトウモロコシ5系統からの組換え遺伝子の検知法

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Original

A Multiplex PCR Method of Detecting Recombinant DNAs from Five Lines of Genetically Modified Maize

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Seven lines of genetically modified (GM) maize have been authorized in Japan as foods and feeds imported from the USA. We improved a multiplex PCR method described in the previous report in order to distinguish the five lines of GM maize. Genomic DNA was extracted from GM maize with a silica spin column kit, which could reduce experimental time and improve safety in the laboratory and potentially in the environment. We sequenced recombinant DNA (r-DNA) introduced into GM maize, and re-designed new primer pairs to increase the specificity of PCR to distinguish five lines of GM maize by multiplex PCR. A primer pair for the maize intrinsic *zein* gene (*Ze1*) was also designed to confirm the presence of amplifiable maize DNA. The lengths of PCR products using these six primer pairs were different. The *Ze1* and the r-DNAs from the five lines of GM maize were qualitatively detected in one tube. The specific PCR bands were distinguishable from each other on the basis of the expected length. The r-DNA could be detected from maize samples containing 0.5% of each of the five lines of GM maize. The sensitivity would be acceptable to secure the verification of non-GMO materials and to monitor the reliability of the labeling system.

Key words: genetically modified maize; recombinant DNA; PCR; multiplex PCR; detection technique

Introduction

Recent years have seen tremendous advances in food biotechnology, including transgenic crop breeding and genetic modifications of organisms used in food production. The general public, however, has shown anxiety over this new technology. A new labeling system for genetically modified crops and processed foods containing them (GM foods) is under consideration in many countries and the development of a practical detection method is required for scientific inquiry by authorities and industries.

In Japan, in April 2000, the commercialization of 29 kinds of genetically modified (GM) crops, including soybean, maize, canola, potato, tomato, cotton, and sugar beet, was authorized by the Ministry of Health and Welfare (MHW) and the Ministry of Agriculture, Forestry and Fisheries (MAFF). A labeling system of GM foods in Japan, established on March 31, 2000, has been enforced and it provides a one-year grace period. The purpose of the new labeling system is to provide information regarding the use of GM technologies and to foster consumers' rights to select their preferred foods. The requirement of labeling includes any products derived from GM crops, which have been assessed for safety by the government, as well as processed foods, which include the five transgenic crops, soy, maize, canola, potato, and cotton, and 24 kinds of processed foods derived from them. This system is applied to foods that include any of the five crops if the crop is among the top three ingredients in terms of weight and represents not less than 5% (w/w) in the food. In this system, foods are classified into three categories (those using GM crops, those using non-genetically modified (non-GM) crops, and those in which the GM crop is not segregated during the foods' production and distribution); labeling is compulsory for "using GMOs" and "not segregated" foods, while it is optional for "non-GMOs". Recently, MHW announced a mandatory requirement for the safety assessment of GM foods effective on May 1, 2000, and any GM food that has no safety assessment shall be neither imported nor sold in Japan on and after April 1, 2001. MHW has also been discussing the necessity for labeling of GM foods from the viewpoint of

public health. Therefore, the development of practical methods of detecting recombinant DNA (r-DNA) or its product protein in foods is required.

Focusing on GM maize, in 1999, three lines of GM maize, which were glyphosate-tolerant GA21, glufosinate-tolerant DLL25, and glufosinate-tolerant and insect-resistant DBT418, were newly authorized for food use in Japan, in addition to four lines (Event 176, Bt11, MON810 and T25) approved already. Some previous reports have dealt with the detection of r-DNA from maize seed; a screening method[1] was presented that could detect the 35S promoter region (P-35S) derived from the cauliflower mosaic virus (CMV), and specific detection methods have been presented for MON810[2] or Event176[3]. Recently, we reported a specific detection method of r-DNAs from four lines of GM maize, including a simultaneous detection of three lines of insect-resistant maize (Bt11, Event176, and MON810), by a multiplex PCR[4]. In the previous report, genomic DNA extracted from samples was partially purified by successive extraction using CTAB buffer and phenol/chloroform (CTAB method). The extracted DNA was well amplified by PCR, while the extraction procedure was found to be time-consuming, and chloroform, which is a toxic solvent, was used. Thus, we examined DNA extraction by using a silica spin column to reduce experimental time and to improve safety in the laboratory and potentially in the environment. Also in the previous report, the primer pair between P-35S and *pat* gene, which was designed for T25, gave PCR bands in Bt11, though the length of amplified band was different from that of T25.

We developed the primer design to detect r-DNAs derived from GM maize authorized for food use, and we detected two lines of GM maize (T25 and GA21), in addition to the three lines (Event176, Bt11, and MON 810) detected in our previous report, by a multiplex PCR. The safety assessment documents of GA21 available to the public did not fully provide information on the inserted DNA sequence. Therefore, we performed PCR of the genomic DNA with primer pairs designed from known sequences of inserted promoters and terminators, and sequenced the r-DNAs introduced into T25 and GA21 to design specific primers for them. In this report, we introduce a multiplex PCR method by which the five lines of GM maize were qualitatively and simultaneously distinguishable.

Materials and Methods

i) Maize (Zea mays) and other cereal samples

GM maize: Seeds of five lines of GM maize; i.e., the progenies of insect-resistant corn Bt11 and Event 176 developed by Novartis Seeds Inc. (Greensboro, NC, USA), insect-resistant MON810 and glyphosate-tolerant GA21 developed by Monsanto Company (St. Louis, MO, USA), and glufosinate-tolerant T25 developed by Aventis CropScience (Lyon, France) were directly imported from the USA. Conventional non-GM maize: Hybrid 1412 dried seeds sold by Dairyland Seed Co. (West Bend, WI, USA) were directly imported from the USA. GM soy: Seeds of glyphosate-tolerant "Roundup Ready Soy" developed by Monsanto Company were directly imported from the USA. Conventional non-genetically modified soy (non-GM soy): dried grain produced in the State of Ohio, USA, in 1998, was directly imported from the USA. A rice (*Oryza sativa*) variety "Kinuhikari", a wheat (*Triticum aestivum*) variety "Haruyutaka", and a barley (*Hordeum vulgare*) variety "Harrington" were used to study the specificity of the designed primer pairs.

ii) Reagents

Tris (hydroxymethyl) aminomethane (Tris): Sigma Chemical Co. (St. Louis, MO, USA). Ethylenediaminetetraacetic acid salts (EDTA): Dojindo Laboratories (Kumamoto, Japan). Agarose: LO3 [TAKARA] manu factured by Takara Shuzo Co., Ltd. (Kyoto, Japan). DNA marker: 100 bp ladder by New England Biolabs Inc. (Beverly, MA, USA). DNA polymerase: AmpliTaq Gold manufactured by PE Biosystems (Foster City, CA, USA); TAKARA Taq manufactured by Takara Shuzo Co., Ltd. Restriction enzymes: *Bst*EII and *Xba*I by New England Biolabs Inc.; *Sse*8387I, *Bsa*AI, and *Sfi*I by Takara Shuzo Co., Ltd. All other reagents: Special grade. Water: purified by CPW-200 (Advantec Toyo Kaisha Ltd., Tokyo, Japan) super water purifier.

iii) Equipment

Electric mill: DM-6, Yu Chi Machinery Co., Ltd. (Chang Hua, Taiwan). Centrifuge: Allegra™ 6KR, Beckman Coulter, Inc. (Fullerton, CA, USA). Electrophoresis equipment: Mupid® II, Advance Co., Ltd. (Tokyo, Japan). Image Analysis Instrument: Molecular Imager® FX System, Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Thermal cycler: GeneAmp PCR System 9700, PE Biosystems. Spectrophotometer: DU-7400, Beckman Coulter, Inc.

iv) Extraction of genomic DNA

Samples were ground in an electric mill. Ground samples (500 mg) were taken for DNA extraction. Genomic DNA was extracted using a silica spin column (DNeasy Plant MAXI kit, Qiagen GmbH, Hilden, Germany). The extraction was performed according to the manufacturer's manual, and genomic DNA was eluted with water from a silica spin column. The DNA concentration of solutions was determined by measuring the UV absorption at 260 nm. The quality of DNA was evaluated from the 260/280 nm and 260/230 nm UV absorption ratios, and by agarose gel electrophoresis. These DNA samples were used for the subsequent PCR-analysis.

v) Oligonucleotide primers

Primers were synthesized by Greiner Japan Co., Ltd. (Tokyo, Japan), purified on a reverse-phase column, then diluted with an appropriate volume of water to a final concentration of 50 μmol/L and stored at -20℃ until use.

To examine the DNA sequences inserted in T25 and GA21, two primer pairs (P35S 1-5′-T35S 1-3′ and rAct pro 1-5′-NOS ter 1-3′ in Table 1) were designed and

Table 1. List of PCR Primers

	Name	Sequence (5′→3′)	Specificity		Amplicon	Reference
A:	PE01 CR02	AGA TTC TTC ACT CCG ATG CAG CCT A CTC TCG GCG TAG ATT TGG TAC A	PEPC pro/sense *cryIA*(*b*)/anti-sense	Event176	0.9 kbp	4)
	CM01 CR02	CAC TAC AAA TGC CAT CAT TGC GAT A CTC TCG GCG TAG ATT TGG TAC A	P-35S/sense *cryIA*(*b*)/anti-sense	Bt11 MON810	1.2 kbp 1.4 kbp	4)
	P35S 1-5′ T35S 1-3′	ATT GAT GTG ATA TCT CCA CTG ACG T ACT AAG GGT TTC TTA TAT GCT CAA CA	P-35S/sense 35S terminator/anti-sense	T25	0.8 kbp	5) 5), 6)
	rAct pro 1-5′ NOS ter 1-3′	ATC TTT GGC CTT GGT AGT TTG ATT GCG GGA CTC TAA TCA TAA	P*r-act*/sense NOS-ter/anti-sense	GA21	2.3 kbp	7), 8) 9)
B:	TR03 TR04	TCT GCC CTA TCA ACT TTC GAT GGT A AAT TTG CGC GCC TGC TGC CTT CCT T	Eukaryote/sense Eukaryote/anti-sense	Eukaryote	137 bp	11)
C:	Ze1 1-5′ Ze1 1-3′	CCT CAG TCG CAC ATA TCT ACT ATA CT CTA GAA TGC AGC ACC AAC AAA	*Ze1*/sense *Ze1*/anti-sense	maize	508 bp	10)
	E176 1-5′ cryIA 1-3′	GTA GCA GAC ACC CCT CTC CAC A TCG TTG ATG TTK GGG TTG TTG TCC	PEPC pro/sense *cryIA*(*b*)/anti-sense	Event 176	343 bp	
	Bt11 1-5′ cryIA 1-3′	CCA TTT TTC AGC TAG GAA GTT C TCG TTG ATG TTK GGG TTG TTG TCC	*adh1*-1S IVS6/sense *cryIA*(*b*)/anti-sense	Bt11	110 bp	
	T25 1-5′ T25 1-3′	GCC AGT TAG GCC AGT TAC CCA TGA GCG AAA CCC TAT AAG AAC CCT	*pat*/sense 35S terminator/anti-sense	T25	149 bp	
	M810 1-5′ cryIA 1-3′	GAG TTT CCT TTT TGT TGC TCT C TCG TTG ATG TTK GGG TTG TTG TCC	*hsp70* int./sense *cryIA*(*b*)/anti-sense	MON810	199 bp	
	GA21 1-5′ GA21 1-3′	ACG GTG GAA GAG TTC AAT GTA TG TCT CCT TGA TGG GCT GCA	OTP/sense *m-epsps*/anti-sense	GA21	270 bp	

A: for DNA sequencing
B: for confirmation of the feasibility of PCR amplification of DNAs extracted from soy, rice, wheat, and barley
C: for multiplex PCR

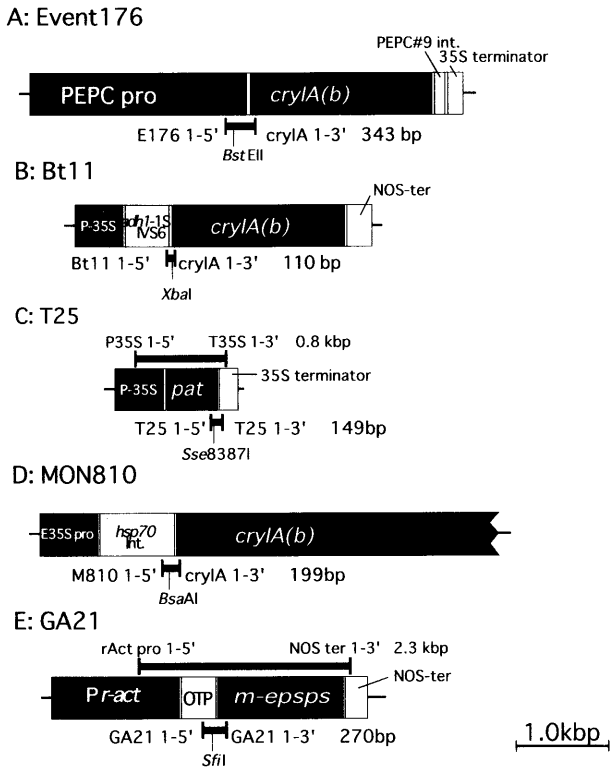

Fig. 1. Schematic diagrams of designed PCR primers in relation to the r-DNA units introduced into the five lines of GM maize (A–E)

The PCR primer pairs were designed to detect Event176, Bt11, T25, MON810, and GA21, respectively. The amplified products of E176 1-5′-cryIA 1-3′, Bt11 1-5′-cryIA 1-3′, T25 1-5′-T25 1-3′, M810 1-5′-cryIA 1-3′, and GA21 1-5′-GA21 1-3′ were confirmed by digestion with the restriction enzymes *Bst*EII, *Xba*I, *Sse*8387I, *Bsa*AI, and *Sfi*I, respectively.

The segments of P35S 1-5′-T35S 1-3′ and rAct pro 1-5′-NOS ter 1-3′ were sequenced.

A: Event 176: The unit of an insect-resistant trait consists of [maize phosphoenolpyruvate carboxylase promoter (PEPC pro)]–[synthetic *cryIA(b)* gene derived from *Bacillius thuringiensis* subsp. *kurstaki* strain HD-1]–[DNA fragment containing the No. 9 intron sequence (PEPC#9 int.) from maize phosphoenolpyruvate carboxylase]–[35S terminator derived from cauliflower mosic virus (CMV)].

B: Bt11: The unit of an insect-resistant trait consists of [CMV P-35S]–[DNA fragment containing the No.6 intron sequence (IVS6) from maize alcohol dehydrogenase 1 gene (*adh1*-1S)]–[synthetic *cryIA(b)*gene]–[NOS-ter derived from *Agrobacterium tumefaciens*].

C: T25: The unit of an herbicide-tolerant trait consists of [P-35S]–[*pat* gene]–[35S terminator].

D: MON810: The unit of an insect-resistant trait consists of [E35S pro derived from CMV promoter with the duplicated enhancer region]–[fragment of DNA (*hsp70* int.) containing the No. 1 intron sequence from maize *hsp70* gene (heat-shock protein)]–[synthetic *cryIA(b)* gene].

E: GA21: The unit of an herbicide-tolerant trait consists of [rice actin promoter containing the No. 1 intron (Pr-*act*)]–[optimized transit peptide sequence (OTP); DNA sequence for chloroplast transit peptide synthesized from the information on peptide sequences of the N-terminal upstream region of ribulose-1,5-bisphosphate carboxylase derived from maize and sunflower]–[point mutated *epsps* gene derived from maize (*m-epsps*)]–[NOS-ter].

synthesized, referring to the DNA Data Bank of Japan (Genbank accession Nos.V00141, S44221, and V00087) and to previous reports about P-35S[5], 35S terminator from CMV[5, 6], rice actin 1 gene promoter (P*r-act*)[7, 8], and NOS terminator (NOS-ter)[9]. For detection of Event 176, Bt11, and MON810, three 5′-primers and one 3′-primer were designed, referring to the sequence results, which were obtained by using two primer pairs (PE01-CR02 and CM01-CR02 in Table 1) in our previous report[4]. A Ze 1-5′-Ze 1-3′ primer pair was synthesized for detection of the intrinsic *zein* gene (*Ze1*)[10] based on Genbank accession No. M23537. A primer pair (TR03-TR04 in Table 1) was synthesized to confirm that the DNAs extracted from rice, soy, wheat, and barley were suitable for PCR, according to the method in Allmann *et al.*[11]. The r-DNA components introduced into the five lines of GM maize are shown in Fig. 1. The synthesized primers are shown in Table 1.

vi) DNA sequencing:

The direct DNA sequencings of PCR products were performed by Nippon Flour Mills Co., Ltd. (Tokyo, Japan). The PCR for sequencing was carried out under the following conditions. The reaction volume of 25 μL contained 25 ng genomic DNA, 0.5 μmol/L of each primer, 230 μmol/L of each dATP, dGTP, dTTP, and dCTP, and 1.25 units of Takara Taq polymerase. Reac-

tions were buffered by addition of the PCR buffer (Takara Shuzo Co., Ltd.) containing 1.75 mmol/L $MgCl_2$. Amplification was performed in a thermal cycler according to the following PCR step-cycle program: pre-incubation at 94℃ for 3 min; 40 cycles consisting of denaturation at 94℃ for 1 min, annealing at 60℃ for 1 min, and extension at 72℃ for 1 min; followed by a final extension at 72℃ for 7 min.

vii) PCR conditions and multiplex PCR conditions

PCR was carried out under the following conditions. The reaction volume of 25 μL contained 25 ng genomic DNA, 0.2 μmol/L of each primer, 1.5 mmol/L $MgCl_2$, 200 μmol/L of each dATP, dGTP, dTTP, and dCTP, and 1.25 units of AmpliTaq Gold DNA polymerase. Reactions were buffered by addition of the PCR buffer II (PE Biosystems). Amplification was performed in a thermal cycler according to the following PCR step-cycle program: pre-incubation at 95℃ for 10 min; 10 cycles consisting of denaturation at 95℃ for 0.5 min, annealing at 63℃ for 1 min, and extension at 72℃ for 1 min; 30 cycles consisting of denaturation at 95℃ for 0.5 min, annealing at 60℃ for 1 min and extension at 72℃ for 1 min; followed by a final extension at 72℃ for 7 min. The multiplex PCR conditions were the same as the PCR conditions, except that the reaction volume of 25 μL contained 0.2 μmol/L of Ze 1-5′, Ze 1-3′, E176 1-5′, Bt 11 1-5′, T25 1-5′, T25 1-3′, M810 1-5′, GA21 1-5′, and GA21 1-3′ primers, and 0.6 μmol/L of cryIA 1-3′ primer. After the PCR amplification, agarose gel electrophoresis was carried out using the PCR mixture and the r-DNA was identified from the length of the amplified DNA fragments. A band of approximately the expected length was confirmed by the profile of agarose gel electrophoresis after appropriate restriction enzyme digestion. Each experiment was repeated at least three times.

viii) Agarose gel electrophoresis

Five microliters of each extracted DNA solution or PCR reaction mixture was electrophoresed at constant voltage (100 V) on a 1.5% (for extracted DNA solution) or 3% (for PCR reaction mixture) agarose gel that had been supplemented with 0.5 μg/mL ethidium bromide in the TAE [40 mmol/L Tris-HCl, 40 mmol/L acetic acid, and 1 mmol/L EDTA (pH 8.0)] buffer solution. The gel was photographed with a Molecular Imager® FX System.

Results and Discussion

1) DNA extraction

We examined whether DNA extraction was suitable for r-DNA detection. The purity and quantity of the extracted DNA were evaluated by agarose gel electrophoresis and by using the 260/280 nm and the 260/230 nm UV absorption ratios; in most cases of maize, the absorption ratio of 260/230 nm was more than 1.7, and that of 260/280 nm was between 1.7 and 2.0. As described in a previous report[4], we extracted DNA by the CTAB method, and in most cases of maize the absorption ratio of 260/230 nm was more than 1.8, and that of 260/280 nm was between 1.8 and 2.0. The absorption ratios, however, fluctuated even among DNA solutions prepared from the same sample (data not shown), as well as among DNA solutions extracted by the CTAB method. To confirm the feasibility of PCR amplification of the extracted DNA, the PCR was carried out using a Ze 1-5′-Ze 1-3′ primer pair (for maize) or a TR03-TR04 primer pair (for soy, rice, wheat, and barley). The Ze 1-5′-Ze 1-3′ primer pair was designed from *Zel*, and we obtained the expected 508 bp amplification products from all maize samples. The TR03-TR04 primer pair is specific for highly conserved eukaryote DNA sequences, as previously described[11], and we obtained the expected 137 bp amplification product from soy, rice, wheat, and barley samples (data not shown). These data clearly showed that the DNA extraction by using a silica spin column, which reduced the experimental time and improved safety in the laboratory and potentially in the environment, provided sufficient DNA for PCR amplification, although the UV absorption ratios of DNA extracted by using a silica spin column was slightly lower than that by the CTAB method. Therefore, we adopted DNA extraction with a silica spin column instead of the CTAB method. Results of agarose gel electrophoresis of typical DNAs extracted from maize by the CTAB method and by using a silica spin column, respectively, are shown in Fig. 2.

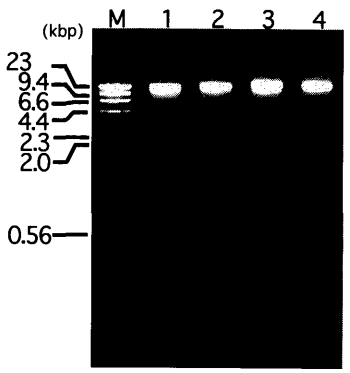

Fig. 2. Agarose gel electrophoresis of typical DNAs extracted from maize by the CTAB method and by using a silica spin column

Lanes 1 and 2: DNAs extracted from non-GM maize and GM maize (MON810), respectively, by the CTAB method; lanes 3 and 4: DNAs extracted from non-GM maize and GM maize (MON810), respectively, by using a silica spin column; M: λDNA *Hin*dIII digestion standard.

2) DNA sequencing and primer design

In the previous report, we introduced a detection method for r-DNAs from four lines of GM maize (Bt11, Event176, MON810, and T25). The method included a multiplex PCR for the three lines of GM maize (Bt11, Event 176, and MON810) possessing the *cryIA(b)* gene and a single PCR for T25 maize. In Japan, the GA21 maize line has been also authorized, as described above. Therefore, we tried to develop a new multiplex PCR

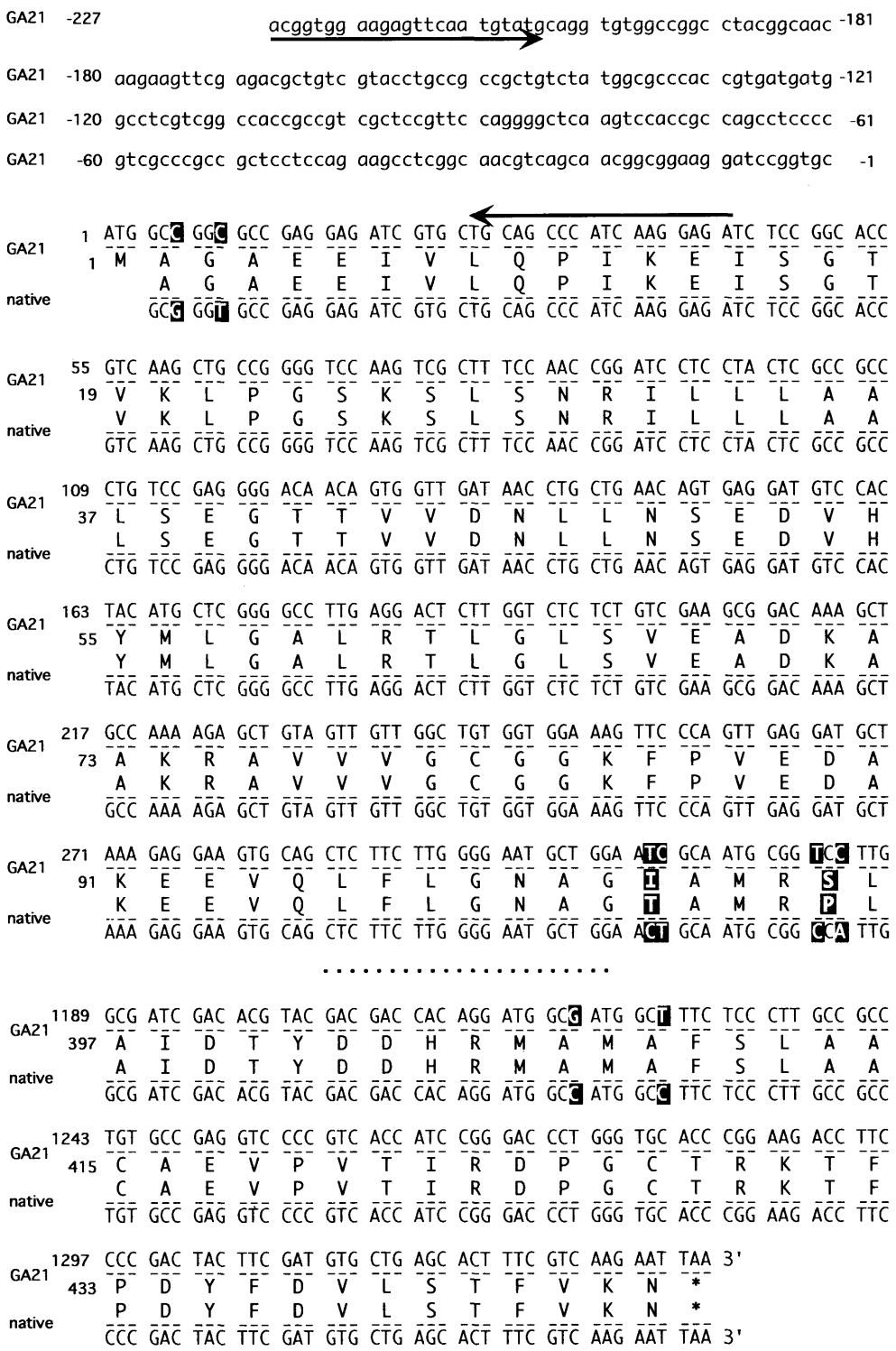

Fig. 3. Partial DNA sequences and derived amino acid sequences of the DNA amplified by PCR using a primer pair of rAct 1-5′-NOS ter 1-3′ for GA21, and the native *epsps* from *Z. mays*

The differences of DNA and amino acid sequences between the *m-epsps* introduced to GA21 and the native *epsps* of *Z. mays* are shown by outline type.

GA21: the sequences of DNA introduced into GA21 and of anticipated *m-epsps* amino acids; native: the sequences of *epsps* from *Z.mays* (accession No. X63374) DNA and of amino acids.

→: indicates the DNA sequence of the primer region of GA21 1-5′ used as a 5′-primer for PCR. ←: indicates the DNA sequence of the primer region of GA21 1-3′ used as a 3′-primer for PCR.

The 325 to 1,188 region of the DNA introduced into GA21 was identical to the native DNA sequence.

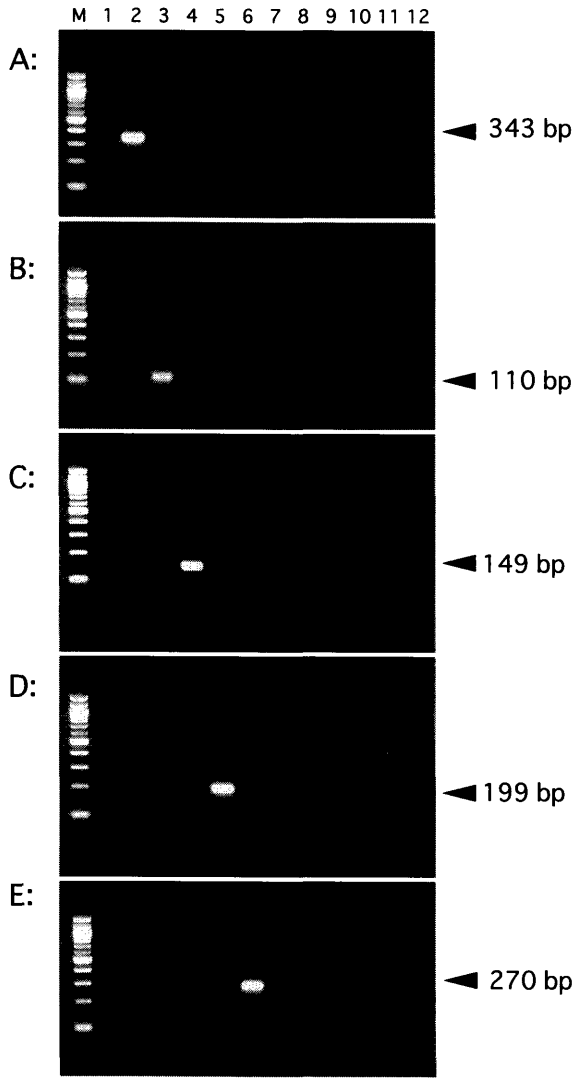

Fig. 4. Agarose gel electrophoresis of PCR products amplified from maize or other cereal DNAs using GM maize specific primer pairs

Arrowheads indicate the expected PCR amplification products.

The pairs of primers for detection of Event176 (A), Bt11(B), T25(C), MON810(D), and GA21(E), respectively, were used.

Lanes 1–6: amplification of maize DNAs from non-GM maize, Event176, Bt11, T25, MON810, and GA21, respectively; lanes 7–11: amplification of non-GM soy, GM soy, rice, wheat, and barley, respectively; lane 12: negative control (no maize DNA); M: 100 bp ladder size standard.

method by which these five lines of GM maize would be qualitatively and simultaneously distinguishable.

In order to design primer pairs for T25 and GA21 detection, sequence data of the inserted DNA were needed. The safety assessment documents, available to the public from the Japan Food Hygienic Association, provide general information about the inserted DNA and expressed protein. However, these documents do not fully describe the inserted DNA sequence. Therefore, we extracted the genomic DNA and performed PCR with primer pairs designed from known sequences of inserted promoters and terminators; i.e., CMV P-35S and 35S terminator for T25, and Pr-act and NOS-ter for GA21.

Our sequence result of the PCR product on T25 suggested that part of the *pat* gene was consistent with the DNA sequence data described in the public information. In the case of GA21, the public information claimed that the introduced 5-enolpyruvylshikimate-3-phospate synthase (m-EPSPS), which is composed of 445 amino acid residues, has about 99.3% or higher homology to the native gene at the amino acid level. The partial DNA sequence of the amplified DNA using a primer pair of rAct 1-5′-NOS ter 1-3′ and the anticipated amino acid sequence are shown in Fig. 3 with the native sequences from mRNA for EPSPS of *Z. mays* (Genbank accession No. X63374). The introduced *m-epsps* gene did not match 8 bases of the sequence of the native *m-epsps* gene, and the difference corresponded to two amino acid mutations (Fig. 3). The differences of amino acids presumably account for the glyphosate-tolerant trait of GA21.

On the basis of the sequence results, we designed specific primer pairs for the r-DNA in each GM maize to amplify an r-DNA segment derived from two exogenous organisms. Two new primer pairs, T25 1-5′-T25 1-3′ and GA21 1-5′-GA21 1-3, were synthesized for T25 and GA21 detection, respectively, and three new primer pairs, E176 1-5′-cryIA 1-3′, Bt11 1-5′-cryIA 1-3′, and M 810 1-5′-cryIA 1-3′ (shown in Table 1 and Fig. 1), were synthesized for Event176, Bt11, and MON810, respectively, as described in Materials and Methods. Because the combination of P-35S and the *pat* gene was not specific for T25, the primer pair CM03-PA01 (used in the previous paper[4]) gave PCR bands both from T25 and Bt11, though the length of the amplified bands was different. On the other hand, the combination of the *pat* gene and the 35S terminator was specific for T25, because the *pat* gene was introduced in the T25 and Bt11 lines with the same promoter and with a different terminator. We can specifically detect the five lines in one tube by using our multiplex PCR method. As shown in Table 1 and Fig. 1, the lengths were different among the expected PCR products for E176 1-5′-cryIA 1-3′, Bt11 1-5′-cryIA 1-3′, T25 1-5′-T25 1-3′, M810 1-5′-cryIA 1-3′, and GA21 1-5′-GA21 1-3′ in GM maize and for Ze 1-5′-Ze 1-3′ as an endogenous control for multiplex PCR. Further, we designed other specific primers for detection of DLL25 and DBT418, which were authorized by MHW and MAFF in Japan (data not shown). However, we cannot prove the efficacy of the designed primers because we are as yet unable to obtain positive control seeds for these lines.

3) Specificity of the designed primer pairs for maize and other cereals

The specificities of the designed primer pairs were confirmed by PCR for genomic DNAs extracted from each line of GM maize and other main cereal crops

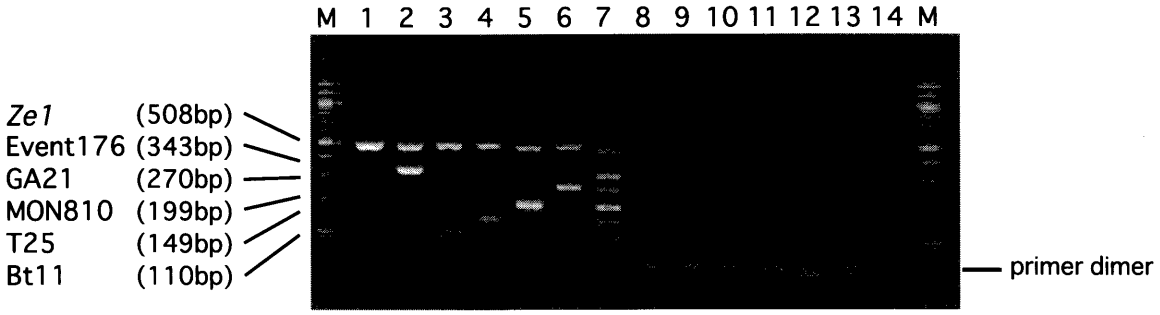

Fig. 5. Specificity of the primer pairs designed for the five lines of GM maize (Event176, Bt11, T25, MON810, and GA21), and for *zein* gene (*Ze1*)

Genomic DNAs extracted from each line of GM maize, non-GM maize, and other cereal crops (soy, rice, wheat, and barley) were amplified with a six primer-pairs mixture in one tube by a multiplex PCR method.

Lines indicate the expected PCR amplification products.

Lanes 1–6: amplification of non-GM maize, Event176, Bt11, T25, MON810, and GA21, respectively; lane 7: amplification of maize containing 20% of each of the five lines of GM maize; lanes 8–12: amplification of non-GM soy, GM soy, rice, wheat, and barley, respectively; lane 13: negative control (no DNA); lane 14: negative control (no primers); M: 100 bp ladder size standard.

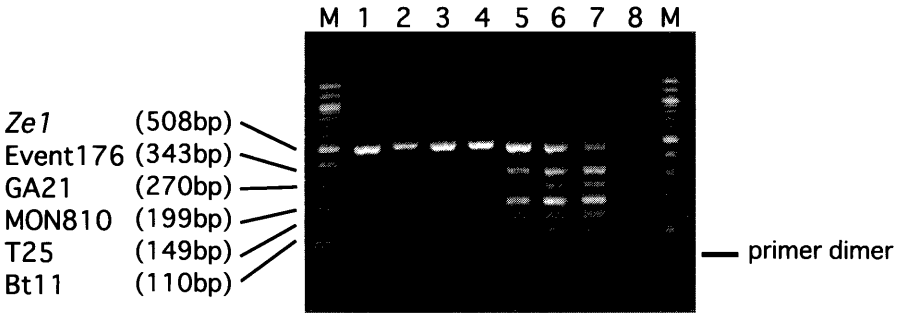

Fig. 6. Sensitivity of a multiplex PCR method using the primer pairs designed for the five lines of GM maize (Event176, Bt11, T25, MON810, and GA21) and for *zein* gene (*Ze1*)

Genomic DNAs extracted from the mixture containing the various amount of the five lines of GM maize in non-GM maize were amplified in one tube by a multiplex PCR method.

Lines indicate the expected PCR amplification products.

Lane 1: amplification of non-GM maize; lanes 2–7: amplification of maize containing 0.05, 0.1, 0.5, 1, 5, 20% of each of the five lines of GM maize, respectively; lane 8: negative control (no DNA); M: 100 bp ladder size standard.

widely used for processed foods. As shown in Fig. 4 A–E, each primer pair was specific for Event176, Bt11, T25, MON810, and GA21, respectively, and no amplifications for other maize, soy, rice, wheat, and barley were observed. These specificities are required to distinguish GM maize from other maize, and to prevent a false positive caused by other main cereal crops used in processed foods. These specific amplifications were accomplished by using primer pairs designed to amplify the region of a trait gene and a functional DNA sequence, such as intron, transit peptide, or terminator sequence, as shown in Fig 1.

4) Specificity and sensitivity of multiplex PCR for maize and other cereals

In the next step, amplification of multiplex PCR using a six primer-pairs mixture in one tube, which contained these five GM maize-specific primer-pairs and a maize-intrinsic primer pair, was investigated for all genomic DNAs extracted from maize, soy, rice, wheat, and barley. In addition, DNA was also extracted from mixtures of ground seeds, in which an equal weight of each of the five lines of GM maize (equivalent to 20% of each of the five lines of GM maize) was included. A Zel 1-5′-Zel 1-3′ primer pair was used for internal control of PCR. As shown in Fig. 5, with DNA extracted from each of the five lines of GM maize, PCR amplified only two different lengths of products that corresponded to the DNA lengths expected for the *Ze1* and each specific sequence in the respective line of GM maize. With DNA extracted from a mixture of the five lines of GM maize, PCR amplified six different lengths of products that corresponded to the DNA lengths expected for the *Ze1*, and specific DNA sequences for Event176, Bt11, T25, MON810, and GA21, respectively. No PCR amplification product was observed with DNAs extracted from soy, rice, wheat, and barley. Thus, the five lines of maize could be distinguished from the other cereals. Further, we carried out digestion of amplified DNA products with restriction enzymes *Bst*EII, *Xba*I, *Sse*8387

I, *Bsa*AI, and *Sfi*I, which correspond to Event176, Bt11, T25, MON810, and GA21, respectively. We confirmed that each amplified DNA corresponded to each r-DNA by specific restriction enzyme digestion. The intensity of the amplicon after agarose gel electrophoresis, however, was not equivalent in the five DNA fragments derived from the respective r-DNAs. The difference in intensity might depend on the DNA sequences of primers under our PCR conditions. In order to investigate the sensitivity, the DNAs were also extracted from mixtures of ground seeds containing 0.05, 0.1, 0.5, 1, 5, and 20% of each of the five lines of GM maize and non-GM maize. The r-DNAs were clearly detected from samples containing above 0.5% of each line of GM maize, as shown in Fig. 6. These amounts of DNA correspond to about 125 pg/tube of genomic DNAs of the five lines of GM maize. The result of PCR from the sample containing 0.1%, however, could be detected, but not stably. This sensitivity would be acceptable to secure the verification of non-GMO materials and to monitor the reliability of the labeling system. Furthermore, we carried out multiplex PCRs to clarify the sensitivities of our method under different conditions of the concentration of Ze1-5′ and Ze1-3′ primers (0.1 and 0.05 μmol/L). The sensitivities of detection were improved until 0.1% of each of GM maize and the amplified bands were clearly shown. However, the amplified band derived from *ze1* was weakened in the sample containing 20% at 0.1 μmol/L of Ze1-5′ and Ze1-3′ primers, and the bands disappeared over 0.1% of each of GM maize at 0.05 μmol/L of Ze1-5′ and Ze1-3′ primers (data not shown). To detect r-DNAs from samples containing less than 0.5% of GM maize, it would be better to modify the concentration of Ze1-5′ and Ze1-3′ primers to 0.1 μmol/L.

Conclusions

We developed a multiplex PCR method to distinguish among five lines of GM maize in one tube. For genomic DNA extraction, we used a silica spin column to reduce experimental time and improve safety in the laboratory and potentially in the environment. The specificity of this PCR method was investigated by using DNAs extracted from maize, soy, rice, wheat, and barley. The expected amplicons were obtained by using DNA extracted from maize, and no amplicon was obtained by using DNAs extracted from soy, rice, wheat, and barley. The sensitivity of this method was also investigated, and the r-DNA could be clearly detected from samples containing above 0.5% of each line of GM maize. Thus, this method should be effective, particularly when the sample includes any of the five lines of GM maize and other cereal crops (such as soy, rice, wheat, and barley). This method should be applied only to maize grain, because heating and physical processes usually degrade the genomic DNA existing in processed foods, and the lengths of the amplicons designed in this report may be too long to detect under those conditions. However, this method might be applicable to processed foods to detect Ze1, because the PCR product from Ze1-5′-Ze1-3′ is the largest DNA fragment.

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