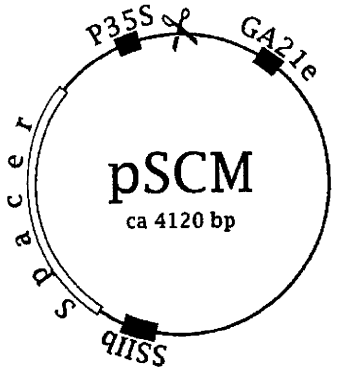

Fig. 1. Schematic illustration of plasmid, designated pSCM, for calibration of duplex real-time PCR

The filled boxes indicate cloned segments including each of the amplification targets. The open box indicates the spacer derived from lambda phase DNA. The pSCM plasmid was digested on the site indicated with a scissor symbol and used for calibration.

25 μL reaction solution for the duplex real-time PCR contained 1 × Universal Master Mix (Applied Biosystems), 50 ng genomic DNA as a template, 0.5 μmol/L of each of the primers, and 0.1 μmol/L of each of the TaqMan® probes. The duplex real-time PCR was performed with the 7900 real-time PCR system or the 7500 real-time PCR system (Applied Biosystems), and all reactions were performed with the 9600 emulation mode and the following step-cycle program: pre-incubation at 50°C for 2 min and 90°C for 10 min, 45 cycles consisting of denaturation at 95°C for 0.5 min and annealing and extension at 59°C for 1 min. Each amplification was performed in parallel in three wells, unless otherwise described.

For the analytical calibration, a dilution series of the plasmid was employed in this study (Fig. 1). The plasmid was extracted from bacterial bulk culture using the QIAGEN® plasmid Mega kit (QIAGEN) and linearized by restriction digestion at sites located outside of the inserted segments. The linearized plasmid DNA was purified by CsCl ultracentrifugation and diluted with TE buffer, including 5 ng/μL of ColE1 plasmid, to five different concentrations, *i.e.*, 20, 125, 1,500, 20,000, and 250,000 copies per 2.5 μL, based on the copy numbers of the maize *starch synthase IIb* gene (*SSIIb*) segment.

The GM contents of the samples were calculated from the proportion of the copy number of the GM-specific segment to that of the taxonomic marker for maize, *SSIIb*, by the following formula:

$$\text{GM content (\%)} = \frac{\text{Copy number of GM-specific segment}}{\text{Copy number of taxonomic marker}} \times \frac{1}{C_f} \times 100.$$

The real-time PCRs for the quantitation of the copy numbers of the GM-specific segment(s) and that of the *SSIIb* segment were performed on the same PCR run. The ratios of the GM-specific sequence to the taxon-specific sequence in the DNA extracted from each genuine GM seed were defined as conversion factors (C_f). We calculated the experimental conversion factors for each of the methods and real-time PCR devices, respectively, and those obtained in this study are listed in Table 2.

Table 2. Experimental conversion factors used in this study

Method	Device	Target GM	C_f[a]
MON810 construct sp.	7700	MON810	0.38[b]
GA21 construct sp.	7700	GA21	2.01[b]
Duplex	7900	MON810	0.33
		GA21	0.50
	7500	MON810	0.30
		GA21	0.45

[a] Conversion factors used in this study were culculated from means of measurements of ten lots with three replications.

[b] These factors were referred to footnote * 3.

For comparison, we analyzed the same samples by means of the construct-specific, simplex real-time PCR methods for MON810 maize or to GA21 maize performed with the 7700 real-time PCR system (Applied Biosystems). These methods employed the construct-specific primer pairs (Table 1) used in our established methods[12] and GM Maize Detection Plasmid Set —ColE1/TE— (Nippon Gene Co., Ltd., Toyama, Japan) for the analytical calibration. An improved SSIIb primer pair (SSIIb03 -5′ and -3′) described elsewhere[16] was used for the construct-specific real-time PCR as well as for the duplex real-time PCR (Table 1).

Analytes for single laboratory evaluation

Four sorts of simulated mixtures of MON810 as targets for the P35S quantitation and GA21 with different commingled levels in a non-GM background, i.e. 0.5% (w/w), 1% (w/w), 5% (w/w) and 10% (w/w) each, were prepared as described in our previous report[13]. Each level of mixture was prepared on a 100 g or 200 g scale, and was divided into aliquots of about 1 g (about 100 or 200 independent packets were prepared). The homogeneity of each simulated mixture sample was confirmed by one-way ANOVA tests[12]. Ten packets chosen at random from each commingled level of samples were subjected to quantitation with the established methods, and each measurement was performed twice for each sample. Table 3 shows the results of one-way ANOVA tests of the logit-transformed means for each duplicate quantitation. These results showed that the between-lot variations were not different from the within-lot variations in each sort of simulated mixture. Thus, the simulated mixtures used in this study were found to be sufficiently homogenous within each sort.

Table 3. Homogeneity of the simulated mixtures[a]

Sample	Target GMO	Mixing level, %	Measured mean, %[b]	95% Confidence limit[b]		*F*-value	*p*-value
				Lower, %	Upper, %		
A	MON810	0.5	0.48	0.44	0.51	0.38	0.92
	GA21	0.5	0.50	0.47	0.53	0.88	0.57
B	MON810	1	1.17	1.13	1.22	2.7	0.067
	GA21	1	1.09	1.05	1.14	1.2	0.40
C	MON810	5	4.47	4.32	4.63	0.47	0.86
	GA21	5	4.84	4.67	5.02	2.7	0.070
D	MON810	10	9.21	8.83	9.60	0.29	0.96
	GA21	10	9.64	9.28	10.01	0.78	0.64

[a] Measurements of ten lots with two replications were transformed using the logit transformation and were analyzed by one-way ANOVA.
[b] Means and 95% confidence limits were calculated based on logistic values and transformed using the inverse logit transformation.

Results and Discussion

Designing of duplex real-time PCR

As the targets of this method, we chose the P35S and an event-specific segment for GA21. The CaMV35S promoter has been found in a very wide range of GM crops, and this has led to the development of many GMO detection methods targeting this segment with real-time PCR technology[12),16)-18)]. We also chose an event-specific segment for GA21 as a target of this method[13)] because GA 21 maize does not contain a P35S segment, and the P35 S and the GA21 maize event-specific (GA21e) segment do not overlap in any GM maize. We could have chosen the P35S and the terminator of the *nopalin synthase* gene (TNOS), which is the most popular terminator used in GM crops. This may be able to detect a wider range of GM maize. On the other hand, there are many events which have both the P35S segment and the TNOS segment. Thus, the method targeting both of the segments would tend to over-estimate the GM content more than the duplex real-time PCR method developed in this study.

The PCR conditions for these methods have been optimized under the simplex real-time PCR conditions. In this study, we first assembled these simplex methods to design a duplex real-time PCR method and examined its performance. For this purpose, we constructed a duplex real-time PCR by assembling two real-time PCR methods for P35S and GA21e. As shown in Fig. 2, the combined primer pairs for P35S and GA21e gave specific amplification for the relevant GM maize and GM soybean, and no amplification was observed in the reactions of non-GM crops. These primer pairs were therefore chosen for the design of the duplex real-time PCR method.

TaqMan probes for their amplification products were also designed, and the fluorescent modifications were carefully considered for the duplex real-time PCR quantitation. In this study, we found a combination consisting of the P35S-TaqFB probe modified with 5-carboxyfluorescein (FAM) at the 5′-end and with Black Hole Quencher 1 (BHQ1) at the 3′-end, and the GA21 es-TaqHB probe modified with 6-carboxy-2′, 4,4′, 5′,7,7′-hexachlorofluorescein (HEX) at the 5′-end and with BHQ1 at the 3′-end (Table 1). Quencher molecules without fluorescence emission are preferable for multiplex real-time PCR methods, because they reduce the risk of interference of fluorescence emission caused by multiple reporters. In fact, other types of probe are also available, such as MGB probe with non-fluorescent quencher (DABCYL). However, this type of probe was not convertible with the currently used probes and required redesign of the oligonucleotide sequences of the probes. Thus, we chose BHQ1 for the quencher molecule. For reporter molecules, we examined six kinds of fluorescent substances and their combinations. We concluded that a combination of FAM as the reporter of the P35S probe and HEX as that of the GA21e probe was optimum for this method (data not shown). VIC also showed good performance, but we chose BHQ1 because of a commercial reason.

Construction of calibrant plasmid and its performance in duplex real-time PCR

We have already developed some real-time PCR methods for the quantitation of GM contents, and plasmid DNAs containing tandemly ligated GM-specific DNA segments and taxon-specific DNA segments have been used as analytical calibrators[12)]. In this study, we constructed a new calibrant plasmid for the duplex PCR method. To construct it, we had to ensure that unintended amplification between the insertions in the plasmid did not occur. Figure 1 shows the schematic diagram of the calibrant plasmid, designated as pSCM. The pSCM plasmid has three DNA segments; 101 bp of the P35S segment, 111 bp of the GA21e segment, and 151 bp of the *SSIIb* segment. The inserted sites were mutually separated by more than a 1 kb interval, and after digestion, the calibrant plasmid was linearized for use (Fig. 1). We confirmed that no unintended amplification was observed with the pSCM plasmid under both the qualitative and quantitative PCR conditions (Figs. 2

and 3).

The duplex and the simplex real-time PCRs were performed with the pSCM plasmids diluted to a concentration ranging from 20 to 250,000 copies per reaction. Figure 3 shows the amplification plots of the duplex real-time PCR (B, D), and the simplex real-time PCRs (A, C) which were the components of the duplex real-time PCR. Each detection plot for P35S (E, F) and GA21e (G, H) demonstrated correlated amplification with the copy number of its target segment, and the efficiency of PCR amplification in the duplex real-time PCR was almost equivalent to that in the simplex PCRs (Fig. 3). In addition, we performed the duplex real-time PCR using two types of real-time PCR devices with different fluorescence detection mechanisms. No significant optical crosstalk between the detection channels was observed on either device (data not shown). Thus, we concluded that the duplex real-time PCR would be a good candidate for a routine method for the screening of agricultural crops.

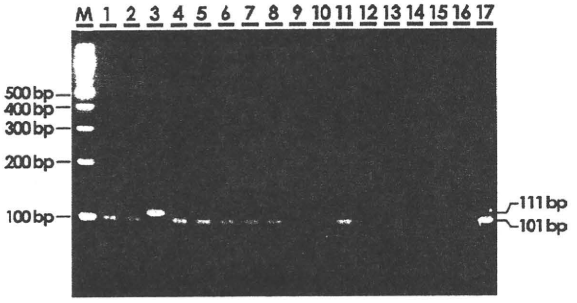

Fig. 2. Specificity of duplex PCR

The qualitative PCR was performed with the combination of primer pairs developed in this study. 111 bp and 101 bp bands were amplification products corresponding to GA21e and P35S segments, respectively. Lanes 1–8, amplification of DNAs extracted from the seeds of NK603, Event176, T25, GA21, MON863, MON 810, TC1507 and Bt11, respectively; lane 9, non-GM maize; lane 10, non-GM soy; lane 11, Roundup Ready® soy; lane 12, rice; lane 13, wheat; and lane 14, barley. Lanes 15 and 16 are the negative controls (no template and no primers, respectively). Lane 17 is an amplification of pSCM plasmid. Lane M shows 100 bp ladder size standards. Each electrophoresis was performed on 3% agarose gel.

Precision of duplex real-time PCR method

To evaluate the precision of the duplex real-time PCR method, a single-laboratory evaluation experiment was designed based on the harmonized guidelines of IUPAC, ISO, and AOAC[20], and conducted. In this study, MON 810 was chosen as a representative of GM maize events containing the P35S segment, because it has been widely grown and has only one P35S segment per GM haploid. Then, we prepared simulated samples containing two events, MON810 and GA21, in four different GM commingle levels (0.5%, 1%, 5%, and 10%). These simulated samples were analyzed by the established real-time PCR methods[12], and by the newly developed duplex real-time PCR method (Tables 4 and 5).

In this study, we employed the 7900 real-time PCR system as one of the analytical devices for the duplex real-time PCR method, which is a successor of the 7700 real-time PCR system used previously[12]. In addition, we also analyzed the same samples by the duplex real-time PCR method with the 7500 real-time PCR system, which has a different optical system than the 7900 system. By the duplex real-time PCR method with the 7900 real-time PCR system, the relative standard deviation (RSD) scores ranged from 2.8% to 8.2% for MON

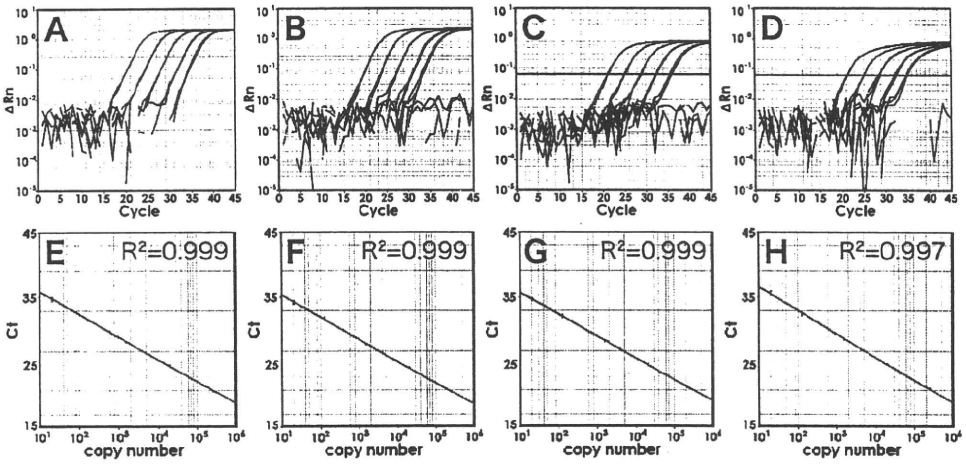

Fig. 3. Amplification plots for dilution series of pSCM plasmid and corresponding standard curves

Typical patterns of amplification plots of a P35S segment are shown in A and B, and those for a GA21 event-specific segment are shown in C and D. Each amplification was performed using the individual simplex real-time PCRs (A, C) or the duplex real-time PCR (B, D) with a 7900 real-time PCR system. The respective calibration curves are shown in the panels below. The correlation factors of the standard curves are indicated at the upper-right corners.

Table 4. Trueness and precision statistics of measurements of the estimated method[a]

Sample	Target GMO	Mixing level, %	Trueness		Precision	
			Measured mean, %	Bias, relative%	SD[b]	RSD[c]
non-GMO	MON810[d]	0	0.00	nd	0.00	nd
	GA21[e]	0	0.00	nd	0.00	nd
A	MON810	0.5	0.45	−0.090	0.042	9.2
	GA21	0.5	0.48	−0.040	0.042	8.8
B	MON810	1	1.17	0.17	0.06	5.4
	GA21	1	1.11	0.11	0.052	4.7
C	MON810	5	4.61	−0.078	0.19	4.2
	GA21	5	4.79	−0.041	0.27	5.7
D	MON810	10	9.63	−0.037	0.43	4.5
	GA21	10	9.40	−0.060	0.49	5.2

[a] Each experiment was performed with ten independent packets. Each real-time PCR was performed with three wells replication, and repeated twice on independent PCR runs. Means of those measurements were used for statistical analyses.
[b] SD: Standard deviation
[c] RSD: Relative standard deviation
[d] Analyses were performed by the construct-specific real-time PCR method for MON810 maize[12].
[e] Analyses were performed by the construct-specific real-time PCR method for GA21 maize[12].

Table 5. Trueness and precision statistics of measurements of the duplex real-time PCR methods

Instrument	Sample	Target GMO	Mixing level, %	Trueness		Precision	
				Measured mean, %	Bias, relative%	SD[b]	RSD[c]
7900	non-GMO	MON810[d]	0	0.00	nd	0.00	nd
		GA21[d]	0	0.00	nd	0.00	nd
	A	MON810	0.5	0.51	0.028	0.037	7.3
		GA21	0.5	0.52	0.032	0.046	8.9
	B	MON810	1	1.03	0.026	0.084	8.2
		GA21	1	0.93	−0.074	0.062	6.7
	C	MON810	5	5.44	0.088	0.24	4.3
		GA21	5	4.70	−0.059	0.13	2.8
	D	MON810	10	11.06	0.11	0.31	2.8
		GA21	10	9.59	−0.041	0.36	3.7
7500	non-GMO	MON810	0	0.00	nd	0.00	nd
		GA21	0	0.00	nd	0.00	nd
	A	MON810	0.5	0.52	0.045	0.053	10.1
		GA21	0.5	0.44	−0.12	0.036	8.1
	B	MON810	1	1.03	0.025	0.066	6.4
		GA21	1	0.87	−0.13	0.075	8.7
	C	MON810	5	4.65	−0.071	0.40	8.7
		GA21	5	3.95	−0.21	0.33	8.4
	D	MON810	10	10.01	0.0012	0.88	8.8
		GA21	10	8.61	−0.14	0.55	6.4

[a] Each experiment was performed with ten independent packets. Each real-time PCR was performed with three wells replication, and repeated twice on independent PCR runs. Means of those measurements were used for statistical analyses.
[b] SD: Standard deviation
[c] RSD: Relative standard deviation
[d] GM-specific segment was analyzed by the duplex real-time PCR.

810 and from 2.8% to 8.9% for GA21 (Table 5). Comparison of these scores with those obtained by the established methods suggested that the precision of the duplex real-time PCR method was almost the same as those of the established methods (Tables 4 and 5). As for results obtained with the 7500 real-time PCR system,

the RSD scores were generally higher than those obtained with the 7900 real-time PCR system, but most of them were found to be below 10% (Table 5). The general analytical uncertainty of the quantitative PCR method was found to be around 25%[4]. These results demonstrated that the precision of the developed method satisfied the criteria of quantitative analysis by the real-time PCR method at the tested range of 0.5 to 10%. The result of single-laboratory evaluation suggested that the limit of quantitation (LOQ) of the duplex real-time PCR method is lower than 0.5% for both MON810 and GA21 maize (Table 5). This LOQ is the same as that seen in our previous real-time PCR methods, which have a GM-specific amplification site per GM haploid[15]. Since the regulatory threshold level for obligatory GM labeling is 5% in Japan, the LOQ is sufficient for a regulatory analytical method. Thus, although future interlaboratory validation is needed, the current results indicate that the duplex real-time PCR would be a good candidate for a routine method for the screening of agricultural crops. Further investigations are necessary, regarding the rapidly increasing stacked varieties.

Conclusion

In this study, we developed a duplex real-time PCR that could quantitatively detect not only a P35S segment but also an event-specific segment for a Roundup Ready maize, GA21. The method using the duplex real-time PCR quantified the contents of GM maize with P35S and GA21 simultaneously, and their sum represented the total content of GM maize in the sample. The result of a single-laboratory evaluation demonstrated that the precision of this method is almost the same as those of the established methods. In addition, this method will reduce both the cost and time requirement of routine GMO analysis by half. Consequently, the duplex real-time PCR method was considered to be a good candidate for a routine screening method for GM maize commingled in agricultural products.

Acknowledgements

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Report

Investigation of Residual DNAs in Sugar from Sugar Beet (*Beta vulgaris* L.)

(Received August 5, 2008)

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Genetically modified (GM) sugar beets have been bred for use as food and animal feed. To evaluate the applicability of GMO analyses to beet sugar products, we investigated residual DNA in eight sorts of in-process beet sugar samples and commercial beet sugar products. Polymerase chain reaction (PCR) analyses with taxon-specific primers indicated that sugar beet DNA was degraded at an early stage of sugar processing, and no PCR amplification was detected from the investigated sugar products because of low DNA recovery and/or PCR inhibition.

Key words: genetically modified (GM); sugar beet (*Beta vulgaris* L.); deoxyribonucleic acid (DNA); polymerase chain reaction (PCR); taxon specific DNA

Introduction

Recombinant DNA technologies have been increasingly used in modern farming and are thought to offer various advantages. The global area of genetically modified (GM) crops exceeded 120 million hectares in 2007, and is expected to continue to rise[1]. GM crops have been authorized for use as food and/or animal feed in many countries based on their own criteria for safety assessment. However, consumers have demanded appropriate information and labeling for foods derived from GM crops. Thus, labeling systems have been introduced for GM foods in the European Union (EU), Korea, Japan, Australia and other countries, and these systems are distinct from each other[2]. In addition, many countries have been seeking ways for the coexistence of cultivation of conventional crops and GM crops. In these situations, scientifically sound GMO detection methods have become more important. Sugar beet is a major agricultural crop, used as the raw material for refined sugar, especially in cool regions. GM sugar beets have also been bred and authorized for food and/or feed applications by many countries. Therefore it is desirable to survey the commercial distribution of GM sugar beets and/or their processed foods. However, it has not been established whether or not sufficient amounts and/or quality of DNAs for DNA extraction-based analyses remain in refined beet sugar products. In this study, we investigated residual DNA in commercial beet sugar products and assessed the appropriateness of GMO analysis methods for processed sugar beet products for regulatory purposes.

Materials and Methods

Materials

Fresh sugar beet and eight sorts of in-process samples of sugar beets were kindly provided by the Japan Beet Sugar Association. The sampling points of the in-process samples are indicated in Fig. 1. Eight sorts of commercial beet sugar samples were purchased from markets in Tokyo and Sapporo in Japan. Nineteen varieties of sugar beets and four plants closely related to sugar beets were provided by the National Agricultural Research Center for the Hokkaido Region, and used for the specificity tests of PCR primers.

DNA extraction

For the PCR experimental controls, DNAs were extracted from leaves or aerial parts of seedlings with a DNeasy® plant Maxi kit (QIAGEN, Hilden, Germany) according to the attached protocol. DNA extraction from in-process samples and beet sugar products was performed using an anion exchange column, Genomic-tip 20/G (QIAGEN), and the experimental pro-

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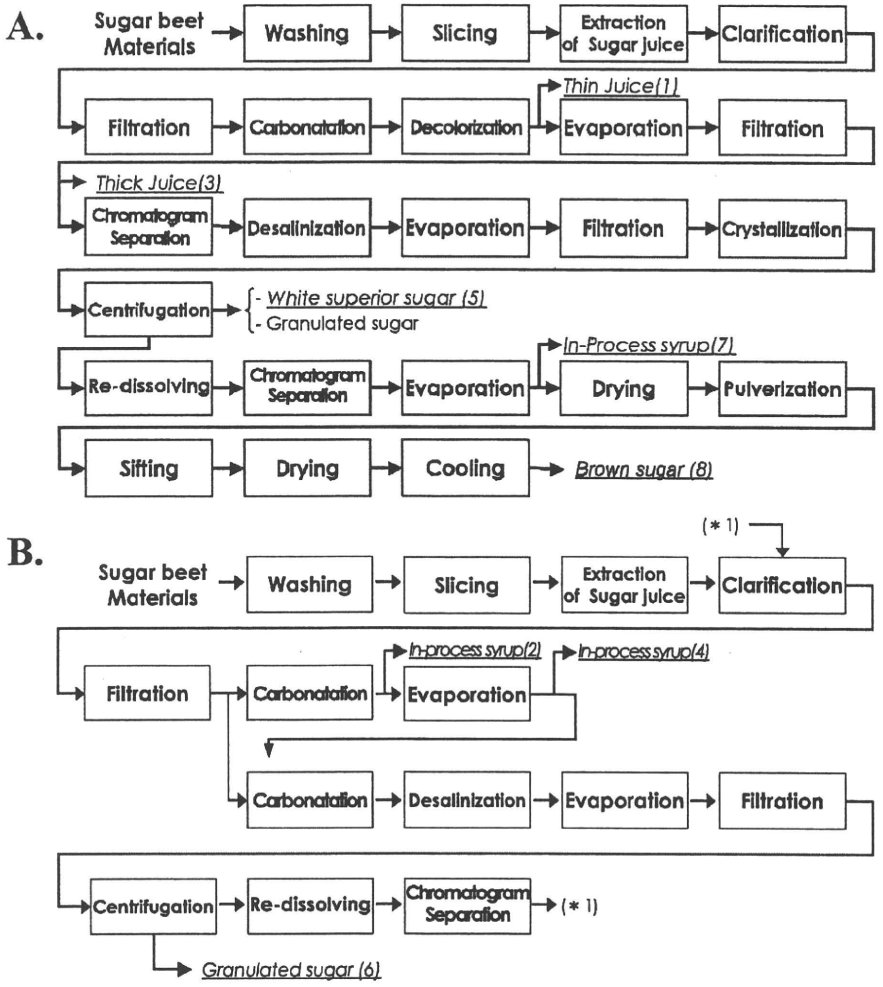

Fig. 1. Flow charts of beet sugar processes

Sugar beet materials are washed and sliced into thin strips called cassettes, which go through a diffuser machine to extract raw sugar juices. The raw juices are clarified and filtered to remove beet pulp. Filtered raw juices are mixed with "milk of lime", which consists of calcium oxide and carbon dioxide gas, and non-sugar components in the juices are precipitated with the calcium carbonate. Then, the supernatant is called thin juice. The thin juice is purified and evaporated to obtain thicker juice. The thick juice goes into a crystallizer tank and fine sugar crystals grow. The crystallized sugar is purified to fine sugar products, such as white superior sugar and granulated sugar. Much sugar remains in the syrup, and then chromatographic separation is performed on the syrup to produce brown sugar. The processes shown here are two examples (flow chart A and B) and there are some differences in processing from company to company. The underlined terms indicated the points at which specimens of the in-process products were taken for use in this study, and the numbers in parentheses correspond to the numbers in Table 1 and Fig. 4.

cedure generally followed the Japanese standard method for GMO analysis on food items with some modifications[3], *1. Extracted DNAs were finally resuspended in 40 μL of sterile distilled water. All extraction experiments were performed in a clean laboratory with designated operators, and two independent extracts were made from the in-process samples and beet sugar products.

*1 Notification No. 110 (Mar. 27, 2001), Department of Food Safety, MHLW, Japan (2001).

Estimation of amount and quality of extracted DNAs

The amount and quality of the extracted DNA solutions was estimated from the ultraviolet (UV) absorption spectrum measured by a UV spectrophotometer, ND-1000 (NanoDrop® Technologies, Wilmington, DE, USA). 1.5 μL of each undiluted DNA extract was directly subjected to UV measurement, and the UV absorptions at 230 nm, 260 nm, and 280 nm were observed. In addition, as the experimental control, we also performed DNA quantitation using the Qant-iT™ PicoGreen® dsDNA quantitation reagent (Molecular Probes, Eugene, OR, USA) and Cytofluore® 2350 (Millipore, Billerica, MA, USA). The experimental proce-

Table 1. Quality of the extracted DNA from in-process samples and beet sugar produsts

#	Type	Sample[a]	260 nm/280 nm[b]	260 nm/230 nm[c]	Conc. (ng/μL)[d]	Conc. (ng/μL)[e]
1	In-process samples	Thin juice	2.0	0.28	13.4	8.36
2		In-process syrup	1.2	0.08	3.3	0.72
3		Thick juice	2.3	0.07	3.0	0.58
4		In-process syrup	1.7	0.09	3.8	0.83
5		White superior sugar	1.2	0.08	2.2	0.36
6		Granulated sugar	1.7	0.09	3.0	−0.16
7		In-process syrup	1.7	0.07	3.1	−0.03
8		Brown Sugar	1.3	0.10	4.3	0.01
9	Commercial beet	Brown sugar (1)	2.8	0.09	4.3	0.39
10	sugar products	Brown sugar (2)	4.0	0.08	3.7	0.85
11		Syrup-type product added oligosaccharide	2.3	0.06	2.3	0.70
12		Granulated sugar (1)	2.7	0.06	2.9	0.72
13		White superior sugar	20.3	0.07	2.7	0.73
14		Brown sugar (3)	2.2	0.06	2.3	0.73
15		Bleached brown sugar	1.2	0.07	3.5	0.68
16		Granulated sugar (2)	5.0	0.06	2.6	0.66

[a] DNA extractions were performed with two independent replications, and UV absorptions are given as the means of them.
[b] The ratios of DNA solution in good condition usually range from 1.7 to 2.0.
[c] The ratios of DNA solution in good condition are usually more than 0.6.
[d] Calculated concentrations estimated from UV absorptions.
[e] Calculated concentrations estimated using the Qant-iT™ PicoGreen® reagent.

dures followed the manufacturers' protocols.

Qualitative PCR analysis

The GeneAmp® PCR system 9700 (Applied Biosystems; ABI, Foster City, CA, USA) was used in the max mode, and the PCR mixture, in a final volume of 25 μL, consisted of 1X PCR buffer II (ABI), 0.2 mM dNTPs (ABI), 1.5 mM $MgCl_2$ (ABI), 0.025 U AmpliTaq® Gold DNA polymerase (ABI), 0.5 μM each primer and DNA sample. Twenty-five ng (2.5 μL of 10 ng/μL) of template DNA, as calculated from the UV absorption at 260 nm, was used for PCR analysis unless otherwise described. When the concentration of extracted DNA was not enough, the maximum volume (17,875 μL) of undiluted DNA extract was used for the reaction. The primer pairs used in this study were as follows: primer pair 1; 5′-GCCCCCAAAAACCCTTCA-3′ and 5′-GGGCAATTTGGTAGGCTTCTT-3′, and primer pair 2; 5′-ATCCCTGCAGCCATCAGTGA-3′ and 5′-ACCAGTAAGCCACTCAACAGTCAA-3′. As an inhibition assay of PCR, we observed amplification from each reaction mixture spiked with 260 pg of extracted DNA from the sugar beet plant (cv. Skane). Twenty-five μL of PCR mixture consisted of 1X PCR buffer II (ABI), 0.2 mM dNTPs (ABI), 1.5 mM $MgCl_2$ (ABI), 0.025 U AmpliTaq® Gold DNA polymerase (ABI), 0.5 μM each primer, 16,875 μL of DNA extraction, and 1 μL of 260 pg/μL spike DNA.

The qualitative PCR reactions was performed on a thermal cycler, the Silver 96-Well GeneAmp® PCR System 9700 (ABI) in Max mode, according to the following step-cycle program: pre-incubation at 95℃ for 10 min; 40 cycles consisting of denaturation at 95℃ for 0.5 min, annealing at 60℃ for 0.5 min and extension at 72℃ for 0.5 min; followed by a final extension at 72℃ for 7 min. After the amplification, PCR products were electrophoresed on 3% agarose gels buffered with Tris-Acetate-EDTA (TAE) solution.

Results and Discussion

Yield and quality of extracted DNA from in-process samples and beet sugar products

DNA was extracted from 1 g of each in-process or beet sugar product sample with the anion exchange column, Genomic-tip 20/G (QIAGEN), which we have been using for the DNA extraction of highly processed foods[3]. The yield and quality of the extracted DNA solution was estimated from the UV absorption spectrum. The UV absorption ratios at 260 nm/280 nm of most samples were out of the optimal range of 1.7 to 2.0, which indicated poor quality of DNA (Table 1). Moreover, absorption ratios of 260 nm/230 nm ranged from 0.06 to 0.28, which implied that sugars contaminated the extracted DNA solutions (Table 1). Based on the UV absorption at 260 nm, the calculated concentrations of extracted DNAs ranged from 2.2 ng/μL (white superior sugar) to 13.4 ng/μL (thin juice) and from 2.3 ng/μL (syrup-type product with added oligosaccharide, and brown sugar #3) to 4.3 ng/μL (brown sugar #1) for in-process samples and commercial products, respectively (Table 1). Following the Japanese standard method for GM analyses, we generally used 25 ng of DNA, *i.e.*, 2.5 μL of 10 ng/μL diluted DNA, for quantitative PCR analysis. Thus, these yields were very low. In addition, the UV absorption measurements indicated that contamination with nucleic acids and/or other substances unrelated to PCR amplification was probably presnt. In fact, fluorometric quantitation of double-

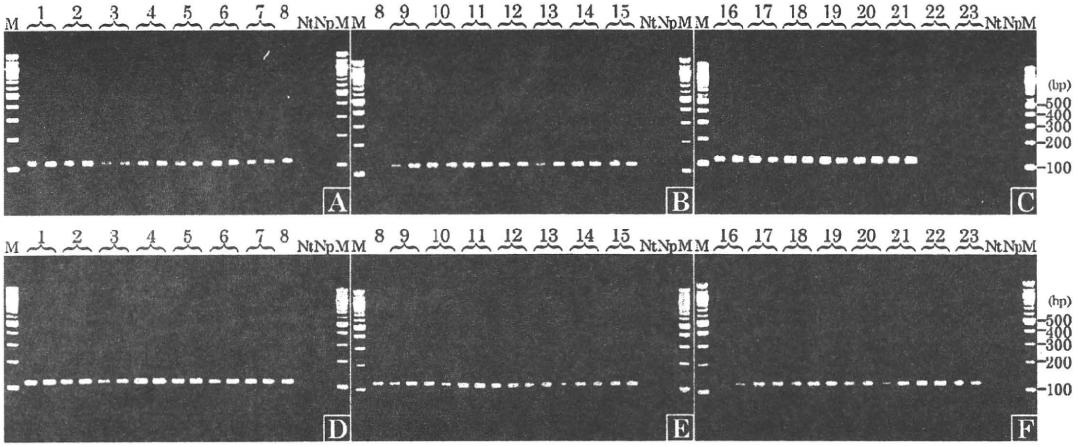

Fig. 2. Specificities of primer pairs used in this study

PCR products amplified with primer pair 1 (A–C) and primer pair 2 (D–F) were electrophoresed on 3% agarose gels. Lanes 1–19, the amplification of sugar beet (*Beta vulgaris* L. subsp. *vulgaris*) DNA extracted from seedlings of cultivar Monomidori, Monohikari, Monoperl, Monowhite, Monohomare, Mighty, Schwerdt, Kabutomaru, Yukihinode, Kitamasari, Etopirika, Nozomi, Skane, Freuden, NK-150, C-110, Amano, Hinderupgaad, and Detroit Dark Red, respectively; lanes 20 and 21, the amplification of chard plant (*Beta vulgaris* L. subsp. *vulgaris* var. *cicla*) DNA extracted from seedlings of line FK-02-09 and FK-02-34, respectively; lane 22, the amplification of chenopodium plant (*Chenopodium amaranticolor*) DNA extracted from seedlings; lane 23, the amplification of quinua plant (*C. quinoa*) DNA extracted from seedlings; Nt, negative control without template; Np, negative control without primers; M, 100 bp ladder size standard. The predicted sizes of the specific amplification products with primer pair 1 and primer pair 2 are 116 bp and 121 bp, respectively.

stranded (ds) DNA using the Qant-iT™ PicoGreen® dsDNA quantitation reagent gave concentrations of extracted DNAs ranging up to 8.36 ng/μL (thin juice), and up to 0.85 ng/μL (brown sugar #2) for in-process samples and commercial products, respectively (Table 1). These results suggested that it was difficult to extract sufficient amounts of DNA at suitable concentrations for PCR analysis from the final products of sugar beets.

Development of sugar beet-specific qualitative PCR method

To detect residual DNA with high sensitivity, we tried to amplify and to sugar beet-specific DNA fragments from the extracted DNAs of the in-process products and the beet sugar products. For this purpose, we designed two pairs of taxon-specific primers, which were elaborated to detect single nucleotide polymorphism (SNP) markers[4]. These primer pairs were specifically able to detect the 19 cultivars of sugar beet investigated in this study (Fig. 2). Amplification was also observed in some reactions with other plants that are closely related with sugar beet, such as chard (*Beta vulgaris* L. subsp. *vulgaris* var. *cicla*), chenopodium (*Chenopodium amaranticolor*), and quinua (*C. quinoa*). No amplification was observed in reactions with DNAs from other plants, *i.e.*, soy, maize, rice, wheat, cotton, oilseed, alfalfa, potato and barley (data not shown). The detection limit of these primer pairs was 5 copies of genomic DNA, calculated from the UV absorption using a *C*-value of 26 pg/2*C*[5] (Fig. 3). Therefore, we concluded that these primer pairs were suitable for use in the following experiments. Moreover, these primer pairs would be suitable as taxon-specific controls in future detection methods for GM sugar beets.

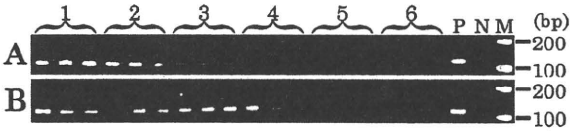

Fig. 3. Estimation of limits of detection (LODs) of sugar beet-specific PCR methods

Successively diluted solutions of genome DNA extracted from leaves of sugar beet plant (cultivar Skane) were prepared and were examined by PCR amplifications with primer pair 1 (A) and primer pair 2 (B). PCR products were electrophoresed on 3% agarose gels. Lanes 1–5, the amplifications of sugar beet DNA extractions containing 500 copies, 100 copies, 20 copies, 10 copies, and 5 copies per reaction, respectively; Lane 6, negative control without template; P, positive control (same as sugar beet genomic DNA samples for 20 copies); M, 100 bp ladder size standard. DNA concentrations were calculated from UV absorption using a *C*-value a 26 pg/2*C*. Each reaction was performed in three replications with independent extractions.

Qualitative PCR analysis of residual DNA in beet sugar

As the template in PCR for in-process samples and beet sugar products, the permissible maximum volume of the undiluted DNA extracts was added to the PCR reactions, and the amount of DNA used for each reaction ranged from 40 ng to 239 ng as calculated from the

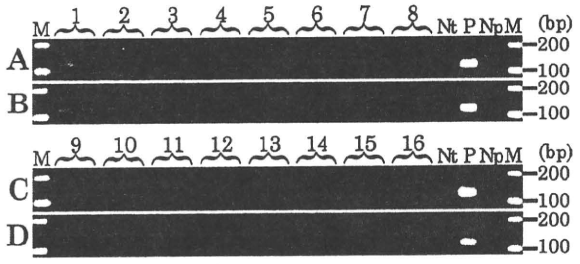

Fig. 4. Analysis of residual DNA in in-process products and commercialized beet sugar product

PCR products amplified with the primer pair 1 (A, C) and the primer pair 2 (B, D) were electrophoresed on 3% agarose gels. Lanes 1–8, detection of sugar beet DNA from the in-process products indicated in Fig. 1; lanes 9–16, detection of sugar beet DNA from commercial beet sugar products, namely brown sugar (1), brown sugar (2), syrup-type product with added oligosaccharide, granulated sugar (1), white superior sugar, brown sugar (3), bleached brown sugar, and granulated sugar (2), respectively. P, detection of sugar beet DNA extracted from seedlings as a positive control; Nt, negative control without template; Np, negative control without primers; M, 100 bp ladder size standard. Each reaction was performed in two replications with independent extractions.

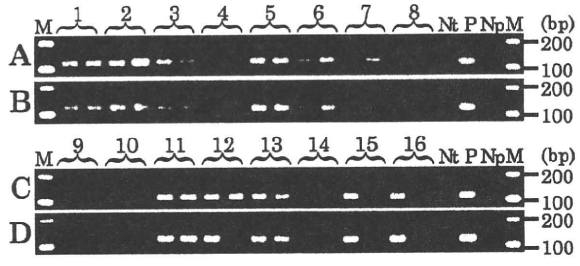

Fig. 5. Inhibition assay

Sugar beet DNA (260 pg) extracted from leaves of sugar beet plant (cv. Skane) was spiked in a reaction mixture containing 16.875 μL of extracts from samples. Amplification was performed with the primer pair 1 (A, C) and the primer pair 2 (B, D), and products were analyzed by electrophoresed on 3% agarose gels after the thermal cycling. Lanes 1–8, detection of sugar beet DNA from in-process products as indicated in Fig. 1; lanes 9–16, detection of sugar beet DNA from commercial beet sugar products, namely brown sugar (1), brown sugar (2), syrup-type product with added oligosaccharide, granulated sugar (1), white superior sugar, brown sugar (3), bleached brown sugar, and granulated sugar (2), respectively. P, detection of sugar beet DNA extracted from leaves as a positive control; Nt, negative control without template; Np, negative control without primers; M, 100 bp ladder size standard. Each reaction was performed in two replications with independent extractions.

UV absorption. In the case of in-process sugars, amplification was observed in the earlier stages of processing, such as thin juice and the thick juice, but no amplification was observed in the case of samples from later stages (Figs. 4A, B). In addition, for inhibition assay of the extracts, we evaluated amplification from samples spiked with 20 copies of sugar beet genome DNA extracted from leaves. The inhibition assays were performed in duplicate for each extract. The results indicated that amplifications were strongly inhibited (2 out of 2) in in-process syrup samples (#4) and brown sugars (#8, #9, #10, and #14) and partially inhibited in in-process syrup samples (#7) and some commercial sugars (#12 and #15) (Fig. 5). Although some samples may contain PCR-inhibitory substances, the results on the in-process samples suggested that the sugar beet DNA was degraded in the early stage of the sugar processing. Thus, it is unlikely residues of DNA are present at measurable levels in commercial beet sugars.

Conclusion

Our results suggested that it is difficult to extract DNA for PCR analyses from processed sugar beets. Although we rely on imported sugar materials, import of raw sugar beets into Japan is forbidden for phytosanitary reasons*2. GM sugar beets generally come into the Japanese market as processed sugars or partially purified sugars. In Japan, the mandatory GM labeling is not required for processed foods that do not contain a sufficient amount and/or quality of marker DNAs or proteins, *e.g.* cooking oil and soy sauce*3. Based on the result of this investigation, the Japanese government has decided that the mandatory GM labeling is not applicable the sugar products. Moreover, we found taxon-specific primer pairs for sugar beet plants, and clarified that these primers were appropriate to use as taxon-specific controls for GMO analysis by PCR. The results will be useful for future development of detection methods of GM sugar beets.

Acknowledgements

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*2 http://www.pps.go.jp / english / law / list 1-(20080412-).html

*3 Notification No. 517 (Mar. 31, 2000), Labeling standard for genetically modified foods. Ministry of Agriculture, Forestry and Fisheries of Japan.

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Original

Development of Multiplex PCR Method for Simultaneous Detection of Four Events of Genetically Modified Maize: DAS-59122-7, MIR604, MON863 and MON88017

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A novel multiplex PCR method was developed for simultaneous event-specific detection of four events of GM maize, *i.e.*, DAS-59122-7, MIR604, MON88017, and MON863. The single laboratory examination of analytical performance using simulated DNA mixtures containing GM DNA at various concentrations in non-GM DNA suggested that the limits of detection (LOD) of the multiplex PCR method were 0.16% for MON863, MIR604, and MON88017, and 0.078% for DAS-59122-7. We previously developed a nonaplex (9plex) PCR method for eight events of GM maize, *i.e.*, Bt11, Bt176, GA21, MON810, MON863, NK603, T25, and TC1507. Together with the nonaplex PCR method, the newly developed method enabled the detection and identification of eleven GM maize events that are frequently included in commercial GM seed used in Japan. In addition, this combinational analysis may be useful for the identification of combined event products of GM maize.

Key words: genetically modified (GM); maize (*Zea mays*); multiplex PCR; DAS-59122-7; MIR 604; MON88017

Introduction

Recombinant DNA technologies have been used in modern farming and have provided many advantages in related industries. The global area of genetically modified (GM) crops exceeded 125 million hectares in 2008, and is expected to continue to rise[1]. GM crops have been authorized for use as food and/or feed in many countries based on those countries' criteria for safety assessment. However, consumers have demanded appropriate information and labeling for foods derived from GM crops. Thus, various labeling systems have been introduced for GM foods in many parts of the world, such as the European Union (EU)*1, Korea*2 and Japan*3, *4. However, each system has a different threshold for the adventitious commingling of GM crops, *e.g.*, the thresholds are 0.9, 3 and 5% in the EU, Korea and Japan, respectively. At the same time, many countries have been trying to implement rules for the coexistence of GM and conventional crops. Consequently, scientifically-sound GM detection methods are essential.

For the detection of GM maize in foods and food materials, PCR-based detection methods, which are able to detect even small amounts of transgenes in raw materials and processed foods, have been routinely used. We have also developed simplex and multiplex qualitative PCR methods[2)-7)], simplex real-time PCR methods[8), 9)] and an individual kernel-based detection method including a qualitative multiplex real-time PCR[10)], *5. Some of these methods have been employed as official analytical methods in Japan*6, *7.

On the other hand, the cost and time required for GM

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*1 Regulation (EC) No. 1829/2003. Official J. Eur. Union L268, 1-23. Off. J. Eur. Union 2003, L268, 1-23 (2003).

*2 Notification No. 2000-31; Ministry of Agriculture and Forestry of Korea (2000).

*3 Notification No. 79 (Mar. 15, 2001); Department of Food Safety, Ministry of Health, Labour and Welfare of Japan (2001).

*4 Notification No. 517 (Mar. 31, 2000); Ministry of Agriculture, Forestry and Fisheries of Japan (2000).

*5 Notification No. 803 (Aug. 3, 2009); Department of Food Safety, Ministry of Health, Labour and Welfare of Japan (2009).

*6 Notification No. 110 (Mar. 27, 2001); Department of Food Safety, Ministry of Health, Labour and Welfare of Japan (2001).

*7 Japanese Agricultural Standard analytical test handbook, available at http://www.famic.go.jp/technical_information/jashandbook/index.html.

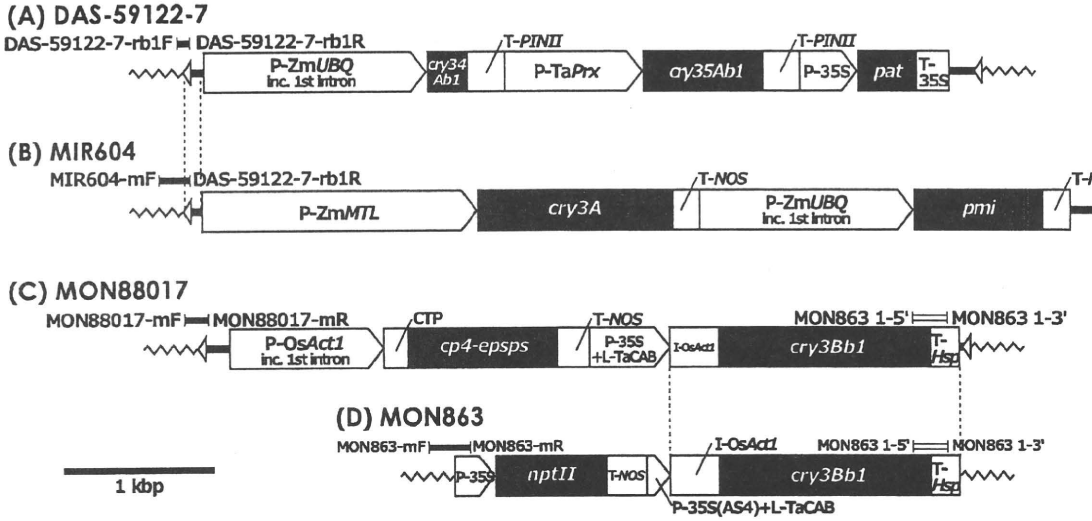

Fig. 1. Schematic diagrams of r-DNAs introduced into GM maize targeted by the multiplex PCR method

The structures of the r-DNAs transformed into DAS-59122-7, MIR604, MON88017, and MON863 are shown in (A)–(D), respectively. The structural trait genes are shown in filled boxes. Promoters are shown in open pentagonal arrows, and terminators and the transport signal sequence are shown in open boxes except I-OsActI, which is a sequence used to increase the expression level of the downstream gene. The lines, open arrowheads, and wavy lines at flanking boxes indicate non-coding vector segments, border sequences of the T-DNA vector, and flanking maize genomic sequences, respectively. The detection regions of the multiplex PCR developed in this study are shown as broad lines, with the primers used for the amplification shown at the ends. The double lines indicate the amplification targets of the previous primer pair for MON863 described in the previous report[5]. A scale bar is shown in the bottom left corner of the panel.

analysis could constitute major bottlenecks in providing consumers with cost-effective labeling (reviewed by Rodoríguez-Lázaro *et al.*[11]). Non-GM foods supply chains, however, need to be secured by affordable detection methods. Thus, the development of cost- and time-effective detection methods is important to ensure freedom of choice of consumers.

Maize is the major agricultural crop to which GM technology has been applied. In Japan, sixteen transgenic events in maize and some of their combined varieties have been authorized as of 2009*[8]. We previously reported a nonaplex (9plex) PCR method which detects eight events of GM maize, *i.e.*, Bt11, Bt176, GA 21, MON810, MON863, NK603, T25 and TC1507[5]. At the time the method was developed, it covered every GM maize which had been used for commercial purposes in Japan. After that, some newly authorized GM maize events, *i.e.*, DAS-59122-7, MIR604 and MON 88017, entered commercial cultivation in the U.S. Then, we attempted to develop a new multiplex PCR method for simultaneous detection of these three events. Additionally, we included MON863 as a target in this method because the detection target of MON863 in the nonaplex PCR method was contained not only in MON 863, but also in MON88017, and the two events could not be discriminated by the nonaplex PCR method (Fig. 1). The newly developed multiplex PCR method amplified four segments specific to the four respective GM maize events and a segment for endogenous maize *starch synthase IIb* (*ssIIb*) gene as an experimental control. Moreover, the analytical performance of the developed method was examined to see if the method would be applicable to detect relevant GM events at the threshold level of adventitious commingling, that is 5% in Japan or a far lower level, 0.9% in EU.

*[8] List of products that have undergone safety assessment and been announced in the official gazette (Apr. 30, 2009); Department of Food Safety, Ministry of Health, Labour and Welfare of Japan (2009). (http://www.mhlw.go.jp/english/topics/food/pdf/sec01-2.pdf)

Materials and Methods

Maize (Zea mays) and other plant materials

Dry progeny seeds of GM maize of Bt11, Event176 and GA21, and a ground sample of MIR604 were supplied by Syngenta Seeds AG (Basel, Switzerland); dry progeny seeds of GM maize of MON810, NK603, MON863, MON88017, MON863×NK603, MON810×MON863, MON810×MON863×NK603, GA21×MON 810, MON810×NK603 and MON810×MON88017 were provided by Monsanto Co. (St. Louis, MO, USA); dry progeny seeds of GM maize of TC1507, DAS-59122-7, DAS-59122-7×NK603, DAS-59122-7×TC1507 and DAS-59122-7×NK603×TC1507 were provided by Pioneer Hi-Bred International (Johnston, IA, USA). Dry progeny seeds of GM maize of T25 were directly imported. DAS-59122-7, MIR604, MON863 and MON 88017 were used as positive controls. Bt11, Event176, GA21, MON810, NK603, T25 and TC1507 were used as non-specific GM maize samples. As experimental controls, QC9651 maize (Quality Technology International, Inc. (QTI), Elgin, IL, USA), Roundup Ready® (RR) soy

Table 1. List of primers for the developed multiplex PCR method

Specificity	Name	Sequence	T_m (℃)		Concentration in muliplex PCR (μmol/L)	Length of amplification (bp)	Ref.
			GC%[a]	NN[b]			
MON863	MON863-mF	5′-TGGTTCGGAGAGCACTTGTTG-3′	50.5	67.5	0.40	281	
	MON863-mR	5′-TTCTTTTTCCACGATGCTCCTC-3′	49.2	66.3	0.40		
MIR604	MIR604-mF	5′-GTGAATGGAGATGGACGGATGC-3′	52.9	69.8	0.15	201	14)
	DAS-59122-7-rb1R	5′-CCTTAATTCTCCGCTCATGATCAG-3′	51.9	66.6	0.30		
MON88017	MON88017-mF	5′-ATCGTGTGACAACGCTAGCA-3′	48.0	64.5	0.50	150	
	MON88017-mR	5′-CATATTGACCATCATACTCATTGCT-3′	49.0	62.6	0.50		
Taxon	SSIIb03-5′	5′-CCAATCCTTTGACATCTGCTCC-3′	51.0	66.7	0.06	114	17)
	SSIIb03-3′	5′-GATCAGCTTTGGGTCCGGA-3′	49.4	67.4	0.06		17)
DAS-59122-7	DAS-59122-7-rb1F	5′-GGGATAAGCAAGTAAAAGCGCTC-3′	51.5	65.7	0.25	86	14)
	DAS-59122-7-rb1R	5′-CCTTAATTCTCCGCTCATGATCAG-3′	51.9	66.6	0.30		14)

[a] Calculated by GC% method.
[b] Calculated by nearest neighbor method.

and non-GM cereal materials, *i.e.*, dry soybeans harvested in Ohio in 1998, rice (*Oryza sativa*) variety Kinuhikari, wheat (*Triticum aestivum*) variety Haruyutaka and barley (*Hordeum vulgare*) variety Harrington (harvested in Japan) were used.

DNA extraction

Seeds of GM maize, *i.e.*, DAS-59122-7, MON863 and MON88017, and combined event products of GM maize were ground with a Multibeads Shocker (Yasui Kikai Co., Osaka, Japan) on a single-seed basis, and DNA of each kernel was extracted with the DNeasy Plant Mini kit (QIAGEN GmbH, Hilden, Germany) following the standard experimental procedure for GM analysis in foodstuffs in Japan*6. Other maize and other plant seed materials were ground with a P-14 seed rotor mill (Fritsch GmbH, Ibar-Oberstein, Germany), and used for DNA extraction. A ground flour sample of MIR604 maize was re-ground with the P-14 seed rotor mill and used for DNA extraction. DNA extraction was performed with the DNeasy Plant Maxi kit (QIAGEN) as described in the standard experimental procedure for GM analysis in foodstuffs in Japan*6,*7. The DNA concentration of solutions was determined by measuring the UV absorption at 260 nm, and the quality of DNA solutions was evaluated in terms of the absorption ratios at 260/280 nm and 260/230 nm. DNA solutions having absorption ratios at 260/280 nm and 260/230 nm of between 1.7 and 2.0, and >1.7, respectively, were used for the subsequent experiments. For the examination of the analytical performance of the developed method, DNA concentrations in extracts were analyzed with the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Invitrogen, Carisbad, CA, USA), using a SpectraMax® M2e-TS luminescence reader (Molecular Devices, Sunnyvale, CA, USA).

Oligonucleotide primers

For primer design, we used publicly available sequence information, *e.g.*, the DNA Data Bank of Japan (DDBJ)*9 and public patent bulletins. Candidate oligonucleotide sequences for primers were designed using the Oligo™ primer analysis program (National Bioscience Inc., Plymouth, NM, USA) or the Primer Express software (Applied Biosystems, Foster City, CA, USA; ABI). Primer pairs used in this study are listed in Table 1. The primers were synthesized by Fasmac Co., Ltd. (Kanagawa, Japan) and purified by HPLC. Each oligonucleotide was diluted to the appropriate concentration for PCR assays with sterilized water and stored at −30℃ until use.

Simplex PCR conditions

The 25 μL reaction solution contained 25 ng genomic DNA, 0.5 μmol/L each primer, 0.2 mmol/L each dNTP (ABI), 1.5 mmol/L $MgCl_2$ (ABI), 1×PCR buffer II (ABI) and 0.625 units of AmpliTaq Gold DNA polymerase (ABI). The reaction mixture was amplified in a thermal cycler, the Silver 96-Well GeneAmp PCR System 9700 (ABI) in Max mode, according to the following step-cycle program: pre-incubation at 95℃ for 10 min; 40 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.

Multiplex PCR condition

In general, the experimental conditions of PCR followed those of our previous multiplex PCR method[5]. The 25 μL reaction solution contained 25 ng genomic DNA, 0.2 mmol/L each dNTP, 1.5 mmol/L $MgCl_2$, 1× PCR buffer II, 1.25 units of AmpliTaq Gold DNA polymerase and nine sorts of oligonucleotide primers at the following concentrations: 0.50 μmol/L each for MON 88017-mF and MON88017-mR, 0.40 μmol/L each for MON863-mF and MON863-mR, 0.30 μmol/L for DAS 59122-7-rb1R; 0.25 μmol/L for DAS59122-7-rb1F; 0.15 μmol/L for MIR604-mF; and 0.060 μmol/L each for SSIIb03-5′ and SSIIb03-3′. The reaction mixture was amplified in a thermal cycler, the Silver 96-Well GeneAmp PCR System 9700, in Max mode, according to

*9 http://www.ddbj.nig.ac.jp/index-e.html

the following step-cycle program: pre-incubation at 95℃ for 10 min; 10 cycles consisting of denaturation at 95℃ for 0.5 min, annealing at 65℃ for 1 min and extension at 72℃ for 1 min; 27 cycles consisting of denaturation at 95℃ for 0.5 min, annealing at 60℃ for 1 min and extension at 72℃ for 1 min; then a final extension at 72℃ for 7 min.

Analysis of PCR product

The PCR products were analyzed by agarose gel electrophoresis 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, USA). A 5 μL aliquot of each PCR product was electrophoresed at a constant voltage (100 V) for 30 min in 1× Tris-Acetate-EDTA (TAE) solution consisting of 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, the gel was scanned with the Molecular Imager FX system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The sequencing analysis of amplified products was performed by Fasmac Co., Ltd.

Examination of analytical performance

In order to examine the limit of detection (LOD) of the developed method, we prepared five dilution series of extracted GM maize DNA solution(s), *i.e.*, DAS-59122-7, MIR604, MON863, MON88017 and a mixture of all of them at equal concentrations, each diluted with non-GM maize DNA solution. Extracted DNA solutions diluted to 10 ng/μL based on measurement with PicoGreen Kit were used as simulated samples. For the simulated samples containing one event of GM maize DNA, each GM maize DNA solution was diluted to 10% (v/v) with non-GM maize DNA solution, and then serially double-diluted with the non-GM maize DNA solution to make solutions containing GM maize DNA at concentrations of 5.0%, 2.5%, 1.25%, 0.63%, 0.31%, 0.16%, 0.078%, 0.039% and 0.020% (v/v). We also prepared simulated samples containing four events of GM maize DNA at the same concentrations, *i.e.*, 5.0%, 2.5%, 1.25%, 0.63%, 0.31%, 0.16%, 0.078%, 0.039% and 0.020% (v/v). The lowest level of dilution, 0.020% (v/v), was expected to contain about 0.9 copies of maize genome haploid per sample, according to the *C*-value of maize[12]. Each dilution series was prepared with two independent replications for use in the examination. In addition, we examined the robustness of the developed method by detecting a target event when another target event coexisted at a higher concentration in a sample.

Results and Discussion

Primer design

In this study, we focused on three events of GM maize, *i.e.*, DAS-59122-7, MIR604 and MON88017, as detection targets. In addition, we added MON863 to the targets, because our previous nonaplex PCR method did not distinguish between MON863 and MON88017. Detection methods for the respective events using PCR amplification have already been described by the developer companies[13)-15)]. However, it is not practical to simply combine these methods because the lengths of some of the amplification products are similar. Thus, we designed sets of primers for the multiplex PCR method listed in Table 1. As shown in Fig. 1, each primer pair was designed to amplify an event-specific segment; more specifically, one primer from each pair was designed to anneal to a terminal region of the recombinant DNA (r-DNA) and the other was designed to anneal to an endogenous genomic DNA region flanking the r-DNA. For the detection of DAS-59122-7, we employed the primer pair provided by its developer[13)]. The sequence analysis of the primer pair showed that the complementary sequence to one of the primers, DAS-59122-7-rb1R, was found not only in the r-DNA of DAS-59122-7 (DDBJ accession no. CS566037) but also in the r-DNA of MIR604 (DDBJ accession no. DI57741). The sequence information indicated that this region was derived from a transformation vector plasmid DNA. Then, we applied this primer for the detection of MIR604, combined with a new primer designed on the flanking genome region (Table 1). For the detection of MON863 and MON88017, we designed new primers specific to the respective events using publicly available sequence information (DDBJ accession no. AR 534233 and DDBJ accession no. DJ058152). We also added a primer pair specific to the *ssIIb* gene as an experimental control for amplification (Table 1). The *ssIIb* gene is a single copy gene in the maize genome, and the primer pair has already been used in our quantitative real-time PCR method[9)]. These five primer pairs were also designed to give amplification products of different lengths. In order to perform multiplex PCR, it was preferable to make the binding affinities of all primers similar. In fact, the melting temperatures (T_m) of these primers ranged from 48.0 to 52.9℃ (GC content method) or from 62.6 to 69.8℃ (nearest neighbor method) (Table 1). Thus, we used these primers for the multiplex PCR method, and the subsequent experiments confirmed their applicability.

Specificities of primers in simplex PCR condition

The specificities of the designed primer pairs were individually confirmed by simplex PCR assays. The DNAs extracted from each of the eleven GM maize events and from other plant materials were used as templates. As shown in Fig. 2, all primer pairs specifically amplified products of the expected lengths from their specific targets in simplex PCR conditions. By contrast, none of these primer pairs amplified products from DNAs extracted from other events of GM maize, non-GM maize and other plant materials, *i.e.*, soy, rice, wheat and barley (Fig. 2). These results indicated that the developed primer pairs could be used for the detection of their respective target events not only in maize materials, but also in mixtures of maize and other cereals. Their specificity in the presence of other GM

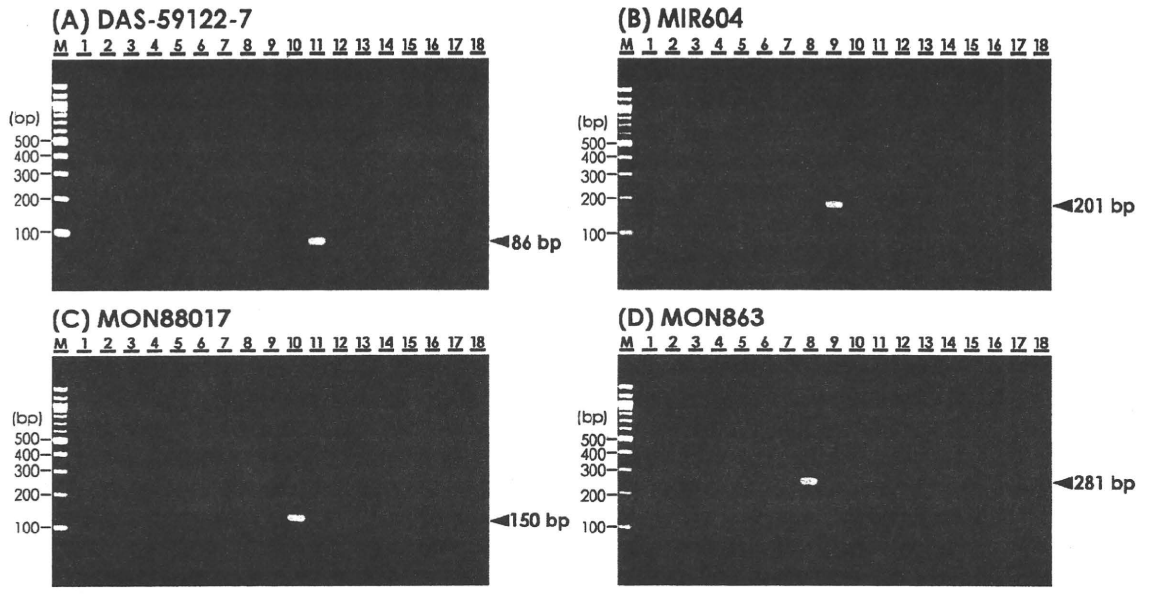

Fig. 2. Specificities of detection primer pairs for the multiplex PCR

Simplex PCR products amplified with the DAS-59122-7 primer pair (A), the MIR604 primer pair (B), the MON 88017 primer pair (C), and the MON863 primer pair (D) were electrophoresed on 3% agarose gel. Lanes 1–12, amplification of maize DNAs extracted from the seeds of each representative variety of maize: NK603, Event176, T25, GA21, MON810, TC1507, Bt11, MON863, MIR604, MON88017, DAS-59122-7, and the non-GM variety, respectively. Lanes 13–17, amplification of DNAs extracted from non-GM soy, Roundup Ready® soy, rice, wheat, and barley, respectively; lane 18, negative control (no DNA); and lane M, 100-bp ladder size standard. Arrowheads indicate the amplified products in the respective PCR reactions; the expected lengths of the products are shown with arrowheads.

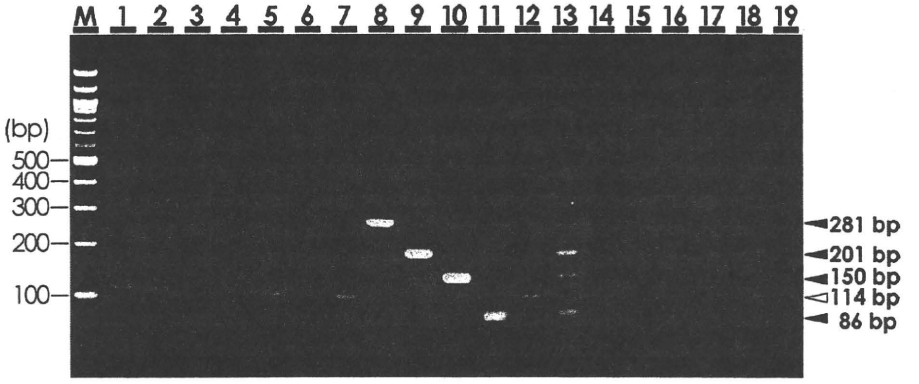

Fig. 3. Specificities of the multiplex PCR

PCR products were electrophoresed on 3% agarose gel. Lanes 1–11, amplification products of DNAs extracted from the seeds of each event of GM maize: NK603, Event176, T25, GA21, MON810, TC1507, Bt11, MON863, MIR 604, MON88017, and DAS-59122-7, respectively. Lane 12, amplification product of non-GM maize; lane 13, amplification product of simulated GMO mixture containing 10% each of four targeted GM events (*i.e.*, MON863, MIR604, MON88017, and DAS-59122-7); lanes 14–18, amplification of DNAs extracted from non-GM soy, Roundup Ready® soy, rice, wheat, and barley, respectively; lane 19, negative control (no DNA); and lane M, 100-bp ladder size standard.

crop events, such as those of alfalfa, cotton, sugar beet, and rapeseed, should be tested in the future, though the major raw GM crops used for foods are soy and maize.

Multiplex PCR

We have already developed a nonaplex PCR method for the detection and identification of eight transgenic maize events. In order to perform two multiplex PCR methods simultaneously, the experimental conditions of the newly developed method were set to be the same as those of the previous method, except for the primers. The optimal concentrations of primers were experimentally adjusted with a simulated sample containing 10% each of DNAs extracted from the four targeted GM events. The primer concentrations were determined to make the intensities of the amplicon bands nearly equi-

Table 2. Single laboratory measurement[a, b]

A. MON863[d]

GMO content[c]	Detection of GMO				Detection of SSIIb	Reliability index			
	MON863	MIR604	MON88017	DAS-59122-7		Sensitivity	Specificity	False positive	False negative
0.63%	21/21 (100%)	0/21 (0.0%)	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	21/21 (100%)	63/63 (100%)	0/63 (0.0%)	0/21 (0.0%)
0.31%	21/21 (100%)	0/21 (0.0%)	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	21/21 (100%)	63/63 (100%)	0/63 (0.0%)	0/21 (0.0%)
0.16%	21/21 (100%)	0/21 (0.0%)	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	21/21 (100%)	63/63 (100%)	0/63 (0.0%)	0/21 (0.0%)
0.078%	18/21 (85.7%)	0/21 (0.0%)	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	18/21 (85.7%)	63/63 (100%)	0/63 (0.0%)	3/21 (14.3%)

B. MIR604[e]

GMO content[c]	Detection of GMO				Detection of SSIIb	Reliability index			
	MON863	MIR604	MON88017	DAS-59122-7		Sensitivity	Specificity	False positive	False negative
0.63%	0/21 (0.0%)	21/21 (100%)	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	21/21 (100%)	63/63 (100%)	0/63 (0.0%)	0/21 (0.0%)
0.31%	0/21 (0.0%)	21/21 (100%)	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	21/21 (100%)	63/63 (100%)	0/63 (0.0%)	0/21 (0.0%)
0.16%	0/21 (0.0%)	21/21 (100%)	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	21/21 (100%)	63/63 (100%)	0/63 (0.0%)	0/21 (0.0%)
0.078%	0/21 (0.0%)	19/21 (90.5%)	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	19/21 (90.5%)	63/63 (100%)	0/63 (0.0%)	2/21 (9.5%)

C. MON88017[f]

GMO content[c]	Detection of GMO				Detection of SSIIb	Reliability index			
	MON863	MIR604	MON88017	DAS-59122-7		Sensitivity	Specificity	False positive	False negative
0.63%	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	0/21 (0.0%)	21/21 (100%)	21/21 (100%)	63/63 (100%)	0/63 (0.0%)	0/21 (0.0%)
0.31%	0/21 (0.0%)	0/21 (0.0%)	20/21 (95.2%)	0/21 (0.0%)	21/21 (100%)	20/21 (95.2%)	63/63 (100%)	0/63 (0.0%)	1/21 (4.8%)
0.16%	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	0/21 (0.0%)	21/21 (100%)	21/21 (100%)	63/63 (100%)	0/63 (0.0%)	0/21 (0.0%)
0.078%	0/21 (0.0%)	0/21 (0.0%)	18/21 (85.7%)	0/21 (0.0%)	21/21 (100%)	18/21 (85.7%)	63/63 (100%)	0/63 (0.0%)	3/21 (14.3%)

D. DAS-59912-7[g]

GMO content[c]	Detection of GMO				Detection of SSIIb	Reliability index			
	MON863	MIR604	MON88017	DAS-59122-7		Sensitivity	Specificity	False positive	False negative
0.63%	0/21 (0.0%)	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	21/21 (100%)	21/21 (100%)	63/63 (100%)	0/63 (0.0%)	0/21 (0.0%)
0.31%	0/21 (0.0%)	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	21/21 (100%)	21/21 (100%)	63/63 (100%)	0/63 (0.0%)	0/21 (0.0%)
0.16%	0/21 (0.0%)	0/21 (0.0%)	0/21 (0.0%)	21/21 (100%)	21/21 (100%)	21/21 (100%)	63/63 (100%)	0/63 (0.0%)	0/21 (0.0%)
0.078%	0/21 (0.0%)	0/21 (0.0%)	0/21 (0.0%)	20/21 (95.2%)	21/21 (100%)	20/21 (95.2%)	63/63 (100%)	0/63 (0.0%)	1/21 (4.8%)

[a] Amplifications were performed using 21 independent PCR runs, and analyses were performed with agarose gel electrophoresis for each experimental condition.
[b] Results of detections were indicated by both fraction, (number of positive trials)/(number of all trials), and percentage (indicated in parentheses).
[c] Simulated samples were prepared as the mixtures of DNA extracts.
[d] Simulated samples containing MON863 only were used as analytes.
[e] Simulated samples containing MIR604 only were used as analytes.
[f] Simulated samples containing MON88017 only were used as analytes.
[g] Simulated samples containing DAS-59122-7 only were used as analytes.

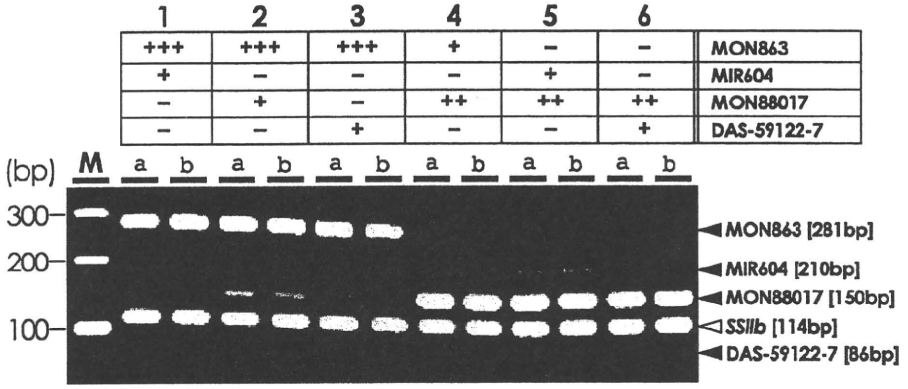

Fig. 4. LOD robustness of multiplex PCR.

To examine LOD robustness, targeted GM DNA from each event at the concentrations near the LODs (indicated by +) were accompanied by another target GM DNA at a much higher concentration (indicated by ++ or +++) and subjected to detection. Two independent simulated samples (a and b) were prepared for each combination, and both results are shown. Lanes 1–3, DNA amplification with simulated DNA mixture of 5% MON863 and 0.16% MIR604, 0.16% MON88017, or 0.078% DAS-59122-7, respectively; lanes 4–6, amplification of simulated DNA mixture of 2.5% MON88017 and 0.16% MON863, 0.16% MIR604, or 0.078% DAS-59122-7, respectively; and lane M, 100-bp ladder size standard. Each PCR analysis was performed in duplicate, and PCR products were electrophoresed on 3% agarose gel.

valent on electrophoresis. Figure 3 shows a typical result of the developed multiplex PCR performed with the optimized primer mixtures. Each of the four targeted events of GM maize gave two amplifications corresponding to the relevant event-specific amplification and a taxon-specific amplification (*ssIIb*), while the other events of GM maize and non-GM maize gave only amplification of *ssIIb* (Fig. 3). Furthermore, the simulated sample containing 10% each of these four events gave all five amplifications corresponding to the four event-specific amplifications and the taxon-specific amplification (Fig. 3). We also subcloned and sequenced the amplification products, and confirmed that these were identical with their target segments (data not shown). On the other hand, the multiplex PCR gave no amplification with soy, rice, wheat, and barley samples. These results indicated that this method is able to detect and identify the four targeted GM maize events, *i.e.*, DAS-59122-7, MIR604, MON863 and MON88017. In addition, the amplification of *ssIIb* is expected with every sample, including maize genomic DNA, and this may be used as an internal control to distinguish true negative results from PCR inhibition or experimental failures for maize samples.

Examination of analytical performance

To verify the reliability of the developed method, the analytical performance was examined. We prepared four serial dilutions containing a DNA solution extracted from each of the four targeted GM maize events and a serial dilution containing all of them as analytes. Preliminary tests suggested that the multiplex PCR method specifically detected samples containing 1.25% or more of target(s) (data not shown). Then, we prepared simulated samples containing target(s) at concentrations ranging from 0.63% to 0.078% for the examination. For the preparation of simulated samples, DNA concentration was measured with PicoGreen dsDNA reagent, which is a sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA and can eliminate the effects caused by single-stranded nucleic acids and other impurities. Referring to previous reports[*10, *11], twenty-one independent multiplex PCR runs were performed for each kind of simulated sample, and the results were analyzed statistically (Table 2). According to the ISO standard for GMO analysis methods on foodstuffs[*12], the LOD value was defined as the lowest level of analytes with the false negative rate of 5% or less in multilaboratory collaborative evaluation. Based on the false negative rates, LODs of the developed multipex PCR method were estimated to be between 0.078 and 0.16% for MON863, MIR604 and MON88017, and at least 0.078% for DAS-59122-7 (Table 2). In contrast, no false positive detections were observed from non-specific targets (Table 2). In addition, we examined whether the multiplex PCR could detect one target event at a

[*10] Report on the in-house validation of an event-specific detection method for event Bt10 using a qualitative PCR assay and verification by restriction analysis. Joint Research Centre, European Commission (2005). (http://gmo-crl.jrc.ec.europa.eu/summaries/Bt10%20validation%20report%20version2.pdf)

[*11] Report on the in-house validation of a detection method for event Bt10 maize using a qualitative PCR assay. Joint Research Centre, European Commission (2005). (http://gmo-crl.jrc.ec.europa.eu/summaries/Bt10%20validation%20report.pdf)

[*12] ISO 24276:2006, Foodstuffs—Methods of analysis for the detection of genetically modified organisms and derived products—General requirements and definitions.

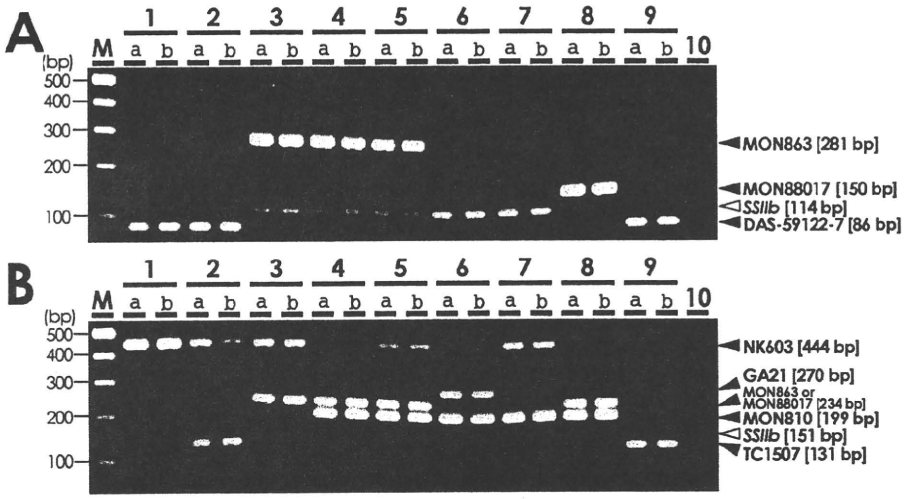

Fig. 5. Identification of combined event GM varieties

Amplifications of DNAs extracted from nine kinds of combined-trait GM maize by the new multiplex PCR method introduced in this study and by the 9plex PCR method from the previous study[5] are shown in A and B, respectively. Simulated samples were prepared in two independent lots for each (indicated as a and b), and are shown in results. Lanes 1–9, amplification of DNAs extracted from the seeds of each combined event product of GM maize, DAS-59122-7×NK603, DAS-59122-7×NK603×TC1507, MON863×NK603, MON863×MON810, MON 863×MON810×NK603, GA21×MON810, MON810×NK603, MON810×MON88017, DAS-59122-7×TC1507, respectively; and lane M, 100-bp ladder size standard. Each PCR analysis was performed in duplicate, and PCR products were electrophoresed on 3% agarose gel.

low concentration in a sample that included another target event at a higher concentration. The results indicated that the multiplex PCR detected each target event at a concentration near the LOD, even in samples containing another target event at a concentration more than fifteen times the LOD (Fig. 4). Although a future interlaboratory collaborative study is required for the determination of LODs, the results implied that the LODs of the developed method are around 0.1% for the four targeted GM maize events.

Detection of combined event products of GM maize using multiplex PCR methods

The high detection specificity for the respective targets indicated that the multiplex PCR method could be used not only for qualitative screening, but also as a profiling tool for combined event products of GM maize. In fact, the preceding multiplex PCR method was able to identify the varieties of GM maize from non-identity-preserved maize samples[16]. Figure 5 shows the results of the two multiplex PCR methods for some combined event products. All the reactions specifically gave amplification(s) corresponding to the respective GM events. Because the acreages of these varieties are rapidly increasing in the United States, the combination of multiplex methods should be a powerful analytical tool for combined event products of GM maize.

Conclusion

In this study, we developed a new multiplex PCR method for the qualitative detection of four events of GM maize, *i.e.*, DAS-59122-7, MIR604, MON863 and MON88017. The developed method was able to detect these four targeted events of GM maize with high specificity. The examination of the analytical performance suggested that this multiplex PCR would enable us to reliably amplify target DNA segments in a simulated GM mixture containing as little as 0.16% (v/v) of MON863, MIR604, and MON88017, and 0.078% of DAS-59122-7, which might be sufficient for the monitoring of their adventitious commingling. In addition, the combination of this method with the preceding multiplex PCR method should serve as a powerful profiling tool not only for simplex events, but also combined events of GM maize. For instance, a combination of these multiplex qualitative PCR methods with individual kernel- or plant-based detection methods would efficiently give profiling data. We consider that these methods are cost- and time-effective detection methods which should contribute to the reduction of the cost of GM food labeling.

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