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Quantitative Detection System for Maize Sample Containing Combined-Trait Genetically Modified Maize

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Various countries have established regulations that stipulate the labeling of agricultural commodities, feed, and food products that contain or are made from genetically modified (GM) material or that contain adventitious GM material in amounts that exceed certain threshold levels. While regulations in some countries refer to GM material on a weight per weight (w/w) percentage, the currently applied detection methods do not directly measure the w/w percentage of the GM material. Depending on the particular method and the sample matrix it is applied to, the conversion of analytical results to a w/w percentage is challenging or not possible. The first rapid PCR system for GM maize detection on a single kernel basis has been developed. The equipment for the grinding of individual kernels and a silica membrane-based 96-well DNA extraction kit were both significantly revised and optimized for this particular purpose, respectively. We developed a multiplex real-time PCR method for the rapid quantification of GM DNA sequences in the obtained DNA solutions. In addition, a multiplex qualitative PCR detection method allows for the simultaneous detection of different GM maize traits in each kernel and thereby for identification of individual kernels that contain a combination of two or more GM traits. Especially for grain samples that potentially contain combined-trait GM maize kernels, the proposed methods can deliver informative results in a rapid, precise, and reliable manner.

Genetically modified organisms (GMO) are products of recombinant DNA technology, which can result in improved functional properties. In the past two decades, tremendous advances have been achieved in genetically modified (GM) crop species including maize. The production of GM crops, especially maize and soybean, has increased in the United States over the past several years.[1] Recently, the production of combined-trait products (stacks) of GM maize, in which two or more different characteristic traits were inserted, has also increased in the United States due to their enhanced production efficiency. The stack varieties of GM maize are actually bred by crossing a plant containing one transgene with individuals harboring another transgene to introduce sequentially new transgenes into the plant.

In some countries, the acceptance of GM foods by consumers is still controversial, and concerns about their safety persist among public opinion. In many countries, the labeling of grains, feedstuff, and foodstuff is mandatory if the 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 GMO material in a nongenetically modified (non-GM) background as the basis for labeling.[2-4] 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 determining the GMO content in foods are based on the polymerase chain reaction (PCR) due to its sensitivity, specificity, and applicability for the analysis of complex food matrixes.[5-18] Furthermore, many real-time PCR systems have been developed to identify and quantify GM maize, GM soybeans, and GM varieties of other agricultural

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(1) James, C. Global status of commercialized transgenic crops: ISAAA Briefs. 2003.
(2) Commission Regulation (EC) 49/2000 of January 10, 2000. Official J. Eur. Communities 2000; Vol. L6, pp 13- 14.
(3) Notification 79 of March 15, 2000, Department of Food Safety, the Ministry of Health, Labour and Welfare of Japan.
(4) Notification 2000-31 of April 22, 2000, Ministry of Agriculture and Forestry of Korea.
(5) Hardegger, M.; Brodmann, P.; Herrmann, A. Eur. Food Res. Technol. 1999, 209, 83- 87.
(6) Jankiewicz, A.; Broll, H.; Zagon, J. Eur. Food Res. Technol. 1999, 209, 77-82.
(7) Vollenhofer, S.; Burg, K.; Schmidt, J.; Kroath, H. J. Agric. Food Chem. 1999, 47, 5038- 5043.
(8) Hupfer, C.; Hotzel, H.; Sachse, K.; Engel, K. H. Z. Lebensm. Unters. Forsch. 1998, 206, 203- 207.
(9) Matsuoka, T.; Kawashima, Y.; Akiyama, H.; Miura, H.; Goda, Y.; Sebata, T.; Isshiki, K.; Toyoda, M.; Hino, A. J. Food Hyg. Soc. Jpn. 1999, 40, 149- 157.
(10) Matsuoka, T.; Kawashima, Y.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. J. Food Hyg. Soc. Jpn. 2000, 41, 137-143.

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commodities.[19-26] Real-time PCR systems are based on the use of fluorescent markers that monitor the formation of the PCR product during each cycle of the reaction. Most commonly, GMO quantification by quantitative real-time PCR methods is calculated from the ratio of the target GMO DNA versus DNA from the respective target plant species.

Since the analyte of these methods is DNA, the immediate results reflect, for example, the ratio of two DNA targets in the sample. Nevertheless, the unit of the reported results should ideally be equivalent to the weight per weight (w/w) percentage of the GM material in the sample. For example, it would preferably reflect the number of GM maize kernels relative to the total number of maize kernels.

Although levels of adventitious commingling of GM materials into a non-GM background for the labeling system refer to GM material on a w/w percentage, the currently applicable detection methods do not directly measure the w/w percentage of GM material. The GM percentages calculated using current quantitative PCR methods are calculated by converting relative copy numbers between a specific recombinant DNA (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 combined-trait GM maize as determined by the currently available methodology is prone 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. A combined-trait GM maize kernel contains the same traits as the two separate GM maize kernels from both individual parent GM lines. Once kernels in a grain sample have been homogenized to perform the analysis with a subsample of the homogenate, combined-trait GM kernels cannot be differentiated from separate kernels of the parent lines. This restriction is intrinsic to the sample homogenization and applies entirely irrespective of the subsequent detection methodology and target analyte. For instance, it applies to the 35S screening quantification by real-time PCR as much as it applies to the detection of biotechnology proteins by immunoassays. Only the analysis of individual maize kernels in a sample can reveal the presence of stacked maize products. In the presence of combined-trait GM maize kernels, a single-kernel analysis is also a prerequisite for determination of the GMO percentage on a w/w or kernel/kernel basis. However, the analysis of individual maize kernels has been thought to be time-consuming and not feasible for samples with hundreds of kernels.

In the present study, we developed the first rapid and simple detection system that delivers informative results by single-kernel analysis of grain samples that could potentially contain combined-trait GM maize kernels.

EXPERIMENTAL SECTION

Maize Samples. Non-GM maize grain was obtained from the Ministry of Health, Labor and Welfare (MHLW) in Japan. Maize seed from MON810, GA21, and a stacked trait product (MON810 × GA21) were kindly provided by the Monsanto Co. (St. Louis, MO) for the positive controls of the GM maize.

Oligonucleotide Primers and Probes. Sets of primer pairs and Taq-Man probes for the construct-specific and universal GM quantification were consistent with those described in our previous paper.[20,21] All the sets of primer pairs were purchased from Fasmac Co., Ltd. (Kanagawa, Japan), and the Taq-Man probes (p35S-Taq, GA21-Taq, SSIIb-Taq) for the detection of the cauliflower mosaic virus 35s promoter sequence (p35S), GA21 specifc sequence, and maize starch synthase IIb gene sequence (SSIIb) are labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethyl-rhodamine (TAMRA) at the 5′ and 3′ ends, respectively, and were also purchased from Fasmac Co., Ltd. In the Japanese official standard real-time PCR methods,[20,21,27] the SSIIb 3 system (SSIIb 3-5′ and SSIIb 3-3′ with SSIIb-Taq) were used as the primers and probe for the quantification of the taxon-specific gene encoding SSIIb, 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) are used for the screening method. The target sequence of p35S-1 system to detect the 35S promoter region derived from cauliflower mosaic virus is widely found in the rDNA of almost all GM events with the exception of GA21, and the GA21-3 system was designed to detect the construct specific sequence GM maize event GA21. The total quantification value obtained p35S-1 and GA21-3 systems is deemed as the estimated total amount of GM maize events.[27] In the multiplex real-time PCR method developed in this study, the SSIIb-TaqV, which is labeled with VIC and TAMRA at the 5′ and 3′ ends, was newly synthesized by Applied Biosystems (AB; St. Louis, MO) and used as a probe for the detection of the SSIIb instead of SSIIb-Taq. The SSIIb-TaqV sequence is 5′-VIC-AG-CAAAGTCAGAGCGCTGCAATGCA-TAMRA-3′.[22]

(11) Matsuoka, T.; Kuribara, H.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. J. Food Hyg. Soc. Jpn. 2001, 42, 24-32.
(12) Matsuoka, T.; Kuribara, H.; Takubo, K.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. J. Agric. Food Chem. 2002, 50, 2100-2109.
(13) Akiyama, H.; Sugimoto, K.; Matsumoto, M.; Isuzugawa, K.; Shibuya, M.; Goda, Y.; Toyoda, M. J. Food Hyg. Soc. Jpn. 2002, 43, 24-29.
(14) Akiyama, H.; Watanabe, T.; Wakui, C.; Chiba, Y.; Shibuya, M.; Goda, Y.; Toyoda, M. J. Food Hyg. Soc. Jpn. 2002, 43, 301-305.
(15) Watanabe, T.; Kasama, K.; Wakui, C.; Shibuya, M.; Matsuki, A.; Akiyama, H.; Maitani, T. J. Food Hyg. Soc. Jpn. 2003, 44, 281-288.
(16) Watanabe, T.; Kuribara, H.; Mishima, T.; Kikuchi, H.; Kodama, T.; Futo, S.; Kasama, K.; Toyota, A.; Nouno, M.; Saita, A.; Takahashi, K.; Hino, A.; Akiyama, H.; Maitani, T.; Kubo, M. Biol. Pharm. Bull. 2004, 27, 1333-1339.
(17) Studer, E.; Rhyner, C.; Luthy, J.; Hubner, P. Z. Lebensm. Unters. Forsch. 1998, 207, 207-213.
(18) Hupfer, C.; Hotzel, H.; Sachse, K.; Moreano, F.; Engel, K. H. Eur. Food Res. Technol. 2000, 212, 95-99.
(19) Vaitilingom, M.; Pijnenburg, H.; Gendre, F.; Brignon, P. J. Agric. Food Chem. 1999, 47, 5261-5266.
(20) Kuribara, H.; Shindo, Y.; Matsuoka, T.; Takubo, K.; Futo, S.; Aoki, N.; Hirao, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. AOAC Int. 2002, 85, 1077-1089.
(21) Shindo, Y.; Kuribara, H.; Matsuoka, T.; Futo, S.; Sawada, C.; Shono, J.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. J. AOAC Int. 2002, 85, 1119-1126.
(22) Yoshimura, T.; Kuribara, H.; Matsuoka, T.; Kodama, T.; Shigematsu, M.; Watanabe, T.; Akiyama, H.; Maitani, T.; Furui, S.; Hino, A.; J. Argic. Food Chem. 2005, 53, 2052-2059.
(23) Hubner, P.; Waiblinger, H. U.; Pietsch, K.; Brodmann, P. J. AOAC Int. 2001, 84, 1855-1864.
(24) Hubner, P.; Studer, E.; Luthy, J. Nat. Biotechnol. 1999, 17, 1137-1138.
(25) Lipp, M.; Brodmann, P.; Pietsch, K.; Pauwels, J.; Anklam, E.; Borchers, T.; Braunschweiger, G.; Busch, U.; Eklund, E.; Eriksen, F. D.; Fagan, J.; Fellinger, A.; Gaugitsch, H.; Hayes, D.; Hertel, C.; Hortner, H.; Joudrier, P.; Kruse, L.; Meyer, R.; Miraglia, M.; Muller, W.; Phillipp, P.; Popping, B.; Rentsch, R.; Wurtz, A.; et al. J. AOAC Int. 1999, 82, 923-928.
(26) Huang, H. Y.; Pan, T. M. J. Agric. Food Chem. 2004, 52, 3264-3268.
(27) Notification 0517001, 2004, Department of Food Safety, the Ministry of Health, Labour and Welfare of Japan.

Determination of GMO Amount Using Japanese Official Standard Real-Time PCR Methods. The percentage of GMO in the grinding model test sample was determined using the Japanese official standard real-time PCR methods[20,21,27] after sufficiently grinding the model test sample kernels (See the method in on-line Supporting Information.). The ratios of the copy number of the transgenic gene and the taxon-specific gene in each genuine seed of a representative variety of the GM event were calculated using the following formula-1 and defined as the conversion factor (C_f).[20-22] The GMO amounts (%) are calculated using the following defined C_f and formula-2. The C_f for MON810 and GA21 were 0.39 and 2.01, respectively.[27]

Formula-1: (C_f) (copies of the transgenic gene in the DNA extracted from GM seeds)/(copies of the taxon-specific gene in the DNA extracted from GM seeds).

Formula-2: GMO amount (%) (copies of the transgenic gene in the DNA extracted from an unknown sample × 100)/(copies of the taxon-specific gene in the DNA extracted from an unknown sample × C_f).

Evaluation of Purity and Concentration of Extracts. The DNA concentration was measured by UV absorption at 260 nm, while the DNA purity was evaluated on the basis of the UV absorption ratios of A_{260}/A_{280} and A_{260}/A_{230}. (All the samples showed an A_{260}/A_{280} ratio ranging from 1.6 to 1.9 and an A_{260}/A_{230} ratio ranging from 1.8 to 2.0.)

Grinding of Individual Single Maize Kernel. We individually ground the maize kernels using a Multi-Beads Shocker (model MB601NIHS, Yasui Kikai Co. Osaka, Japan) with an improved tube, which was developed in this study. The grinding of individual single maize seeds was performed using the Multi-Beads Shocker with the new tube holder (type SH-123) at 2500 rpm for 1 min and repeated for 1 min after the tube holder was reversed. To remove the powders and broken pieces of the other kernels, the kernels were washed with 1%sodium dodecyl sulfate, rinsed three times with distilled water (DW) and dried at 40 °C for 40 min in the incubation box before grinding the kernels as described above. The modified Multi-Beads Shocker with the new tube holder (model MB601NIHS), modified sample tube (ST-0350MZ), and distruption medium (metal corn) (MC0316MZ) in this study is commercially available from Yasui Kikai Co. (website: http://www.yasuikikai.co.jp/)

DNA Extraction from Each Maize Kernel Using DNeasy 96 Plant Kit. We modified the volume of the several extraction buffers for use and the procedure in the DNeasy 96 Plant kit protocol to simultaneously extract the maize genomic DNA from the finely ground individual powders. The modified points are as follows. Buffer AP1 (preheated to 65 °C) and RNase A (final concentration, 100 μg/mL) were combined to make a working solution. One milliliter of the working solution was directly added to each modified sample tube containing the ground maize powder described in the previous section. 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. The tubes were next incubated for 30 min at -20 °C, and then centrifuged for 20 min at 3000 rpm using Metalfuge (MBG100, Yasui Kikai Co.). A 400-μL aliquot of each supernatant was then carefully transferred to new microtubes (LF tube; Prescribe Genomics Co., Tsukuba, Japan). The microtubes were centrifuged for 5 min at 12 000 rpm. After transfer of the solution to the DNeasy 96 plate and aspiration, 800 μL of buffer AW was carefully added to each sample. The washing was repeated three times. A 800-μL aliquot of 100%ethanol was then added to each sample. For the elution of DNA from each well of the DNeasy 96 plate, 75 μL of DW (preheated to 65 °C) was added to each well. The plate was resealed and incubated for 5 min at room temperature and aspirated until each DNeasy membrane was dry. The elution was repeated twice. The entire protocol can be seen in the on-line Supporting Information.

Multiplex Real-Time PCR Conditions. The amplification curves of the target sequence was monitored using a fluorescent dye, which was labeled for the designed oligonucleotide probes, using the ABI PRISM 7900HT sequence detection system (AB). The reaction volume of 25 μL contained 2.5 μL of the sample genomic DNA solution, 12.5 μL of Universal Master Mix (AB), 0.5 μM primer pair, and 0.2 μM probe (except for the case of p35S, 0.1 μM probe). The reaction conditions were made for the following PCR step-cycle program: 2 min at 50 °C, and 95 °C for 10 min and 45 cycles, 30 s at 95 °C, and 1 min 30 s at 59 °C.

If the amplification curves for the GMO detection or taxon gene detection were clearly observed after 15 cycles, we considered the sample as positive. If the amplification curve for the GMO detection or taxon gene detection was not observed after 15 cycles, we considered the sample as negative.

In this study, the GM Maize Detection Plasmid Sets ColE1/TE s (Nippon Gene Co., Tokyo, Japan) was used as the positive control. This plasmid set contained six concentrations (including the ColE1 plasmid as nontemplate control) of the reference plasmid pMul5, which is inserted into all the amplification products of p35S, GA21, and SSIIb, diluted with the TE buffer (pH 8.0) including 5 ng/μL ColE1 plasmid.[20,21] The ColE1 plasmid contained none of the amplification products of p35S, GA21, and SSIIb. The positive controls were prepared using the two concentrations of the plasmid set such as 250 000 and 1500 copies/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 NTC as the negative control and positive control (two concentrations of the plasmid set). The other 90 reaction vessels were individually examined for the genomic DNA samples extracted from single maize kernels.

Multiplex Qualitative PCR Conditions. Multiplex qualitative PCR detection was performed according to our previously reported method with some modifications.[11] The reaction mixture for the PCR was prepared in a PCR reaction tube. The reaction volume of 25 μL contained 25 ng of genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 0.5 μmol/L 5′ and 3′ primers, and 0.625 unit of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The reactions were buffered with PCR buffer II (AB) and amplified in a thermal cycler (GeneAmp PCR System 9700; AB) according to the following PCR step-cycle program: preincubation at 95 °C for 10 min, denaturation at 95 °C for 0.5 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 0.5 min. The cycle was repeated 40 times followed by a final extension at 72 °C for 7 min.

In addition, after PCR amplification, agarose gel electrophoresis of the PCR product was carried out according to previous

Table 1. Overestimation of GMO Amounts Using the Conventional Real-Time PCR Methods in the Sample Containing the Stacked-Trait Product[a]

	contents of GM maize	
	mean (%)	SD[b]
stack (MON810xGA21)	15.1	1.0
MON810 and GA21	12.2	0.3
MON810	5.9	0.4
GA21	6.1	1.3

[a] All experiments were performed three times. In this experiments, the SSIIb 3 (SSIIb 3′ 5′ and SSIIb 3′ 3′ with SSIIb-Taq) were used for quantification of the taxon-specific sequences, and P35S-1 (P35S 1′ 5′ and P35S 1′ 3′ with P35S-Taq) for the detection of MON810 and GA21-3 (GA21 3′ 5′ and GA21 3′ 3′ with GA21-Taq) were used for the quantification of the transgenic specific sequences in the GM maize sample. [b] SD standard deviation.

studies.[11-14] The amplification products (7.5 μL) of each specific PCR were submitted for electrophoresis at a constant voltage (100 V) on a 3% Takara L03 agarose gel (Takara Co., Ltd., Tokyo, Japan) in the TAE (40 mmol/L Tris-HCl, 40 mmol/L acetic acid, and 1 mmol/L EDTA, pH 8.0) buffer solution. After gel electrophoresis, the gel was stained in DW containing 0.5 μg/mL ethidium bromide for 30 min and then washed in DW for 30 min. The gel was photographed using a Chemi-lumi Image Analyzer with the Diana system as the analytical software (Raytest, Straubenhardt, Germany).

RESULTS

Overestimation of GMO Content in Samples of Combined-Trait Products Using Real-Time PCR Method. A model test sample containing 180 kernels was prepared using 9 kernels of a variety of stack GM maize (YieldGard Corn Borer with Roundup Ready corn 1; MON810 x GA21 (YieldGard, Roundup Ready, and GA21 are registered trademarks of Monsanto Technology LLC. MON810 x GA21 was obtained from the Monsanto Co.) and 171 kernels of non-GM maize. The percentage of GMO in the model test sample was determined using the Japanese official standard real-time PCR methods[20,21,27] after sufficiently grinding the model test sample kernels. As shown in Table 1, the value was determined to be ~15.1% though the value should be closer to 5% on a weight per weight basis. A second 180-kernel test sample was prepared using 9 kernels of MON810, 9 kernels of GA21, and 162 kernels of non-GM maize. The percent GMO of this sample was determined to be 12.2%. These results clearly suggested that the GMO level determined using the conventional real-time PCR methods could be overestimated in the sample containing the stack variety. This is because the GMO percentage is calculated on the haploid basis, which is defined by the formulas as described in our previous reports and in the Experimental Section.[20,27]

Grinding of Individual Maize Kernel. To rapidly grind maize kernels into a fine powder, we made several modifications to the grinder (Multi-Beads Shocker).[28] The program for the Multi-Beads Shocker was upgraded so that the maximum speed of the instrument could reach 2500 rpm. The diameter of the chamber

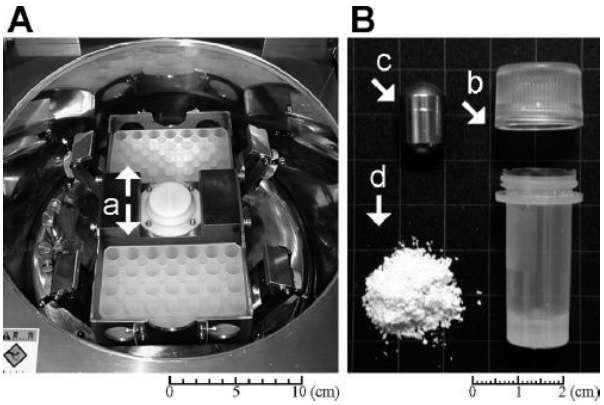

Figure 1. Newly designed Multi-Beads Shocker (A) and sample tube with metal corn (B). Multi-Beads Shocker was redesigned, and tubes and metal corn were modified to improve the efficiency as described in the text. (A) Inside the chamber of Multi-Beads Shocker; (B) sample tube with metal corn; (a) sample holder, (b) tube, (c) metal corn, and (d) ground maize after disruption.

was widened to 340 mm for easy handling of the sample holder (Figure 1A). In addition, we redesigned the sample holder, sample tube, and disruption medium (metal corn) for better performance. The safety plate for the sample tubes was modified so that it could be secured using a torque wrench for high-speed operation (Figure 1A). A new sample holder was made to accommodate a 24-well plate for the convenience of handling a large number of samples (Figure 1A-a). The sample holder was made of a synthetic resin (plastic) in place of metal so that the instrument could be operated at a higher speed (Figure 1A-a). The diameter of the sample tube was changed from 10.5 to 12 mm so that an individual kernel was easily placed in the tube (Figure 1B-b). Furthermore, the shape of the metal corn was rounded at the both ends as shown in Figure 1B-c in order to prevent them from adhering to the inner cap and also to increase the grinding efficiency. The structure of the inner cap was also changed to a round shape, and an O-ring was placed in the tube cap to prevent sample leakage (Figure 1B-b). When 48 kernels were ground in this remodeled equipment, all the kernels were uniformly ground into a fine power in 2 min with a high efficiency regardless of their size and shape (Figure 1B-d).

DNA Extraction from Each Maize Kernel Using DNeasy 96 Plant Kit. To simultaneously extract the maize genomic DNA from the finely ground individual powders described above, we applied the 96-well extraction kits and developed suitable extraction conditions. First, we examined the silica membrane-type kit (DNeasy 96 Plant Kit, Qiagen) and magnet-type kit (Wizard Magnetic 96 DNA Plant System, Promega). The silica membrane-based method appeared to be significantly better than the magnetic-type method in terms of the content and quality of the extracted genomic DNA. We then modified the volume of the several extraction buffers for use and the procedure in the DNeasy 96 Plant Kit as described in the Experimental Section.

To investigate the yield and quality of the genomic DNA individually extracted from the maize kernels, we evaluated the concentration and purity of the genomic DNA from 12 maize kernels. As shown in Table 2, the yields from the individual maize kernels in all the products ranged from 104.5 to 125.0 ng/μL with

(28) Katou, Y.; Kanoh, Y.; Bando, M.; Noguchi, H.; Tanaka, H.; Ashikari, T.; Sugimoto, K.; Shirahige, K. Nature 2003, 424, 1078-1083.

Table 2. Yield and Quality of Genomic DNA in Four Kinds of Maize Using the Modified Extraction Method[a]

	non-GM		MON810		GA21		stack (MON810xGA21)		total	
	mean	RSD (%)	mean	RSD (%)	mean	RSD (%)	mean	RSD (%)	mean	RSD (%)
DNA conc (ng/ μL)	123	15.1	125	8.8	104.5	10.5	115.7	12.3	117	13.6
260/ 280 ratio	1.77	1.18	1.75	0.59	1.77	1.06	1.75	0.57	1.76	0.99
260/ 230 ratio	1.94	5.94	1.86	3.65	1.89	3.89	1.9	2.84	1.9	4.39

[a] Each value represents the mean and relative standard deviation (RSD (%)) of 12 kernel determinants. The UV absorption ratios of 260 nm/ 280 nm and 260 nm/ 230 nm ratios represent A_{260}/A_{280} and A_{260}/A_{230}, respectively, of genomic DNA individually extracted from maize kernel samples.

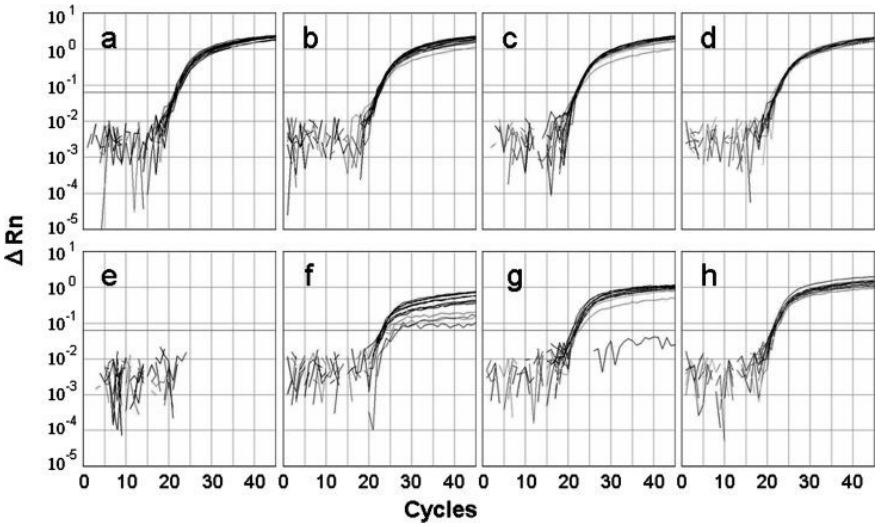

Figure 2. Amplification curves of the 12 genomic DNA solutions per one kind of maize (total of 48 test solutions) using the multiplex real-time PCR method. (a) SSIIb detection of non-GM maize, (b) SSIIb detection of MON810 maize, (c) SSIIb detection of GA21 maize, (d) SSIIb detection of stacked maize (MON810 × GA21), (e) p35S detection and GA21 specific detection of non-GM maize, (f) p35S detection of MON810 maize, (g) specific detection of GA21 maize, and (h) p35S and GA21 specific detection of stacked maize (MON810 × GA21).

an average of 117.0 ng/ μL. All of the GM and non-GM maize varieties had A_{260}/A_{280} ratios between 1.6 and 1.9.

Multiplex Real-Time PCR Qualitative Detection of Genomic DNA from GM Maize Kernel. To identify the extracted genomic DNA from the GM maize kernels, the development of the multiplex real-time PCR method was evaluated. The cauliflower mosaic virus p35S was introduced into maize products MON810, Bt11, T25, and Event 176, but not introduced into GA21.[10,11] Therefore, the two sets of primer and probe for both the p35S detection and GA21 specific detection were considered to be mixed in one reaction tube to detect the genomic DNA from the GM maize kernel. In addition, to check the validity of the extracted genomic DNA for PCR, the set of primer pair and probe for the detection of the SSIIb, which is a the maize taxon-specific gene, would be considered to be added to one tube and simultaneously detected. However, as described in a previous paper, P35S-Taq, GA21-Taq, and SSIIb-Taq were labeled with FAM dye.[20,21] Therefore, we redesigned the SSIIb probe labeled with VIC dye (SSIIb-TaqV) and then used this probe.

With three pairs of primers (p35S-1, GA21-3, SSIIb-3) and three probes (p35S-Taq, GA21-Taq, SSIIb-TaqV) in one reaction, the interaction among the primers and probes appeared to cause a decrease in the PCR efficiency. The efficiency of SSIIb amplification was more significantly affected than the other two (data not shown). Therefore, we increased the concentration of the SSIIb primers (SSIIb-3) and probe (SSIIb-TaqV) higher than those of the other two primer pairs and probes and also increased the time from 1 to 1 min 30 s as the annealing step.

As expected, the amplification curves of both p35S-Taq and GA21-Taq labeled with the FAM dye were successfully observed and the amplification curves of SSIIb-TaqV labeled with the VIC dye were also simultaneously detected. These results suggested that detection of target DNA sequences from the GM maize kernels and confirmation of the validity of the extracted genomic DNA for PCR can be simultaneously performed in one run. Furthermore, we examined whether the amplification curves of SSIIb and those of p35S and GA21 could be consistently observed in 12 genomic DNA solutions extracted from non-GM, MON 810, GA21, and a stack variety of MON810 × GA21 maize kernels. As shown in Figure 2, we confirmed that all the amplification curves of SSIIb and those of p35S and GA21 can be obtained in all the relevant events of the examined maize under the developed multiplex real-time PCR conditions irrespective of a stacked GM event trait or single GM event trait.

In addition, we examined the end-point analysis of the stack maize product and reference plasmids using the developed multiplex real-time PCR method. As shown in Figure 3, the genomic DNA from MON810 × GA21 can be clearly identified

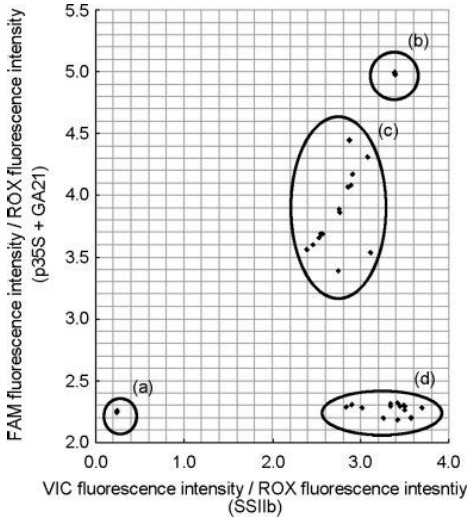

Figure 3. End-point analysis of the stacked GM maize sample, non-GM sample and reference plasmids using the multiplex real-time PCR. (a) nontemplate control (ColE1 plasmid) as a negative control, (b) amplification of 250 000 copies of reference plasmid as a positive control, (c) amplification of genomic DNA from stacked maize kernels (MON810 × GA21), (d) amplification of genomic DNA from non-GM maize kernels. The GM Maize Detection Plasmid Set- ColE1/TE (Nippon Gene Co.) was used as the positive control and the negative control. This plasmid set contained six concentrations (including the ColE1 plasmid as nontemplate control) of the reference plasmid pMul5, which is inserted into all the amplification products of p35S, GA21, and SSIIb, diluted with the TE buffer (pH 8.0) including 5 ng/μL ColE1 plasmid. The ColE1 plasmid contained none of the amplification products of p35S, GA21, and SSIIb. In a negative control, the ColE1 plasmid was also used as the nontemplate control for the analysis. In the reaction plate, the multiplex real-time PCR was performed in duplicate using six reaction vessels for the negative control and positive control (two concentrations of the plasmid set). The other 90 reaction vessels were individually examined for the genomic DNA samples extracted from single maize kernels.

from those of the non-GM maize samples and reference plasmids using the end-point analysis of the developed multiplex real-time PCR method as a complement confirmation.

Verification of Proposed Detection Method for a Stacked Maize Product. The detection method developed for stacked maize products was used to detect the three types of 5% model test samples containing 9 kernels of transgenic maize seeds (MON810 or GA21 or MON810 × GA21) and 171 kernels of non-GM maize. As expected, we detected the target DNA sequence derived from the respective GM maize (9 kernels) in the three types of 5% model test samples based on the amplification curves. We showed the typical amplification curves of the p35S system for MON810 seeds and non-GM maize samples in Figure 4, and the end-point analysis result of the model test sample containing the MON810 maize seeds as a complement confirmation in Figure 5. As shown in Figure 4, when the amplification curves were clearly observed after 15 cycles, we can denote the sample as GM positive (Figure 4a). In contrast, when the amplification curve was not observed after 15 cycles, we can denote the sample as GM negative (Figure 4b). Therefore, we set up the clearness of the amplification curves after 15 cycles of the real-time PCR as the threshold for the discrimination of GM or non-GM maize kernel.

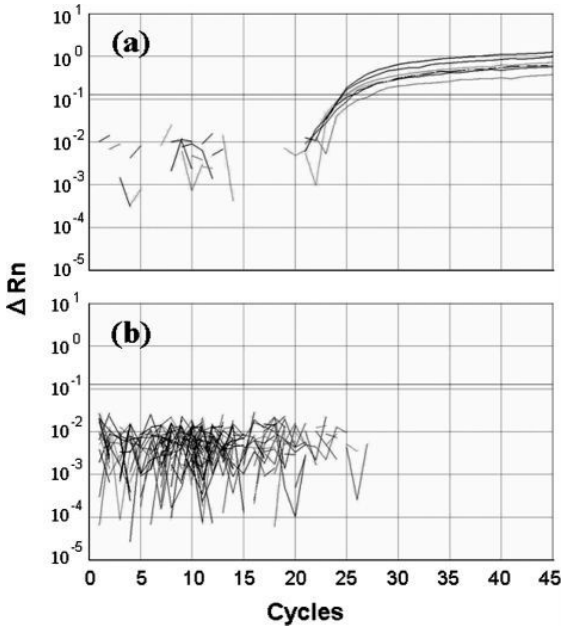

Figure 4. Typical amplification curves of the model test sample containing the MON810 maize seeds using the multiplex real-time PCR. (a) Typical amplification curves of the MON810 maize seeds in the model test sample, (b) Typical amplification curves of the non-GM maize grains in the model test sample.

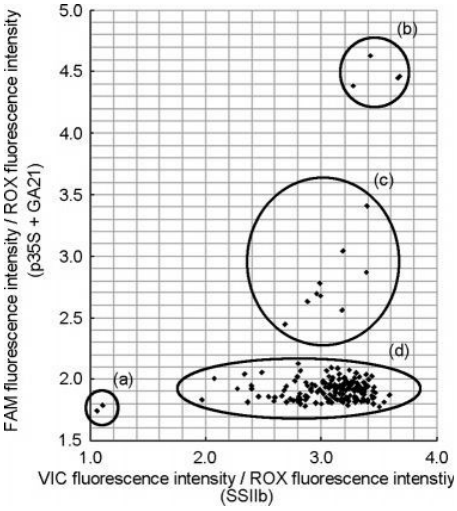

Figure 5. End-point analysis of the model test sample. (a) no-template control as a negative control, (b) amplification of 250 000 copies of standard plasmid as a postitive control, (c) amplification of genomic DNA from MON810 maize seeds, and (d) amplification of genomic DNA from non-GM maize kernels. All the conditions were as in Figure 3.

Confirmation of Stacked Maize Kernel Using Multiplex Qualitative PCR Detection. Finally, we identified whether the genomic DNA from each kernel contains the stacked traits or a single trait using the multiplex qualitative PCR detection method published in a previous report.[11] As shown in Figure 6, each GM trait was identified as expected. For MON810 × GA21, the two amplification bands for MON810 and GA21 were simultaneously detected by the agarose gel electrophoresis analysis in one extraction.

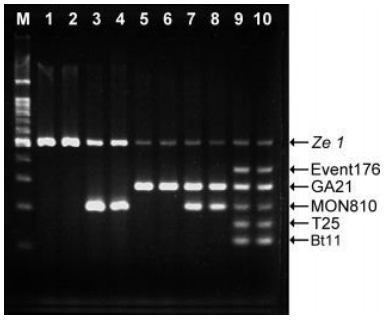

Figure 6. Agarose gel electrophoresis of the PCR products amplified from genomic DNA extracted from individual maize kernels. Arrowheads indicate the expected PCR amplification products. *Ze1* represents the amplification product from maize taxon-specific gene, *zein* gene. Event 176, GA21, MON810, T25, and Bt11 represent the PCR amplification product from each GM line, respectively. Lanes 1 and 2: amplification of genomic DNA from non-GM maize kernel. Lanes 3 and 4: amplification of genomic DNA from MON810 kernel. Lanes 5 and 6: amplification of genomic DNA from GA21 kernel. Lanes 7 and 8: amplification of genomic DNA from MON810 × GA21 kernel. Lanes 9 and 10: amplification of the mixed genomic DNA of five lines (event 176, GA21, MON810, T25, and Bt11). M: 100-bp ladder size standard.

DISCUSSION

After the introduction of new labeling system for GM foods in Japan and other countries, many food industries in the countries have been obliged to switch to non-GM materials largely to meet demand from retailers and GMO are excluded from the market except for special products, such as edible oil and animal feed. To monitor the commingling level of GMO in non-GM materials controlled by an identity-preserved handling system, the screening system using the combination of quantification systems for the p35S region and GA21 specific region is the most practical method at the first step for the maize grains in the United States and Canada. A possibility, however, which the relative GMO amount measured from the copy numbers of SSIIb and GM target sequences is not consistent with the actual GMO mixing ratio (%) in grains, would arise by development of stacked maize products.

Several GM maize evevts 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), 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, tolerance to PPT (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, six stacked maize products (MON863 × NK603, MON810 × GA21, MON810 × T25, TC1507 × NK603, MON863 × MON810, MON863 × MON810 × NK603) have already been authorized in Japan. All the authorized GM maize events have p35S except for the GA21. Then, if the stacked GM maize variety is included in the grains, the measured percent GMO might be overestimated by the doubly quantified target sequences. The percent GM could be expressed on a kernel basis, which is based on discrimination of GM or non-GM for single kernels or on a haploid basis as described in the Results.

We have described the first rapid detection system for the amount of GMO in a sample containing a GM stacked maize product. We first improved the grinding equipment and the tube for grinding some maize kernels in terms of the tube size and the inconsistent fineness of the ground maize powder. We improved the sample tube shape as well as the disruption medium (metal corns) and sample holder in order to grind, irrespective of the maize kernel size. Furthermore, we improved the performance of the blender by enhancing the durability of the equipment and by replacing the motor with a high-speed one. Using this improved equipment and tube, we confirmed that 48 individual maize kernels could be ground in a few minutes. In addition, we examined the genomic DNA extraction step for a single ground maize powder. We optimized a rapid extraction method using the modified silica membrane-based 96-plate extraction kits. Although we examined the magnetic-based 96-plate extraction kit, the silica membrane-based method performed better than the magnetic-based method in terms of the content and quality of the extracted genomic DNA (data not shown).

We also developed a multiplex real-time PCR method for the rapid qualitative detection of the genomic DNA extracted from a maize kernel. This developed method can individually discriminate a stacked or single GM trait of GM maize kernels authorized in Japan and simultaneously evaluate the quality of the extracted genomic DNA for PCR in one run. We can discriminate GM or non-GM kernels by the amplification curves from the multiplex real-time PCR as shown in Figure 2 and Figure 4 irrespective of the stacked GM event trait or single GM event trait. The end-point analyses as in Figures 3 and 5 could give supplemental information for the judgment. Furthermore, we confirmed that it can be determined as to whether the genomic DNA from each kernel contains the stacked traits or a single trait using the multiplex qualitative PCR detection method published in a previous report.[11] The multiplex real-time PCR detection method may be applicable for the detection of all authorized GM maize kernels because this method can qualitatively detect both p35S and GA21. The detection system we have developed is also applicable to other combined-trait maize products. For example, Monsanto's triple stack maize product (YieldGard Plus with Roundup Ready, MON863 × MON810 × NK603), which incorporates Bacillus thuringiensis (Bt) toxins for corn borer and rootworm and tolerance to glyphosate, should be detectable. In addition, this detection system could be applicable for the detection of other GM crops such as wheat and soybeans.

We consider that it can clearly discriminate GM or non-GM kernel using the judgment system, though a false positive might occur. Therefore, for only the positive samples, it would be necessary to confirm the results using a multiplex qualitative PCR detection as in Figure 6. In addition, since the multiplex real-time PCR would be very sensitive, carryover contamination from one kernel to another during the grinding process and the DNA extraction step might occur. Therefore, we considered that the grinding process and the DNA extraction step should be carefully performed. Furthermore, we would also improve the experimental conditions of multiplex real-time PCR to clearly discriminate GM and non-GM kernels in future.

Many quantitative methods for GMO analyses have been developed.[6] Undoubtedly, the most widely used methods are

based on PCR and, more particularly, the quantitative real-time PCR.[7] GMO quantification in mixed food samples using these quantitative real-time PCR methods is based on the calculation using the ratio of GMO (copies of the recombinant DNA sequence) versus the plant species-specific DNA content (copies of the taxon-specific gene), calculated on a haploid basis. The calculated value is converted into a percent GMO content (weight to weight). This conversion is based on the assumption that there is a direct 1:1 relationship between the endogenous gene and the GM gene. However, there are many biological factors that can affect this 1:1 relationship, such as GM gene copy number, DNA degradation, DNA endoreduplicaion, outcrossing versus inbreeding, and variability in the genome.[29] Therefore, it is necessary to attempt to convert the GMO content using the conversion factor such as the value defined by formula-1 in the Japanese current official method[20,21] In addition, as we have already described, the GMO content of maize samples containing a stacked-trait maize product could be overestimated using the conventional real-time PCR if the definition of the threshold values for the labeling regulation are on a weight per weight basis.

In the consideration of the feasibility of testing individual maize kernels, for example, when GMO mixing percent is clearly less than the threshold levels that each country have set up, it would be attained by the measurement of the conventional real-time PCR method using p35S-1 andGA21-3 systems. Therefore, we assume that it should be a reasonable way to determine the GMO content using the combination of the grinding maize sample and the conventional real-time PCR method as a screening method. The individual maize grain testing using the method system developed in this report would be applied only when GMO mixing percent larger than around or over the threshold levels is measured by the conventional real-time PCR method. It will be necessary to clarify acceptable uncertainty in consideration of the level of a risk and method applicability by statistical approach in order to consider how many grains to inspect. It will be a practical way to determine percent GMO using the conventional real-time PCR method as a screening monitor at the first step and the individual maize kernel testing for ~200 grains using the detection system developed in the present study. In Japan, we have already been monitoring the GMO labeling at the quarantine inspection centers using the real-time PCR method. If a sample's GMO content was over 5% the material's labeling would be corrected by Ministry guidance. Therefore, the definition of threshold values is very important and remains controversial in Japan. However, we believe that the application of single-kernel testing should be considered from the viewpoint of traceability.

(29) Lipp, M.; Shillito, R.; Giroux, R.; Spiegelhalter, F.; Charlton, S.; Pinero, D.; Song, P. J. AOAC Int. 2005, 88, 136- 155.

CONCLUSION

We have successfully developed a simple detection system that delivers informative results by the single-kernel analysis of grain samples that could potentially contain combined-trait GM maize kernels. The present detection method system is a novel, rapid, precise, and reliable technique for the quantitative analysis of GM samples containing stacked-trait products. Approximately 180 maize kernels can be individually detected within a couple of days. This proposed method system could accurately monitor the labeling system in a reliable manner and can be useful for governmental regulation.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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