Improvement of the group testing method to evaluate GM maize content

研究ノート

Improvement of the group testing method to evaluate GM maize content

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Abstract

The use of genetically modified (GM) stacked-event products combining two or more GM parental events, especially in maize, has been increasing. This situation has made real-time PCR-based quantitative methods prone to overestimation of the content of GM organisms (GMOs) relative to the actual weight proportion. To solve this problem, we previously proposed a group testing method enabling accurate weight-based evaluation of GMO content in maize grains. In the present study, this method was slightly modified and validated in-house in order to enhance its reliability and user friendliness. Additionally, we developed a testing method for identification of GM events in the lysate samples used for the group testing. First, we confirmed specificity of the modified method by means of authentic GM and non-GM materials. Evaluation of the limit of detection suggested that the modified method reliably detected a single GM kernel in a group sample composed of 20 maize kernels. Then, we conducted a blind test using simulated group samples. All of the testing results matched the presence/absence of GM kernels in the simulated samples. Furthermore, identification of GM events in the group testing samples was successfully achieved by DNA purification from the residual lysates and subsequent real-time PCR array analyses.

Key words: GMO, group testing, validation

Introduction

In order to give consumers the freedom of choice between genetically modified (GM) and conventional food products[1)], food-labeling regulations regarding GM organisms (GMOs) were implemented in 2001 in Japan. According to these regulations, "non-GMO" labeling is permitted only when final products are made from non-GMO materials grown and distributed within an identity-preserving handling system. In this system, unintentional commingling of GMOs is allowed up to 5% of content for maize and soybeans. Therefore, quantitative GMO analyses for maize and soybeans are needed to monitor the validity of food labeling.

In recent years, crop varieties with stacked GM events (resulting from crossing of two or more single GM events) have been widely used, especially in maize products[2)]. Although the most commonly used method for quantitative GMO analysis is real-time PCR, assessment of GMO

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content of stacked-event products by real-time PCR leads to overestimation relative to the actual weight-based GMO content[3]. This is because a kernel of a stacked-event product contains the PCR target sequences corresponding to two or more single-event kernels. Because of the increasing use of stacked-event products, it has become virtually impossible to accurately measure GMO content by means of the currently available methodology.

To solve this problem, Akiyama *et al.* developed an individual-kernel detection method that involves multiplex real-time PCR analysis of the DNA extracted from individual ground maize kernels[3]. This detection system has already been adopted as an official GM maize detection method in Japan. Afterwards, we developed a group testing method[4], as an alternative, to make evaluation of the kernel-based GMO content easier. Recently, Noguchi *et al.* reported a trait-specific real-time PCR method that allows for quantification of GM maize content irrespective of stacked GM maize commingling in ground samples[5]. In the trait-specific method, the real-time PCR-quantified herbicide tolerance genes *cp4-epsps* and *pat* and their copy numbers were converted into GMO content on the basis of surveillance of the actual GM event distribution. Certainly, this method may be useful for analysis of GMO content in ground maize samples including stacked-event GM grains. Nevertheless, to make the trait-specific method less biased, extensive surveillance of GM events in commercially distributed grain samples as previously reported[6) 7)], should be conducted on a continuous basis. This approach is expected to decrease feasibility of the method. Thus, the group testing is a promising method for quantification of GM maize irrespective of the stacked GMO commingling.

In the group testing, a predetermined number of groups is taken from a larger bulk sample, while each group contains a defined number of kernels; then GMO content is evaluated statistically on the basis of qualitative results on multiple small pools of grains (Fig. 1). The developed method enabled efficient evaluation of GMO content on a weight/weight basis irrespective regardless of the presence of stacked-event products. The previously developed testing method consisted of a sample pretreatment step (in which a group of 20 maize kernels was ground in a lysis buffer by means of a household food processor) and a subsequent PCR assay step, in which the lysed sample was directly analyzed as a DNA template by qualitative PCR. For the qualitative PCR analysis, we set up two duplex real-time PCR assays, a GM maize screening assay, and an experimental control assay. The GM maize screening assay was designed to detect the 35S promoter region (P35S) and NOS terminator region (TNOS) widely introduced into commercially available GM maize events. The experimental control assay is intended to confirm that the reaction mixture contained sufficient amounts of extracted DNA, and there is no PCR inhibition. The target sequences of the experimental control assay were designed to detect both the starch synthase IIb gene from *Zea mays* (SSIIb, as the endogenous reference DNA) and an artificial sequence in small amounts of plasmid DNA (as an internal positive control; IPC).

In the previously conducted collaborative trial of the group testing method[4], we encountered PCR inhibition

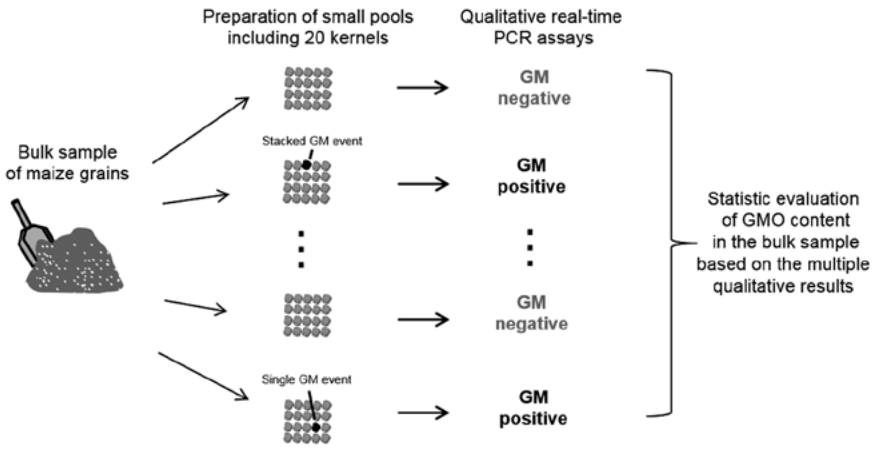

Fig. 1 An outline of the group testing strategy.

in one sample out of 216 samples, and just this one PCR result had to be eliminated from the analysis. To make the group testing method more practical, the frequency of elimination of samples due to PCR inhibition should be reduced, and this approach will also decrease the number of extra analyses. In order to make the group testing robust, we recently developed a novel real-time PCR master mix whose efficiency is not affected by PCR inhibitors from maize grains. We found that this master mix reagent enabled direct real-time PCR analysis of crude cell lysates of a wide range of foods and agricultural products[8]. In the present study, we attempted to enhance the method's reliability and user friendliness by means of some modifications including adoption of the master mix reagent. We also validated the modified method. Additionally, we developed a method for GM event identification after the group testing.

Materials and Methods

1. Materials

We used the following representative GM maize events: Bt11, DAS-59122-7, Event176, GA21, MIR162, MIR604, MON810, MON863, MON88017, MON89034, NK603, TC1507, T25, and 3272. F1-generation seeds or ground materials of Bt11, Event176, GA21, MIR162, MIR604, and 3272 were kindly provided by Syngenta Seeds (Basel, Switzerland); F1-generation seeds of MON810, MON863, MON88017, MON89034, and NK603 were kindly provided by Monsanto (St. Louis, MO, USA); and F1-generation seeds of DAS-59122-7 and TC1507 were kindly provided by Pioneer Hi-Bred International (Johnston, IA, USA). F1-generation seeds of T25 were imported from the USA. Dry seeds of conventional maize were imported from the USA and used as non-GM maize. For preparation of purified DNA extracts, we used the DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany) as described in the "JAS analytical test handbook."[9] DNA concentration in the solutions was determined by measuring ultraviolet (UV) light absorbance at 260 nm. All DNA extracts were diluted to the concentration of 20 ng/μL and then used for the subsequent PCR analyses. The purity of each genuine GM and conventional seed was confirmed by the real-time PCR array as reported[10, 11].

To prepare the DNA template for the IPC reaction, a pUC19 plasmid harboring the artificial sequence Art was constructed and named pART as we reported previously[4]. After linearization by a restriction enzyme and purification by ethanol precipitation, the theoretical copy number of the pART solution was calculated from the theoretical molecular weight of the plasmid and the mass-based DNA concentration estimated by the UV spectroscopy. The solution was diluted to a given concentration with a 5 ng/μL ColE1 plasmid solution in Tris/ethylenediaminetetraacetic acid (EDTA) buffer (Nippon Gene, Tokyo, Japan) on the basis of the theoretical copy number. Then, we evaluated the copy number of the Art sequence by digital PCR analysis using the QuantStudio 3D Digital PCR System (Life Technologies, Carlsbad, CA, USA). We prepared the reaction mixture in the total volume of 30 μL, which included 27 pmol of primers IPC 1-5′ (5′-CCGAGCTTACAAGGCAGGTT-3′) and IPC 1-3′ (5′-TGGCTCGTACACCAGCATACTAG-3′), 7.5 pmol of IPC 1-Taq probe (5′-TAGCTTCAAGCATCTGGCTGTCGGC-3′), 5 μL of a DNA template, and 15 μL of QuantStudio 3D Digital PCR Master Mix (Life Technologies). IPC 1-Taq was labeled with the dyes 6-carboxyfluorescein (FAM) and black hole quencher 1 (BHQ1) at the 5′ and 3′ termini, respectively. We loaded 14.5 μL of the reaction mixture into each Digital PCR 20K chip, and two chips were prepared as duplicates. Then, the chips were subjected to thermal cycling on a GeneAmp PCR System 9700 (Life Technologies). The thermal cycling conditions were as follows: 10 min at 96°C, 39 cycles of 2 min at 60°C and 30 s at 98°C, then 2 min at 60°C and an indefinite hold at 10°C. Fluorescence scanning of the chip was performed by means of the QuantStudio 3D Digital PCR instrument (Life Technologies), and the raw data were analyzed in the QuantStudio 3D AnalysisSuite software (Life Technologies). The pART solution whose copy numbers were estimated at 17–23 copies/μL was used as the DNA template in the IPC reaction.

2. The modified method of group testing

We modified the conditions of the real-time PCR analysis in the previously reported group testing method[4]. We chose a newly developed real-time PCR master mix reagent, DirectAce qPCR Mix (Nippon Gene), as the PCR enzyme and buffer. The primer pair SSIIb 3-5′ and SSIIb 3-3′ was used as a substitute for the primer pair SSIIb 1-5′ and 1-3′. In the previous testing method, theoretically estimated 40 copies of the pART plasmid were used as the DNA template for IPC reaction. Meanwhile, in this study, the concentration of the pART plasmid solution was maintained

at 17–23 copies/μL, and 1 μL of the solution was added to the reaction mixture. The modified testing protocol was as follows:

Step 1[4]. A grain counter plate (For 100 Soybeans; Fuji Kinzoku, Tokyo, Japan) is ready, and only 20 holes are made available by covering the plate with a sheet of a cellophane film or aluminum foil. Twenty maize kernels are scooped as one group with the plate and are put into glass vessels (sample capacity, 75 mL) of a Milser 800-DG household food processor (Iwatani, Tokyo, Japan). An analyst can determine the number of groups in one experiment depending on the analytical purpose.

Step 2[4]. One liter of lysis buffer is prepared from 20 mL of a 1 mol/L Tris-HCl buffer solution (Nacalai Tesque, Kyoto, Japan), 10 mL of a 0.5 mol/L EDTA solution (Nacalai Tesque), 80 mL of a 5 mol/L NaCl solution (Nacalai Tesque), 30 mL of a 10% sodium dodecyl sulfate (SDS) solution (Nacalai Tesque), and ultrapure water. Twenty milliliters of the lysis buffer is added to each glass vessel. Each group is ground for 20 s by means of the household food processor. The lysates in glass vessels are incubated for 10 min at room temperature, and then the glass vessels are vigorously shaken by hand. After 10 min of static standing for separation of the solid and liquid phases, 50 μL of the supernatant is transferred to a plastic tube. The sample of each supernatant is diluted twofold with sterile distilled water. The diluted solution is centrifuged at more than 1,000 × *g* on a personal benchtop centrifuge for 1 min, and then the supernatant is used as a DNA template for the following PCR assay.

Step 3. The DNA templates are analyzed by the two duplex real-time PCR assays, i.e., the GM maize screening assay and experimental control assay. In the GM maize screening assay, the reaction mixture (25 μL) consists of 12.5 pmol of primers P35S 1-5′ (5′-ATTGATGTGATATCTCCACTGACGT-3′), P35S 1-3′ (5′-CCTCTCCAAATGAAATGAACTTCCT-3′), TNOS 2-5′ (5′-GTCTTGCGATGATTATCATATAATTTCTG-3′), and TNOS 2-3′ (5′-CGCTATATTTTGTTTTCTATCGCGT-3′); 2.5 pmol of probes P35S-Taq (5′-CCCACTATCCTTCGCAAGACCCTTCCT-3′) and TNOS-Taq (5′-AGATGGGTTTTTATGATTAGAGTCCCGCAA-3′); 2.5 μL of a DNA template; 12.5 μL of 2 × DirectAce qPCR Mix (Nippon gene); and 0.5 μL of the ROX Passive Reference solution enclosed with the DirectAce qPCR Mix. In the experimental control assay, the reaction mixture (25 μL) consists of 12.5 pmol of primers IPC 1-5′, IPC 1-3′, SSIIb 3-5′ (5′-CCAATCCTTTGACATCTGCTCC-3′), and SSIIb 3-3′ (5′-GATCAGCTTTGGGTCCGGA-3′); 2.5 pmol of probes IPC 1-Taq and SSIIb-Taq (5′-AGCAAAGTCAGAGCGCTGCAATGCA-3′); 1 μL of the IPC plasmid solution; 2.5 μL of a DNA template; 12.5 μL of 2 × DirectAce qPCR Mix; and 0.5μL of ROX Passive Reference solution. The oligonucleotides as PCR primers and TaqMan probes were both synthesized by Fasmac (Atsugi, Japan). P35S-Taq, TNOS-Taq, and IPC 1-Taq were labeled with the dyes FAM and BHQ1 at the 5′ and 3′ termini, respectively. For SSIIb-Taq, hexachlorofluorescein (HEX) was used in place of FAM. The thermal cycling conditions were as follows: 10 min at 95°C and 45 cycles of 15 s at 95°C and 1 min at 65°C in 9600 emulation mode. All real-time PCR analyses are performed on a 7900HT real-time PCR instrument (Life Technologies).

Step 4[4]. Data analysis is conducted in the Sequence Detection Software, version 2.3. The manual threshold cycle (C_t) mode (thresholds: 0.256 for FAM and 0.064 for HEX) and manual baseline mode (start of baseline, 3; end of baseline, [15]) are set in the "Delta Rn vs. Cycle" view of the "Amplification Plot" option. When the C_t values are below 40, the reaction result is labeled positive. If the result of SSIIb or IPC detection by the experimental control assay is found to be negative, the group in question is rejected. If both SSIIb and IPC yield positive results, the group is labeled either GM positive or GM negative depending on the result of the GM maize screening assay.

3. Evaluation of the inhibition tolerance of real-time PCR analysis

In accordance with the modified method, 20 kernels of a non-GM maize material were ground, and crude extract was obtained. Then, the crude extract was analyzed as a DNA template by real-time PCR in the experimental control assay as described above except for the amount of crude extract in the PCR mixtures. We added the crude extract in the amounts of 2.5, 3.3, 4.2, and 5 μL corresponding to 1-, 1.33-, 1.67-, and 2-fold amounts relative to those defined in the protocol, respectively. For comparison with the previously reported method[4], we also performed a real-time PCR analysis where BIOTAQ HS DNA polymerase (Shimadzu, Kyoto, Japan) and 12.5 μL of 2 × Ampdirect Plus buffer (Shimadzu) were

used instead of 2 × DirectAce qPCR Mix.

4. Evaluation of specificity of real-time PCR under the modified conditions

The samples of genomic DNA extracted from genuine GM and non-GM maize materials were analyzed by real-time PCR under the modified conditions. C_t values were determined in triplicate for each DNA sample in one PCR run.

5. Evaluation of the limit of detection (LOD) of the modified group testing method

We prepared simulated group samples consisting of one GM and 19 non-GM kernels. As the GM material, F1-generation seeds of MON810 were used. As the non-GM material, 15 kinds of maize samples were used. Two group samples were prepared for each kind of non-GM material; thus, 30 samples were prepared. The samples were analyzed according to the modified group testing method.

6. The method for identification of GM events after the group testing

We used a DNA extraction kit, GM quicker (Nippon Gene), for DNA purification from the residual cell lysate generated during the group testing. First, 600 µL of each supernatant of a lysate sample was transferred into a 1.5-mL microcentrifuge tube, and then 4 µL of an RNase A solution (100 mg/mL) was added, and the mixture was vortexed vigorously. After that, the mixture was incubated for 5 min at room temperature. Then, 75 µL of GE2 buffer was added, and the mixture was vortexed vigorously. After standing still on ice for 5 min, the mixture was centrifuged for 5 min at 13,000 × *g*, and 400 µL of the supernatant was transferred to a 1.5-mL microcentrifuge tube. Next, 50 µL of GB3 buffer and 200 µL of ethanol were added, and the mixture was vortexed. The mixture was transferred to a silica membrane spin column and centrifuged for 30 s at 13,000 × *g*. After removal of the filtrate, 600 µL of GW buffer was added to the spin column, and the spin column was centrifuged for 1 min at 13,000 × *g*. After removal of the filtrate, the spin column was recentrifuged for 1 min at 13,000 × *g* and placed in a collection tube. The DNA retained in the column was eluted by addition of 50 µL of sterile water, incubation for 3 min at room temperature, and centrifugation for 1 min at 13,000 × *g*. The resulting DNA extracts were diluted to 20 ng/µL with sterile water on the basis of UV absorbance measurement, and then the diluted samples were subjected to the real-time PCR array analysis targeting for GM maize events (Bt11, GA21, Event176, MON810, MON863, NK603, T25, TC1507, DAS-59122-7, MIR604, and MON88017) and the endogenous reference gene of maize, SSIIb, as reported previously[10, 11].

7. The blind test of group testing and GM event identification

For preparation of blinded samples, authentic materials of MON810, MON863, MON88017, NK603, and non-GM maize were used. We prepared groups consisting of either one GM and 19 non-GM kernels or 20 non-GM kernels. Groups including a MON810 MON863, MON88017, or NK603 kernel were named A, B, C, and D, respectively. Twenty group samples were defined as a set of blinded samples. Ten groups were intended for the first screening, and three non-GM groups and seven GM groups (groups A, B, C, or D) were randomly mixed and numbered from one to 10.

Another 10 groups were intended for additional analysis, and four non-GM groups and six GM groups (groups A, B, C, or D) were randomly mixed and numbered from 11 to 20. In total, 13 groups in the set of blinded samples contained GM kernels. We prepared three sets of blinded samples.

An analyst (who was given no information on the samples) performed group testing according to the testing protocol described above. Ten groups were analyzed in one experiment using the modified method. After the group testing, the analyst performed the GM event identification as described above.

Results and Discussion

1. Modifications of the group testing method

We recently developed a real-time PCR master mix, the DirectAce qPCR Mix, which allows for stable DNA amplification even from crude cell lysates[8]. In the present study, we introduced the master mix reagent into the group testing method. Because the reagent was optimized for TaqMan real-time PCR analysis and is tolerant of PCR inhibitors, we expected a decrease in the number of false negative results caused by PCR inhibition. We indeed confirmed the improvement of tolerance to PCR inhibitors

in a crude maize extract (Fig. 2). Under the modified PCR conditions, DNA amplification was observed, even though the crude maize extract was present in the 1.67-fold amount relative to the previously reported method. Additionally, the use of the master mix reagent made preparation of the reaction mixture simpler because DNA polymerase, magnesium ions, deoxyribonucleotide triphosphates, and the buffer are already mixed in the reagent.

In our previous study[4], calculated 40 copies of the pART plasmid were used as a DNA template in the IPC reaction. In contrast, in this study, the concentration of the pART plasmid solution was maintained between 17 and 23 copies/μL by the digital PCR analysis, and then 1 μL of the solution was added to the reaction mixture. A reduction in the copy number of the target sequence was expected to make the IPC reaction more sensitive to PCR inhibition.

In the official testing method notified from the Consumer Affair Agency[12], the primer set SSIIb 3-5′ and 3-3′ is used for detecting a maize endogenous reference gene. Additionally, the primer set was recently incorporated into the "JAS Analytical Test Handbook." In order to improve the user friendliness by means of the same reagent for the existing testing methods, we chose the primer set SSIIb 3-5′ and 3-3′ for the group testing.

2. Specificity of real-time PCR under the modified conditions

In the previous study[4], we evaluated specificity of real-time PCR assays by means of 11 GM maize events. Since then, however, several more GM events have been approved. We used 14 GM maize events for the specificity evaluation in the present study. DNA extracts from genuine materials were analyzed by the real-time PCR assays under the modified conditions (Fig. 3). P35S and/or TNOS regions were detected for all GM maize events, and the C_t values of detection roughly corresponded to the total copy numbers of P35S and TNOS regions in each event. Non-specific detection was not observed among non-GM maize samples, as expected. On the basis of these results, we concluded that the real-time PCR under the modified conditions can detect commercially available GM maize events and stacked-event products derived from these single events.

3. Evaluation of LOD of the modified group testing

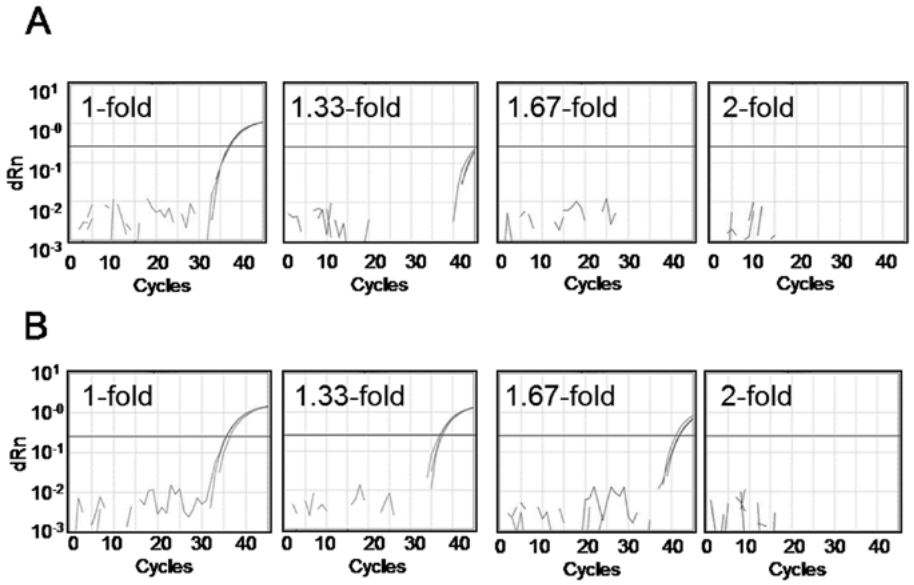

Fig. 2 Evaluation of PCR inhibition by crude maize extract.

We performed an experimental control assay with various amounts of crude maize extract, and the DNA amplification curves in the IPC reaction are shown. The amount of crude maize extract that was analyzed in the testing protocol was defined as "one-fold" and the multipliers indicating the actual added amounts are shown in each panel. A, the previously reported PCR conditions; B, the modified PCR conditions (this study).

method

The LOD of the modified method was assessed by means of simulated group samples containing a single GM kernel among 20 kernels (Fig. 4). This condition yields the smallest amount of GM DNA in the crude cell lysate of grains and makes it possible to test whether the smallest amount of GM DNA results in accurate PCR detection. To prepare the simulated samples, we selected F1-generation seeds of MON810 as a GM material because it has only a single copy of P35S in its diploid genome. As non-GM material, we used various varieties of maize to evaluate the LOD by taking into consideration differences due to the varieties if any. All of the reactions showed the expected DNA amplification, and all of the simulated samples were determined as GM positive. The false negative rate was estimated to be 0%, which fulfilled the criterion for the LOD for qualitative GMO detection methods according to the ISO standards regarding GMO analysis[13]. Additionally, we found that the DNA amplification was not affected by the differences among maize varieties. Thus, the modified method was confirmed to show sufficiently enough performance for reliable detection of a single GM kernel in a group sample.

4. Design of the method for GM event identification following group testing

The P35S and TNOS regions introduced into the GM crops were originally derived from a common plant virus and a soil bacterium, respectively. This situation implies that the positive results of the P35S and TNOS detection may be attributed to the presence of the virus and bacterium in the analytical samples. When the group testing targeting P35S and TNOS was performed, if a bulk sample was contaminated by the virus and/or bacterium, then the sample may be rejected erroneously. In order to implement the group testing for a regulatory inspection, it is necessary to provide a method that precludes false positive detection caused by the contamination with viruses or bacteria. We therefore needed the testing method to identify GM events in group samples. The method is expected to be applied to the samples whose GMO content was estimated to be above 5% by the group testing. We investigated the conditions for DNA extraction from the residual lysate samples, and a suitable procedure was established as described in the

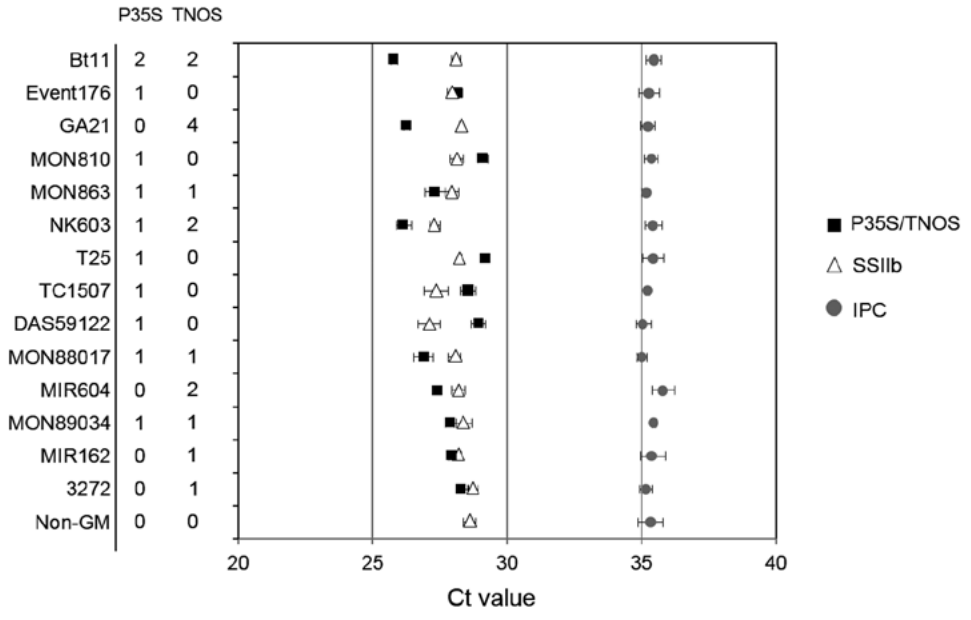

Fig. 3 Evaluation of specificity of real-time PCR in the modified method.

We analyzed various DNA templates by the GM maize screening assay (P35S/TNOS) and by the experimental control assay (IPC/SSIIb). The mean Ct values of three measurements and their standard deviations are shown.

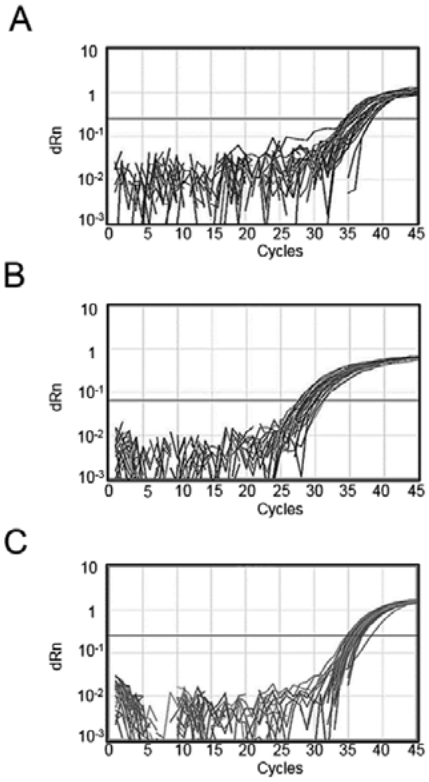

Fig. 4 LOD evaluation of the modified method.

DNA amplification curves for 30 group samples are shown. A, P35S/TNOS detection; B, SSIIb detection; C, IPC detection.

Materials and Methods section. For PCR analysis of the resulting DNA samples, we adopted the real-time PCR array method that we reported previously[10, 11]. The real-time PCR array is a multi-target detection system using 96-well PCR plates to which the primer and TaqMan probe mixtures are added (into the predefined wells). In this study, we implemented the SSIIb-specific detection and event-specific detection for Bt11, GA21, Event176, MON810, MON863, NK603, T25, TC1507, DAS-59122-7, MIR604, and MON88017. Nevertheless, we could change the detection targets depending on a situation because the real-time PCR array is highly flexible in terms of detection targets.

This is the first report on a method for GM event identification after group testing.

5. The blind test of group testing and GM event identification

To evaluate reliability of the entire testing process, we carried out a blind test of the group testing and of the subsequent GM event identification.

As an official testing method in Japan[12], the individual kernel detection method has already been adopted to determine whether GMO content in a bulk sample exceeds 5%. The testing procedure requires analysis of 90 kernels for the first screening. If there are three or more GM kernels among the first 90 kernels tested, another set of 90 kernels must be tested. If the total number of GM kernels in the two tests (180 kernels) is nine or less, then the GMO content of the bulk sample is below 5% and is acceptable[4]. According to the published statistics[14, 15], we previously determined the testing conditions and acceptance criteria of the group testing at the same accuracy of analysis as in the individual kernel detection method. The defined testing conditions and criteria were as follows: A group is comprised of 20 maize kernels, and 10 groups are analyzed for the first screening. If seven or more groups are found to be GM positive in the first screening, an additional set of 10 groups will be analyzed. If the total number of GM-positive groups in the two tests (20 groups) combined is 12 or less, then GMO content of the bulk sample is determined to be below 5%[4]. For the blind test, we prepared three sets of 20 group samples (10 groups for the first screening and 10 groups for the additional analysis) as blind samples to perform the group testing in accordance with the fixed testing conditions as shown above. In order to evaluate the method performance of the GM event identification, we used four representative GM events (MON810, MON863, NK603, and MON88017) as GM materials. Additionally, all sets of blind samples were designed to be rejected based on the acceptance criteria and the samples were destined for the GM event identification.

An analyst in our laboratory who did not know the sample composition performed the group testing according to the modified method described above. All of the results showed the expected positive/negative determinations corresponding to the presence/absence of a GM kernel in each group (Table 1). The results indicated that the method certainly detected the presence of GM kernels and that there was no cross-contamination among the group samples. During the group testing, it is necessary to unmistakably detect a GM kernel in a group sample for accurate evaluation of GMO content. We concluded that the modified method shows adequate performance, and that the analyst who precisely conducts the experiment can obtain accurate results without cross-contamination.

Afterwards, the same analyst performed GM event identification only on the test-positive samples as determined by the group testing. The average concentration of the resulting DNA extracts was 96.1 ng/μL, and the standard deviation was 10.0 ng/μL. The DNA extraction method was found to yield a sufficient amount of DNA for the subsequent PCR analysis. We then performed the real-time PCR array analysis of each DNA extract. The representative results of the real-time PCR array are shown in Fig. 5. In this method, when the amplification curve crossed the threshold line, this situation was labeled as a positive result. Each sample showed DNA amplification curves both in the SSIIb

Table 1. Results of the blind test.

Set 1	Sample number																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
GM kemel admixed	–	B	–	D	A	–	D	B	A	C	–	C	A	D	–	A	C	–	–	B
Result of group testing	–	+	–	+	+	–	+	+	+	+	–	+	+	+	–	+	+	–	–	+
Result of GM event identification	NA	B	NA	D	A	NA	D	B	A	C	NA	C	A	D	NA	A	C	NA	NA	B

Set 2	Sample number																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
GM kemel admixed	B	A	C	–	–	–	C	A	D	C	–	A	–	D	B	B	–	B	D	–
Result of group testing	+	+	+	–	–	–	+	+	+	+	–	+	–	+	+	+	–	+	+	–
Result of GM event identification	B	A	C	NA	NA	NA	C	A	D	C	NA	A	NA	D	B	B	NA	B	D	NA

Set 3	Sample number																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
GM kemel admixed	–	–	D	–	A	C	B	D	A	A	–	–	–	D	–	C	B	C	C	B
Result of group testing	–	–	+	–	+	+	+	+	+	+	–	–	–	+	–	+	+	+	+	+
Result of GM event identification	NA	NA	D	NA	A	C	B	D	A	A	NA	NA	NA	D	NA	C	B	C	C	B

+, positive detection –, negative detection A, MON810. B, MON863. C, NK603. D, MON88017. NA, not analyzed.

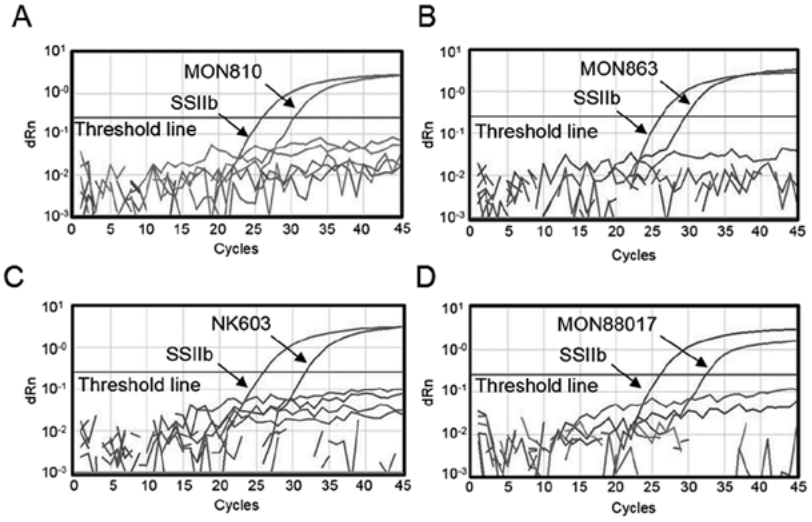

Fig. 5 Representative results of the real-time PCR array analysis for GM event identification.

A, a group sample containing a MON810 kernel; B, a group sample containing a MON863 kernel; C, a group sample containing a NK603 kernel; D, a group sample containing a MON88017 kernel.

detection assay as a positive control and in the respective event-specific assay corresponding to the GM event admixed in the samples. All the results of the GM event identification in the blind test completely matched the admixed GM events in the respective simulated samples, suggesting that our method of GM event identification was also reliable.

Conclusion

In this study, the group testing method was improved to enhance reliability and user friendliness. A method of GM event identification after the group testing was also developed. These methods were validated on the basis of the results of in-house evaluation and a blind test. They are expected to be applicable to evaluation of GMO content in batches of maize grains for verification of food labeling.

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遺伝子組換えトウモロコシの混入率評価を目的とするグループテスティング法の改良

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要　旨

複数の遺伝子組換え（GM）系統の掛け合わせによるスタック品種の使用拡大に伴い，リアルタイムPCRによる定量検知法は，GMトウモロコシの混入率を実際よりも過大に評価する傾向にある．この問題を解決するため，トウモロコシ穀粒中の組換え体混入率を正確に評価することができるグループテスティング法をこれまでに提案している．本研究では，既報の分析法の利便性および信頼性を向上させるため，分析法を改変し，室内妥当性確認を行った．また，グループテスティング後にGM系統を特定する試験法を確立した．最初に，トウモロコシの各種GM系統及び非組換え体の純粋な試料を用いて，改変法の反応特異性を確認した．また，検出限界の評価を行い，改変法が20粒中に１粒含まれるGM穀粒を確実に検出できることを確認した．次に，擬似混入試料を用いたブラインド試験を実施した．全ての擬似混入試料で，試料中のGMの有無に対応した分析結果が得られた．また，グループテスティングで使用した細胞溶解液からDNAを精製し，それをリアルタイムPCRアレイ法で分析することで，グループテスティングの試料に含まれていたGM系統の正確な特定が可能であった．

キーワード：遺伝子組換え，グループテスティング，妥当性確認