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Individual Detection of Genetically Modified Maize Varieties in Non-Identity-Preserved Maize Samples

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In many countries, the labeling of grains and feed- and foodstuffs is mandatory if the genetically modified organism (GMO) content exceeds a certain level of approved GM varieties. The GMO content in a maize sample containing the combined-trait (stacked) GM maize as determined by the currently available methodology is likely to be overestimated. However, there has been little information in the literature on the mixing level and varieties of stacked GM maize in real sample grains. For the first time, the GMO content of non-identity-preserved (non-IP) maize samples imported from the United States has been successfully determined by using a previously developed individual kernel detection system coupled to a multiplex qualitative PCR method followed by multichannel capillary gel electrophoresis system analysis. To clarify the GMO content in the maize samples imported from the United States, determine how many stacked GM traits are contained therein, and which GM trait varieties frequently appeared in 2005, the GMO content (percent) on a kernel basis and the varieties of the GM kernels in the non-IP maize samples imported from the United States were investigated using the individual kernel analysis system. The average (± standard deviation) of the GMO contents on a kernel basis in five non-IP sample lots was determined to be 51.0 ± 21.6%, the percentage of a single GM trait grains was 39%, and the percentage of the stacked GM trait grains was 12%. The MON810 grains and NK603 grains were the most frequent varieties in the single GM traits. The most frequent stacked GM traits were the MON810 × NK603 grains. In addition, the present study would provide the answer and impact for the quantification of GM maize content in the GM maize kernels on labeling regulation.

KEYWORDS: Combined-trait genetically modified maize; multiplex real-time PCR; multiplex qualitative PCR; capillary gel electrophoresis

INTRODUCTION

Genetically modified (GM) crops developed by recombinant DNA (r-DNA) technology are grown in the United States, Canada, and several other countries and are widely consumed worldwide as food and feed. Over the past two decades, the production of GM crops, especially maize and soybeans, has increased in the United States (*1*). Recently, the production of combined-trait (stacked) products of GM maize, in which two or more different characteristic traits are inserted, has also increased in the United States due to their enhanced production efficiency.

Under these circumstances, there has been increasing interest and concerns about this technology among consumers, and perception gaps have emerged between consumers and scientist/authorities. Numerous opinions have been expressed, but the arguments surrounding GM crops and their processed foods generally focus on two topics: safety and labeling (*2*–*5*). In terms of this, many countries and international organizations have been discussing new labeling systems focusing on product information intended for the general public (*6*–*9*). Consequently, the labeling of grains, feed, and foodstuff is mandatory if the genetically modified organism (GMO) content exceeds a certain level of approved GM varieties. For instance, the European Union, Japan, and Korea have set threshold values of 0.9, 5, and 3%, respectively, of the GMO material in a non-GM

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background as the basis for labeling (*10–12*). In Japan, non-GM crops are segregated as non-GM material and imported from the United States by an identity-preserved (IP) handling system that requires document certification from U.S. farms to Japanese processing traders. However, the unintentional mixing of GM products in non-GM materials is inevitable. Accordingly, the enforcement of these threshold values has created a demand for the development of reliable GMO analysis methods.

Most of the developed analytical methods for the detection of GMO in foods are based on the Polymerase Chain Reaction (PCR) for the analysis of complex food matrices (*13–27*).

Furthermore, many real-time PCR systems have been developed to identify and quantify the GM maize, GM soybeans, and GM varieties of other agricultural commodities (*28–35*). The threshold levels for the unintentional mixing of GM materials and non-GM materials required for the labeling system are based on a weight per weight (w/w) percentage. The GM percentages calculated using the current quantitative PCR methods are calculated by converting the relative copy numbers between a specific rDNA sequence and a taxon-specific DNA sequence into a w/w percentage using appropriate reference materials. The GMO percentage in a maize sample containing the stacked GM maize as determined by the currently available methodology is likely to be overestimated as compared to the actual w/w percentage of GM maize in the sample because the relative copy numbers are calculated on a haploid basis (*36*). However, there has been little information on the determination of stacked GM maize. To solve this problem, we previously developed a rapid and simple detection system that delivers informative results by the individual kernel analysis of grain samples that could potentially contain stacked GM maize kernels. The detection system includes the developed multiplex qualitative PCR method for simultaneously detecting eight GM maize events. Some researchers have also developed multiplex PCR methods as simultaneous detection methods of multi-GM maize events using capillary gel electrophoresis (CGE) (*37, 38*). The combined use of multiplex PCR and CGE is a rapid and prospective method for multiple samples. In particular, the automated multichannel CGE could be a beneficial tool for the high-throughput analyses of amplified DNA fragments (*39*).

The present study was designed to clarify the GMO content of non-IP maize samples that may contain GM maize, investigate how many stacked GM traits are contained therein, and determine which GM trait varieties frequently appeared in 2005. We investigated the GMO content (percent) on a kernel basis and the varieties of GM kernels in the non-IP maize samples imported from the United States using the previously developed individual kernel analysis system, including the multiplex real-time PCR method (*36*), and coupled it to the multiplex qualitative PCR method (*27*) followed by multichannel CGE system analysis.

EXPERIMENTAL PROCEDURES

Maize Samples. The nongenetically modified (non-GM) maize grain and non-IP maize samples were obtained from the Ministry of Health, Labour and Welfare (MHLW) in Japan. Event 176 and Bt11 seeds were kindly provided by Syngenta (formerly Novartis Seeds). The T25 maize seeds were kindly provided by Bayer Crop Science. The TC1507 seeds were kindly provided by Pioneer Hi-Bred International, Inc. Furthermore, the MON810 seeds, MON863 seeds, GA21 seeds, NK603 seeds, and six stacked maize seeds (MON863 × NK603, MON810 × GA21, MON810 × T25, MON863 × MON810 and MON863 × MON810 × NK603) were kindly provided by the Monsanto Co., and TC1507 × NK603 was kindly provided by Pioneer Hi-Bred International, Inc., for the positive controls of the GM maize.

Oligonucleotide Primers and Probes for Multiplex Real-Time PCR Method. Sets of primer pairs and Taq-Man probes for the construct-specific and universal GM quantification were those described in our previous paper (*36*). The SSIIb-3 system (SSIIb 3-5′ and SSIIb 3-3′ with SSIIb−TaqV) was used as the primer and probe for the detection of the taxon-specific gene encoding the maize starch synthase IIb gene sequence (SSIIb) in the multiplex real-time PCR method, whereas the p35S-1 system (P35S 1-5′ and P35S 1-3′ with P35S-Taq) and GA21-3 system (GA21 3-5′ and GA21 3-3′ with GA21-Taq) were used for the multiplex real-time PCR method. All sets of primer pairs and the Taq-Man probes (p35S-Taq and GA21-Taq) for the detection of the cauliflower mosaic virus (CaMV) 35s promoter sequence (p35S) and GA21 specific sequence, respectively, were purchased from Fasmac Co., Ltd. (Kanagawa, Japan). SSIIb−TaqV, which is labeled with VIC and TAMRA at the 5′ and 3′ ends, was synthesized by Applied Biosystems (St. Louis, MO) and used as a probe for the detection of SSIIb. The target sequence of the p35S-1 system to detect the 35S promoter region derived from CaMV is widely found in the recombinant DNA of almost all GM events with the exception of GA21. The GA21-3 system was designed to detect the construct-specific sequence GM maize event GA21 (*36*).

Grinding of Individual Maize Kernels. One hundred and eighty kernels from each of the five non-IP maize samples (total of 900 kernels for 5 lots) were randomly sampled. To remove the broken pieces of the other kernels in order to avoid their contamination, the nonground kernels were washed with 1% sodium dodecyl sulfate (SDS), rinsed three times with distilled water in a beaker, and dried at 40 °C for 40 min in the incubation box (DO-300A, AS ONE Co., Osaka, Japan) before they were ground. Each maize kernel and metal corn as a disruption medium (MC0316MZ, Yasui Kikai Co.) were placed in a sample tube (ST-0350MZ, Yasui Kikai Co.), and the tube was closed using the attached cap (ST-0350MZ, Yasui Kikai Co.). Twenty-four sample tubes were arrayed in the tube holder (type SH-123, Yasui Kikai Co.). Two tube holders can be accommodated in a multibead shocker (model MB601NIHS, Yasui Kikai Co. Osaka, Japan; http://www.yasuikikai.co.jp/ company/e_index.html) at a time. The maize kernels were ground by vigorously shaking the tubes and the metal corns using a multibead shocker at 2500 rpm for 1 min and then repeated for 1 min after the tube holder was reversed (*36*).

DNA Extraction from Each Maize Kernel Using DNeasy 96 Plant Kit. Genomic DNA extraction from the finely ground individual powders was performed using the DNeasy 96 Plant kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol with the following modification. Buffer AP1 (preheated to 65 °C) and RNase A (final concentration = 100 μg/mL) were combined to make the working solution. One milliliter of the working solution was added to each sample tube containing the ground maize powder, which is described under Grinding of Individual Maize Kernels. The tubes were capped and incubated for 30 min at 65 °C (inverted 10 times at intervals of 10 min). A 170 μL aliquot of buffer AP2 solution was then added to each solution. The tubes were sealed to avoid leakage during shaking and then vigorously shaken for 15 s. The tubes were next incubated for 10 min at −20 °C and then centrifuged for 20 min at 3000 rpm using a Metalfuge (MBG100, Yasui Kikai Co.). A 400 μL aliquot of each supernatant was carefully transferred to new microtubes. The collection microtubes were centrifuged for 5 min at 12000 rpm. Each supernatant was carefully transferred to new microtubes, and 1.5 volumes (typically 600 μL) of buffer AP3/E was added to each sample. One milliliter of each sample was carefully transferred to the DNeasy 96 plates. The DNeasy 96 plate was sealed with tape. The plate was aspirated until each DNeasy membrane was dry. After removal of the tape, 800 μL of buffer AW was carefully added to each sample. The plate was again sealed with the tape and aspirated until each DNeasy membrane was dry. The washing was repeated three times. A 800 μL aliquot of 100% ethanol was then added to each sample. The plate was aspirated for 15 min to dry each DNeasy membrane. After removal of the tape, the DNeasy 96 plate was placed in the correct orientation on a rack of elution microtubes, and then 75 μL of distilled water (DW) (preheated to 65 °C) was added to each sample. The plate was resealed and incubated for 5 min at room temperature and then aspirated until each DNeasy membrane was dry.

Multiplex Real-Time PCR Conditions. To simultaneously detect the genomic DNAs from the individual GM maize kernels along with the confirmation of the validity of the extracted genomic DNAs, multiplex real-time PCR analyses were performed according to a previous paper (*36*). The amplification curves of the target sequence were monitored using a fluorescent dye, which was used to label the designed oligonucleotide probes, using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). The reaction volume of 25 μL contained 2.5 μL of the sample genomic DNA solution, 12.5 μL of Universal Master Mix (Applied Biosystems), 0.5 μM primer pair, and 0.2 μM probe (except for the case of p35S, 0.1 μM probe). The PCR step-cycle program was as follows: 2 min at 50 °C and 95 °C for 10 min followed by 45 cycles of 30 s at 95 °C and 1.5 min at 59 °C.

If the amplification curves for the GMO detection could be clearly observed after 15 cycles, we considered the sample to be positive for GMOs; otherwise, it was considered to be negative, because we adopted the clarity of the amplification curves after 15 cycles of the real-time PCR as the threshold for the discrimination of the GM or non-GM maize kernel in a previous study (*36*). In this study, the "GM Maize Detection Plasmid Set - ColE1/TE -" (Nippon Gene Co., Tokyo, Japan) was used as the positive control. This plasmid set contained six concentrations of the reference plasmid pMul5, into which is inserted the amplification products of p35S, GA21, and SSIIb, diluted with the TE buffer (pH 8.0) including 5 ng/μL of the ColE1 plasmid (*31, 36*).The ColE1 plasmid contained none of the amplification GM products and was used as the negative control. The positive controls were prepared using the two concentrations of the plasmid set with 250000 and 1500 copies per plate. In the negative control, the ColE1 plasmid was also used as the nontemplate control for the analysis. In the reaction plate, the real-time PCR was performed in duplicate using two reaction vessels for the no-template control as the negative control and positive control (two concentrations of the plasmid set). The other 90 reaction vessels were used for the genomic DNA samples extracted from the single maize kernels.

Multiple Qualitative PCR Conditions. To identify which GM trait varieties are contained in the genomic DNAs extracted from individual kernels, a multiple qualitative PCR detection was performed according to our previously reported method with some modifications (*27*). The reaction mixture for the PCR was prepared in a 96-well plate. The reaction volume of 25 μL contained 25 ng of the genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 0.2 μmol/L of the 5′ and 3′ primers, 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 15 primers at the following concentrations: 0.2 μmol/L for M810 1-5′, NK603 1-5′ M863 1-5′, Bt11 1-5′, and CryIA 1-3′; 0.1 μmol/L for T25 2-5′, T25 2-3′, GA21 1-5′, GA21 1-3′, TC1507 1-5′, and TC1507 1-3′; 0.05 μmol/L for Event 176 1-5′; and 0.045 μmol/L for SSIIb 1-5′ and SSII 1-3′. The reactions were buffered with PCR buffer II (Applied Biosystems) and amplified in a thermal cycler, the Silver 96 well GeneAmp PCR System 9700 (Applied Biosystems) in 9600 mode, according to the following PCR step-cycle program: preincubation at 95 °C for 10 min, 10 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min; 27 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 7 min. The cycle was repeated 40 times followed by a final extension at 72 °C for 7 min.

Capillary Gel Electrophoresis. The PCR amplified products of multiple samples were analyzed using an automated multicapillary electrophoresis system, HDA-GT12 system (*39*) (eGene Inc., Irvine, CA), with a 12-capillary gel cartridge (GCK5000F), to rapidly and simultaneously resolve the samples. The HDA-GT12 system is an automated DNA fragment analyzer, capable of the rapid and simultaneous analysis of 12 samples at a high resolution, and allows hands-free sample analysis from a 96-well plate. Samples are electrokinetically injected into capillary channels and separated with internal calibration markers (CM: CM1 lower, CM2 upper) in every run for automatic lane alignment in an array of 13 cm long fused silica capillary columns. Ethidium bromide, included in the GCK5000F gel cartridge, is used as an intercalator dye, which intensely fluoresces in the presence of dsDNA using a green light-emitting diode (peak wavelength = 525 nm) as the excitation source. BioCalculator, software integrated in the system, controls the scheduling and separation and calculates the size of the DNA fragments on the basis of the relative migration time of each peak of the reference DNA fragments.

The 96-well plate after PCR amplification was placed in the HDA system. After the Biocalculator's AL400 method was selected, aliquots (a few nanoliters) of the PCR products in the 96-well plate were automatically injected into the capillary channels at 8000 V for 20 s and separated at 5000 V for 400 s under ambient temperature with the calibration markers, CM1 and CM2 (50 and 1000 bp DNA fragments, respectively). The separations were aligned by CM1 and CM2 and estimation of the DNA fragment size based on the registered data, which refers to PCR amplification products from each of the eight GM event seeds estimated by the Biocalculator.

Table 1. GMO Content on a Kernel Basis in Five Non-IP Maize Samples

non-IP maize sample lot	kernel number non-GM	kernel number GM	GMO content (%)
A	41	139	77.2
	total = 180		
B	53	127	70.6
	total = 180		
C	106	74	41.1
	total = 180		
D	129	51	28.3
	total = 180		
E	112	68	37.8
	total = 180		
av	441	459	51.0
	total = 900		

RESULTS

GMO Content in Non-IP Maize Samples Using Multiplex Real-Time PCR Method. We randomly sampled 180 kernels from each of the five non-IP maize samples and performed single-kernel analyses using the multiplex real-time PCR method. The multiplex real-time PCR method allows us to individually discriminate GM maize or non-GM maize and simultaneously evaluate the quality of the extracted genomic DNA for PCR in one run. As shown in **Table 1**, the GMO contents on a kernel basis in the five non-IP maize samples were 77.2, 70.6, 41.1, 28.3, and 37.8%, respectively, and their average ± standard deviation was 51.0 ± 21.6%. This result indicates that the non-IP maize samples are highly contaminated with GM maize, showing a highly variable GMO content. **Figure 1** shows the end-point analyses of the multiplex real-time PCR. Considering the amplification curves and the end-point analyses, we could clearly discriminate the non-GM kernels and GM kernels on the basis of the results of the amplification curves for all but 2 of a total of 900 kernels.

GM Trait Analysis of GM Grains Using Multiplex Qualitative PCR Method. Next, we attempted to identify, using the multiplex qualitative PCR detection method (*27*) coupled to the multichannel CGE, the HDA system, the traits of which are present in the genomic DNA from each kernel and whether these are present as single traits or stacked genes. **Figure 2A** shows a typical electropherogram of amplification products from eight reference single GM seeds. As expected, each GM trait of the eight reference single GM seeds was clearly separated on the electropherogram. In addition, six reference stacked seeds (GA21 × MON810, NK603 × MON863, NK603 × MON810, MON863 × MON810, NK603 × MON863 ×

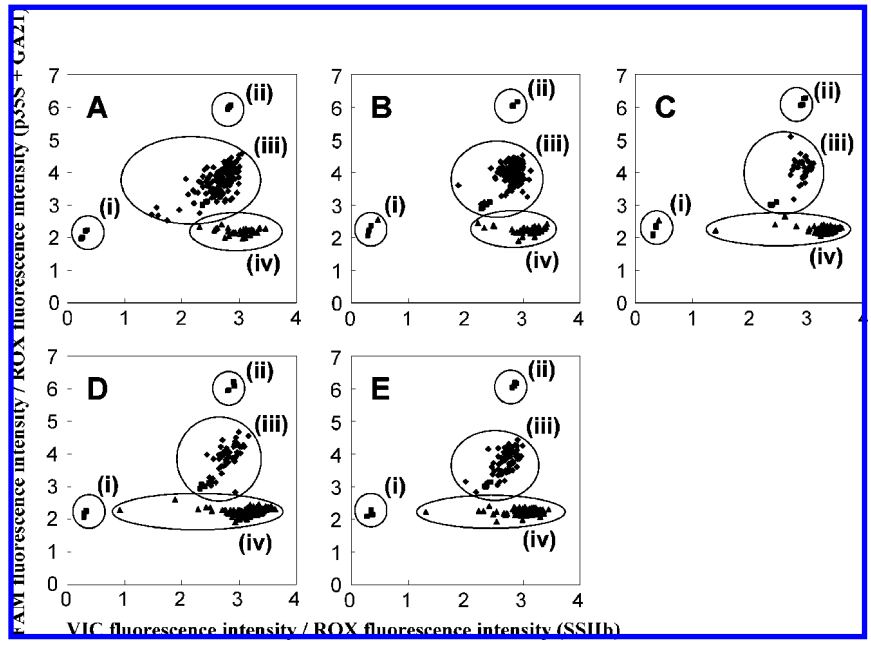

Figure 1. End-point analyses of the five non-IP maize samples and reference plasmids using the multiplex real-time PCR. **A**−**E** show the result of each non-IP maize sample lot: (i) nontemplate control (ColE1 plasmid) as the negative control; (ii) amplification of 250000 copies of reference plasmid as the positive control; (iii) amplification of genomic DNA extracted from GM maize kernels; (iv) amplification of genomic DNA from non-GM maize kernels.

MON810, and NK603 × TC1507) were also identified by the simultaneous detection of the corresponding GM traits. The electropherogram of the NK603 × MON810 reference seed is shown in **Figure 2B**.

The genomic DNAs extracted from 900 kernels of 5 non-IP maize grain samples were individually analyzed using the multiplex qualitative PCR detection method with the multichannel CGE of the HDA system. **Figure 3** shows typical electropherograms of the amplification products from the genomic DNAs extracted from individual non-IP maize grain. As shown in **Table 2**, although the GMO content of each non-IP maize sample widely varied, the tendency of the contaminated GM trait variety population appears to be similar among the samples. The populations of the non-GM grains, single GM trait grains, and stacked trait grains in the 900 kernels are shown in **Table 2**. The percentage of the GM grains is 51%, and, in the GM grains, the percentage of the single GM trait grains is 39% and that of the stacked GM trait grains is 12%. These results show that the ratio of the stacked GM trait grain population was comparatively high in the GM grains of the non-IP maize samples in 2005. The percentages of the single GM trait variety population and that of the stacked GM trait variety population also are shown in **Table 2**. The MON810 grains (55.7%) and NK603 grains (21.1%) were mainly detected in single GM traits, followed by the Bt11 grains (9.4%), TC1507 grains (4.6%), and MON863 (4.3%). Most of the detected stacked GM traits were MON810 × NK603 (87.6%), although MON810 × T25 grains (5.6%) and MON863 × NK603 grains (5.6%) were also present. In addition, the MON810 × MON863 grain was also detected, although only in a single grain. These results showed that MON810 and NK603 were most frequently detected among the single GM traits and that MON810 × NK603 was the most frequently detected stacked GM trait.

DISCUSSION

According to the Japanese GM labeling system, foods are classified into three categories as follows: (1) If GM materials are intentionally used in foods, they should, without exception, be mandatorily labeled with the phrase "GM Materials Used". (2) If the raw materials for these foods are not segregated from the GM materials, they should be mandatorily labeled with "GM Ingredient Not Segregated". (3) To be labeled "Non-GM"(volunteer system), the processor must manage their raw materials with "identity-preserved" (IP) from the U.S. farm for processing in Japan. However, some unintentional mixing of GM products in non-GM materials is inevitable and could be acceptable only when proper confirmation of the IP handling has been conducted. Consequently, the threshold level for the unintentional mixing of the GM constituents (soybean and maize) is 5% in Japan. The maize and soybean imported from the United States are segregated as non-GM by the IP handling system for the GM labeling system. Crop samples segregated as non-GM by the IP handling system are monitored to assess the validity of the GM labeling at the quarantine inspection centers using the real-time PCR method. If a sample's GM content is over 5%, the material's labeling would be corrected with guidance from the Japanese Ministry of Health, Labour and Welfare. Therefore, the definition of the threshold values is a very important issue and remains controversial in Japan.

To date, several GM maize events have been authorized for import into Japan. The GM traits include resistance to feeding damage by the European corn borer [Event 176 and Bt11 from Syngenta (formerly Novartis Seeds) and MON810 from Monsanto Co.] resistance to corn rootworm (e.g., MON863 from Monsanto Co.), tolerance to the herbicide phosphinothricin (PPT) (e.g., T25 from Bayer Crop Science), resistance to the European corn borer (TC1507 from Pioneer Hi-Bred International, Inc., Mycogen Seeds/Dow Agro Sciences LLC), and tolerance to the herbicide glyphosate (e.g., GA21 and NK603, Monsanto Co.). Furthermore, seven stacked maize products (MON863 × NK603, MON810 × NK603, MON810 × GA21, MON810 × T25, TC1507 × NK603, MON863 × MON810, and MON863 × MON810 × NK603) have already been authorized in Japan. If a stacked GM maize variety is present among the grains, the measured GM percentage might be overestimated by the measurement method. The GM percentage

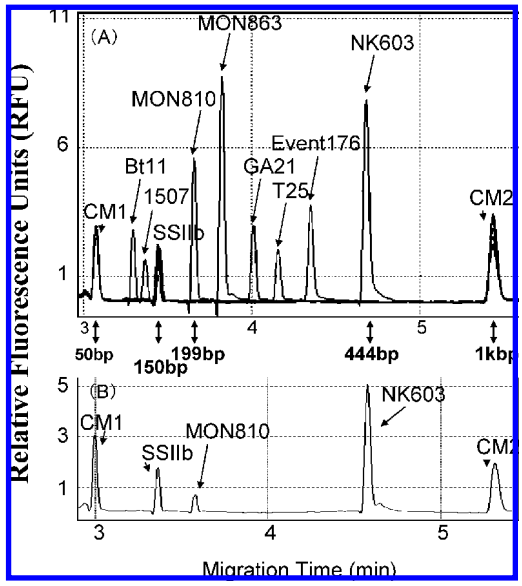

Figure 2. Typical electropherograms of amplification products from eight reference single GM seeds (**A**) and MON810 × NK603 reference seed (**B**). The PCR amplified products of multiple samples were analyzed by an automated multicapillary electrophoresis system, HDA-GT12 system (eGene, Inc.) using a 12 capillary gel cartridge (GCK5000F). (**A**) SSIIb represents the amplification product from the maize taxon specific gene; *SSIIb* gene (150 bp), Bt11 (110 bp), TC1507 (131 bp), MON810 (199 bp), MON863 (199 bp), GA21 (270 bp), T25 (311 bp) Event 176 (343 bp), and NK603 (444 bp) represent the PCR amplification product from each GM line, respectively. (**B**) Arrowheads indicate the PCR amplification product from the respective reference GM event seed. The 96-well plate after PCR amplification was placed in the HDA system. Aliquots (a few nanoliters) of the PCR products (25 μL) were automatically injected into the capillary channels at 8000 V for 20 s and separated at 5000 V for 400 s at ambient temperature with calibration markers, CM1 (50 bp) and CM2 (1000 bp). Estimation of the DNA fragments size based on the registered data, which refers to PCR amplification products from each of the eight GM event seeds, was performed by the Biocalculator.

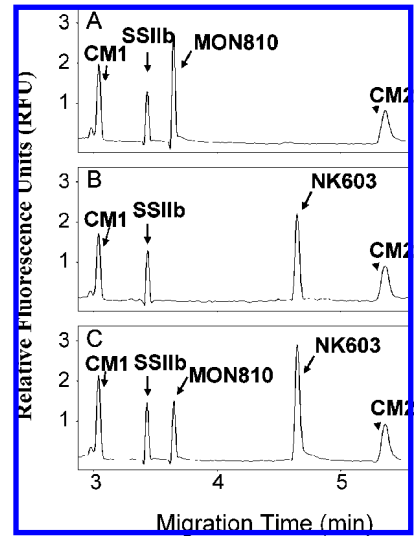

Figure 3. Typical electropherograms of amplification products from genomic DNAs extracted from typical individual maize kernels. **A**−**C** represent typical electrophregrams of amplification products from three individual maize kernels. **A**, **B**, and **C** are identified as MON810, NK603, and NON810 × NK603, respectively, in the GM trait analysis. Arrowheads indicate the identified PCR amplification products from the maize taxon specific gene, *SSIIb* gene, MON810 kernel, and NK603 kernel. SSIIb represents the identified PCR amplification product from the *SSIIb* gene. MON810 and NK603 represent the identified PCR amplification products from the MON810 kernel and NK603 kernel, respectively. CM1 (50 bp) and CM2 (1000 bp) are calibration markers.

Table 2. Population of Individual Kernels in GM Grains of Non-IP Maize Samples

	kernel number						
	nonsegregated sample lot						
GM trait	A	B	C	D	E	total	percentage
Bt11	10	10	7	2	4	33	9.4
1507	2	8	0	2	4	16	4.6
MON810	36	36	51	34	39	196	55.7
MON863	7	3	3	1	1	15	4.3
GA21	2	1	2	1	0	6	1.7
T25	8	1	0	1	2	12	3.4
NK603	33	22	7	3	9	74	21.1
single GM	98	81	70	44	59	352	39.0
MON810 × NK603	36	43	3	6	6	94	87.6
MON810 × T25	2	2	0	0	2	6	5.6
MON810 × MON863	1	0	0	0	0	1	0.9
MON863 × NK603	2	1	1	1	1	6	5.6
stack GM	41	46	4	7	9	107	12.0
non-GM	41	53	106	129	112	441	49.0
						900	100

can be expressed on a kernel basis, which is based on discrimination of the GM or non-GM for single kernels, or an a haploid genome basis. Therefore, we have previously developed a rapid and simple detection system that delivers informative results on the basis of a single-kernel analysis of grain samples and could potentially contain stacked trait GM maize kernels.

In this study, we determined the GMO content of the non-IP maize samples on a kernel basis. As expected, the results indicate that GMOs are present at a high level in the range from 30 to 80%. We used the multiplex qualitative PCR detection method coupled to the HDA multichannel CGE system. Consequently, we showed that, among an analyzed total of 900 kernels, the percentage of GM grains is 51%, the percentage of single GM trait grains is 39%, and that of the stacked GM trait grains is 12% in the GM grains. This result showed that the stacked GM traits, as well as the single GM traits, are present at high levels in the non-IP maize samples imported from the United States in 2005. This evidence implies that the measured GMO percent in the maize sample using the conventional real-time PCR method might be overestimated because the target sequences of a stacked GM maize variety are doubly or triply quantified on the basis of the determination using the haploid. Furthermore, this evidence suggests that only the individual kernel testing as shown in the present study provides informative and traceable results for the labeling regulation on a weight per weight basis.

We also clarified that MON810 grains (55.7%) and NK603 grains (21.1%) were mainly detected in the single GM trait variety, followed by the Bt11 grains (9.4%), TC1507 grains (4.6%), and MON863 (4.3%). The MON810 × NK603 grains (87.6%) were detected in the stacked GM trait variety, followed by the MON810 × T25 grains (5.6%) and the MON863 × NK603 grains (5.6%). The National Agricultural Statistics Service (NASS) reported that the single-trait GM maize planted area and the stacked GM trait variety planted area in the United States during 2005 were 52 and 9%, respectively (*40*). We

estimated that the present multiplex qualitative PCR analyses can almost cover all of the GM maize events cultivated in 2005, because it has been reported that other authorized GM maize events such as DBT41 and DDL25 are not cultivated anymore. Considering this report and these results, these results are reasonable and probably reflect the total GM grain mixing.

It would be necessary to clarify the acceptable uncertainty in consideration of the level of a risk and method applicability by a statistical approach to consider how many kernels to test as the threshold for when a declaration is made that GM or non-GM is 5%. Therefore, we analyzed them using SeedGalc7.1 software provided by the International Seed Testing Association (ISTA;www. seedtest.org) to model the proposed testing plan. The proposed testing plan has two stage tests for the individual kernel analysis. The testing plan scheme uses 90 kernels for the first screen. If there are 2 or more GM kernels in the first 90 kernel testing, another set of 90 kernels is tested. If the total GM kernels combining the two tests (180 kernels) is 9, the lot sample is determined to be a non-GM sample. In this case, the analytical data showed that the plan can keep producer confidence levels at 98.9% (and producer risk at 1.1%), although the consumer confidence level is at 83.4% (and consumer risk at 16.6%). For the feasibility of testing individual maize kernels and the hazard of GM grains, we consider that it would be a practical way to sequentially determine the percent GMO using the conventional real-time PCR assay as a screening monitor in the first step and the proposed testing plan composed of two stages for individual maize kernel testing using the present detection system as the second step.

In conclusion, we were the first to successfully determine the GMO content in non-IP maize samples imported from the United States using the single-kernel detection system that we had previously developed and coupled it to the multiplex qualitative PCR method followed by the multichannel CGE system analysis. In addition, we analyzed the GM trait varieties in GM maize kernels using the multichannel CGE system analysis. The total time needed to perform the GMO content analysis and the varieties of GM kernels for 180 maize kernels is 3 days for a sample, although the GMO content analysis would take only 2 days. The present study would provide the answer and impact for the quantification of the GM maize content in GM maize kernels on the labeling regulation. It will be necessary to clarify the acceptable uncertainty for considering the level of risk and the applicability of the detection method.

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