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A novel chromogenic method for determining the genetically modified soybean content in soybean powder with primer extension

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Abstract

A novel method for determining the Roundup Ready soybean (RRS) content in soybean powder was developed. The RRS DNA-specific oligodeoxyribonucleotide immobilized on a plastic plate was used as the primer of the primer extension reaction (PEXT) with the RRS specific PCR product obtained from the DNA extracts of soybean powder as the templates. The PEXT product was labeled with biotin and visualized by chromogenic reaction using avidin and biotin-conjugated alkaline phosphatase. This method could detect 10^9 copies of the RRS specific DNA sequence in assay solution, and good correlation ($r = 0.99$) was observed between the logarithm of copy number and the color intensity up to 10^{12} copies of RRS specific DNA sequence. By optimizing PCR conditions for amplifying the RRS specific sequence, this method could detect the RRS content in the soybean powder between 0.1 and 5.0%.

Keywords : arrayed primer extension, DNA microarray, genetically modified organism, Roundup Ready soybean

I Introduction

The amount of genetically modified organisms (GMOs) used as food materials and fodders have been increased. However, there is controversy concerning food safety, environmental risks, and ethical issues, and the GMO contents in food materials and fodders are strictly regulated by governments[1-3]. Therefore, development of accurate and convenient methods for determining GMO contents in food materials and fodders is expected. Polymerase chain reaction (PCR) technique is the most convenient method for detecting the DNA sequence characteristic for GMOs. However, quantitative PCR requires an expensive instrument. PCR coupled with enzyme-linked immunosorbent assay (PCR-ELISA) and DNA microarrays have been used for detecting GMOs[4-8]. However, these methods still have some issues regarding quantitative determination of GMO contents in food materials and are mainly applied to qualitative detection.

We previously reported a chromogenic method for detecting the DNA sequences specific for GMOs using multiplex PCR and primer extension reaction (PEXT) coupled with chromogenic reaction, which allowed relatively quick detection of the GMO specific sequences[9]. In this study, we extend the method to determine the content of Roundup Ready soybean (RRS), a GM soybean, in soybean powder.

II Materials and Methods

1. Standard specimen of RRS powder

Certified reference materials (ERM-BF-410 consisting of dried soybean powder with 0, 0.1, 0.5, 1 and 5% RRS line event 40-3-2) certified by the IRMM (Institute for Reference Materials and Measurements, Geel, Belgium) were purchased from Fluka (Buchs, Switzerland).

2. Preparation of DNA extract from soybean powder

DNA was extracted from soybean powder with DNeasy

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Plant Mini Kit (Qiagen, Hilden, Germany) according to the previous procedure[10]. The DNA concentration in the extract was calculated from the absorbance at 260 nm.

3. Amplification of the RRS specific sequence by PCR

A reaction mixture (25 μl) contained 12.5 μl of the FastStart Universal Probe Master (Roche, IN, USA), 0.5 μM each of primers for the RRS specific sequence[10] and 50 ng of DNA specimen extracted from soybean powder. PCR was done in an EZC-96 thermal cycler (Asahi Techno Glass, Chiba, Japan). The thermal cycle program was as follows: 2 min at 50°C, 10 min at 95°C, and subsequent amplification of the DNA for cycles of 30 s at 95°C and 60 s at 59°C. Based on the results from the preliminary experiments (see Results and Discussion) for determining the relationship between the reaction cycles and the amount of products, the reaction cycle in RRS was fixed at 32.

4. Preparation of the PCR product

The PCR product was prepared by using MonoFas DNA Purification Kit I (GL Sciences, Tokyo, Japan). The DNA concentration of the preparation was determined with Quant-iT PicoGreen dsDNA HS Assay kit (Invitrogen, Paisley, UK), and the copy number of the RRS specific sequence was calculated by quantitative PCR. The serial dilutions of the purified PCR product were used to clarify dose-dependent detection of PCR product of RRS specific sequence using PEXT coupled with chromogenic reaction.

5. Quantitative PCR

To ascertain the quantitative amplification of the RRS specific sequence in the DNA extract from soybean powder by PCR, the relationships between PCR cycle number or the GM content (%) in soybean powder and copy number of RRS specific sequence in the post-PCR mixture were investigated using quantitative PCR. The post-PCR mixture and the purified PCR product described above were appropriately diluted with nuclease free water due to bringing levels within the range of the standard curve, and were served as the specimen for quantitative PCR. The reaction mixture (25 μl) contained 12.5 μl of FastStart Universal Probe Master, 0.2 μM of probe, 0.5 μM each of primers, and 2.5 μl of the diluted specimen. The probe and primer set for RRS were the same as those used in the previous experiments[10]. PCR was done with a Prism 7000 Real-time PCR system (Applied Biosystems, CA, USA). The thermal cycle program was as follows: 2 min at 50°C, 10 min at 95°C, and subsequent amplification of the DNA for 40 cycles of 30 s at 95°C and 60 s at 59°C. A standard curve was prepared from the GM Soybean (RRS) Detection Plasmid Set (Nippon Gene, Tokyo Japan). The standard curves were linear in the range form 125 to 250000 copies ($r > 0.99$).

6. Immobilization of the oligodeoxyribonucleotides on a plastic plate

The sequences of the oligodeoxyribonucleotides immobilized on a plastic plate as the primers specific for Lel, which was taxon-specific DNA sequence of soybean and was generally used as a positive control and reference in PCR and real-time PCR methods detecting GM soybean, and RRS were described previously[9]. These oligodeoxyribonucleotides were immobilized onto the plastic plate (25 mm × 5 mm) coated with S-Bio® PrimeSurface® (Sumitomo Bakelite, Hyogo, Japan) according to the previous procedure[9].

7. PEXT coupled with chromogenic reaction using biotin-conjugated alkaline phosphatase

The purified PCR product in dose-dependent detection of PCR product of RRS specific sequence and post-PCR mixture of the RRS specific sequence amplified from the DNA extracts from soybean powder in dose-dependent detection of GM content in soybean powder were used as the template for the PEXT using the oligodeoxyribonucleotides immobilized on the plastic plate as the primers. Aliquots (2.5 μl) of these specimens were subjected to the PEXT coupled with chromogenic reaction using biotin-conjugated alkaline phosphatase[9]. The colored spots developed on the plate were scanned with an MP 600 scanner (Cannon, Tokyo, Japan). The value of color intensity of spot was calculated with the relative intensity of spot to background, which was obtained from the same-size area around the spot, and these intensities were measured by using Scion Image (Scion Corporation, MD, USA).

Ⅲ Results and Discussion

1. Chromogenic detection of RRS specific DNA fragment by PEXT coupled with biotin-conjugated alkaline phosphatase

We previously show the detection of the RRS and Lel (a typical constitutive gene occurring in soybean) specific sequences by multiplex PCR amplification and PEXT coupled with the chromogenic reaction[9]. Also in a single PCR amplification, the color development was observed only when the PCR product of RRS or Lel specific sequence was applied correctly to each corresponding primer in PEXT (data not shown). Thus the PEXT/chromogenic method enables specific detection of target DNA sequences.

To obtain evidence for the quantitative correlation between color intensity and copy number of target DNA, dependency of the color development on the copy number of the RRS

specific sequence was examined. The DNA fragment corresponding to the RRS specific sequence was prepared by PCR amplification from DNA extract from soybean powder containing 5% of RRS and was purified. A series of DNA specimens containing of known amounts (10^8, 10^9, 10^{10}, 10^{11} and 10^{12} copies in 2.5 μl) of the fragment were prepared. Aliquots (2.5 μl) of these DNA specimens were subjected to the PEXT coupled with chromogenic reaction, and results were summarized in Fig. 1. The color intensity of back ground (0 copy) was 0.93 ± 0.04 and no significant color development (0.97 ± 0.07) was observed with 10^8 copies of the fragment. However, significantly high color development (1.08 ± 0.07) was observed by 10^9 copies of the fragment, indicating that this method could detect the RRS specific sequence over 10^9 copies in the DNA specimen. Result shown in Fig. 1 also indicates that the color intensity was dependent linearly on the logarithm of copy number between 10^{10} to 10^{12} copies (color intensity = 1.22 × $\log_{10}$ copy number - 10.71; r = 0.99, see Fig. 1B). These results indicate that this method can quantify the RRS specific sequence between 10^{10} to 10^{12} copies in the DNA specimen.

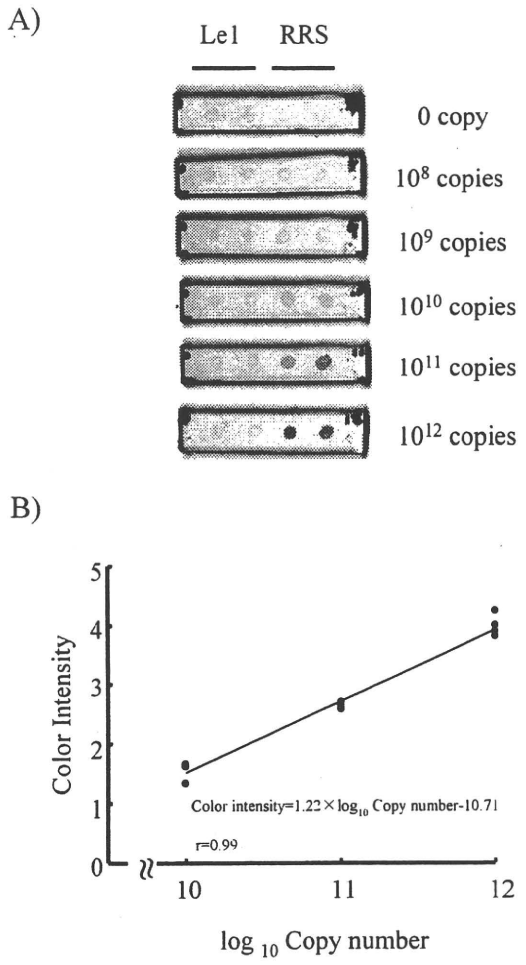

Fig. 1. Dose-dependent detection of PCR product of RRS specific sequence using PEXT coupled with chromogenic reaction

(A) Image of the PEXT of each copy number of RRS specific sequence. The positive signals appear as gray spots. The dots in upper corners denote the position of the plate.

(B) Relationship between the logarithm of copy number of RRS specific sequence and color intensity of spot for RRS in the PEXT. Line represents the least squares regression line (n = 4).

2. Quantitative amplification of the RRS specific sequence by PCR

Detection of RRS specific sequence by the PEXT/chromogenic method requires at least 10^9 copies of the sequence and 10^{10} to 10^{12} copies of the sequence is necessary for quantitative determination (Fig. 1). Accordingly, quantitative amplification of the RRS specific sequence in soybean DNA extracts was essential for applying this method to determine the RRS content in soybean powders. Preceding reports[4-6] described that detection of GMOs by the methods using PCR-ELISA and DNA microarrays might be insufficient for accurate quantitative detection, because of insufficient quantitative accuracy of PCR used for amplifying the target sequences. Then, we made precise examination regarding the correlation between the number of reaction cycles and the amount of product in the PCR amplification of the RRS specific sequence in the DNA extracts from a standard soybean powder containing 5% of RRS. In this experiment, amount of the RRS sequence in the post-PCR mixture was determined by quantitative PCR as described in Experimental section and results are summarized in Table 1. The amplification ratio shown in Table I indicated that the PCR amplification proceeded almost linearly up to 32nd reaction cycle. Then, the relationship between the known GM content in soybean powder and the copy number of the

Table 1. Relationship between PCR cycle number and PCR product amount

PCR cycle	Copy number of RRS specific sequence (/μl)		Amplification ratio
24	$2.3 \times 10^8 \pm 8.7 \times 10^7$	>	3.8
26	$8.8 \times 10^8 \pm 2.8 \times 10^8$	>	3.7
28	$3.3 \times 10^9 \pm 2.7 \times 10^8$	>	3.8
30	$1.2 \times 10^{10} \pm 5.2 \times 10^9$	>	3.0
32	$3.7 \times 10^{10} \pm 4.6 \times 10^9$	>	1.6
34	$5.9 \times 10^{10} \pm 1.1 \times 10^{10}$	>	1.7
36	$9.8 \times 10^{10} \pm 6.2 \times 10^9$	>	1.4
38	$1.3 \times 10^{11} \pm 2.1 \times 10^{10}$	>	1.4
40	$1.8 \times 10^{11} \pm 1.3 \times 10^{10}$		

DNA sample extracted from soybean powder containing 5% of GM soybean was used. The PCR product amounts were indicated as copy number of RRS specific sequence per post-PCR mixture (μl). Values are means ± SD (n = 4).

RRS specific sequence obtained with 32 cycles of PCR was examined using the standard soybean powder containing 0.1, 0.5, 1 and 5% RRS soybean. As shown in Fig. 2, a good linear correlation (copy number = $1.37 \times 10^9 \times$ GM content (%) + 1.04×10^{10}; r = 0.99) was observed between the RRS content and the copy number of RRS specific sequence obtained by the amplification with 32 cycles of PCR. It was also pointed out that the copy number of the RRS specific sequence obtained from the soybean powder containing 0.1% RRS was 1.7×10^9 per μl of the post-PCR mixture, which was enough for the detection by the PEXT/chromogenic method described in the preceding session. Based on these results, it was expected that RRS content in soybean powder might be determined by the method consisting of PCR amplification of the RRS specific sequence and determination of the amplified sequence with PEXT coupled with chromogenic reaction.

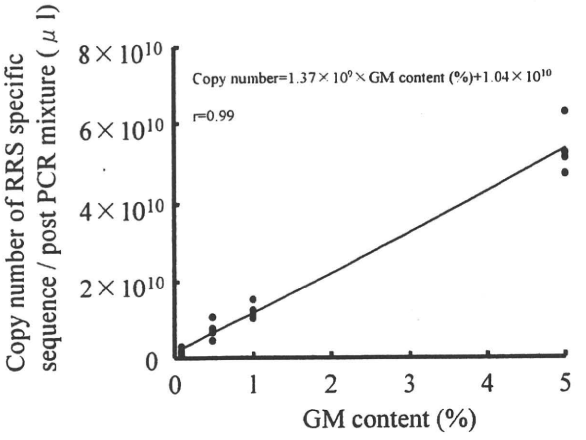

Fig. 2. Relationship between the GM content (%) in soybean powder and copy number of RRS specific sequence in the post-PCR mixture
Line represents least squares regression line (n = 4).

3. Determination of RRS content in soybean powder

To confirm the practicability of present method for determining the RRS content in soybean powder, overall process of this method, extraction of DNA from soybean powder, amplification of RRS specific sequence by 32 cycles of PCR and determination of the amplified RRS specific sequence by PEXT coupled with the chromogenic reaction, was done by using a set of soybean powder containing known amounts of RRS. The color intensity of the control (nuclease free water) was 1.09 ± 0.02, and the color intensity of the RRS-free soybean powder (1.12 ± 0.05) was slightly higher than the control. The color intensity of the soybean powder containing 0.1% of RRS (1.23 ± 0.07) was significantly ($p < 0.05$) higher than the intensities of control and was increased depending on the RRS contents of the specimen (Fig. 3). On

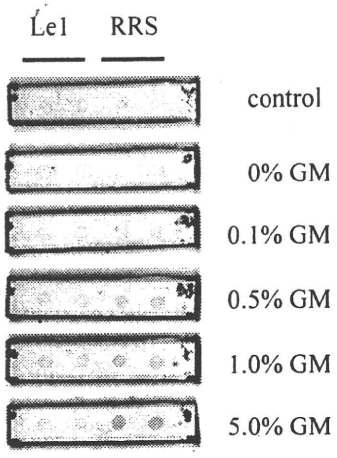

Fig. 3. Dose-dependent detection of GM content (%) in soybean powder using PCR amplification and PEXT coupled with chromogenic reaction
Image of the PEXT resulted from each soybean powder containing GM soybean. The control is nuclease free water. The positive signals appear as gray spots. The dots in upper corners denote the position of the plate.

the other hand, the color intensities observed in the control experiment with the Le1 specific sequence and the PCR products amplified by 27 cycles of reaction enabled liner amplification of the Le1 sequence were independent of the RRS contents of the same soybean powder (data not shown). Since Le1 is a constitutive gene occurring in soybean, the RRS content-dependent increment of the color intensity caused by the RRS sequence-specific PEXT coupled with chromogenic reaction must represent the RRS content of the specimen. It was, however, pointed out that the absolute value of the color intensity was affected by the drift of background color observed on the RRS-free specimen. Therefore, the color intensity of RRS-free specimen was subtracted from the color intensity of the RRS-containing specimens, and the resulting corrected values were plotted against the logarithm of RRS content (Δ color intensity = slope × $\log_{10}$ GM content (%) + intercept) (Table 2). In 8 independent experiments, these plots gave quite good linear correlation (r = 0.95-0.99), and the representative regression equations for the plots were expressed. However, the values of the slope and the intercept of the regression line showed some deviation. These deviations were due to the deviation of color intensity among repetitive experiments. For example, relative standard deviations (RSDs) of the color intensity obtained from 8 independent experiments were 34 and 24% for the soybean powder containing 1 and 5% of RRS.

Based on these observations, it can be concluded that the present method consisting of PCR amplification and PEXT coupled with the chromogenic reaction is useful as a rapid method for determining RRS content in soybean powder.

Table 2. Relationship between the logarithm of GM content (%) in soybean powder and Δ color intensity (for 0% GM soybean) of spot for RRS in the PCR amplification and PEXT coupled with chromogenic reaction

No.	Correlation (r)	Intercept	Slope
1	0.99	0.43	0.41
2	0.97	0.54	0.52
3	0.98	0.38	0.41
4	0.97	0.43	0.45
5	0.98	0.70	0.55
6	0.99	0.55	0.51
7	0.95	0.72	0.66
8	0.96	0.78	0.66
Mean	0.97	0.57	0.52
SD	0.01	0.15	0.10

The results of the least-squares regression analyses of the data from 8 independent experiments are shown.

In this determination, the use of a set of standard sample containing 0.1 and 5.0% of RRS is essential for calibration.

4. General discussion

In this study, we have developed a method for determining GM content in soybean powder by using PCR amplification and PEXT coupled with chromogenic reaction. This method not required special equipment, such as real-time PCR device, laser scanner, electrophoresis apparatus and UV-image analyzer. The plastic plate immobilizing oligodeoxyribonucleotides is stable at 4°C for 2 months (data not shown), and can be provided ready for use. The determination process of PCR products consisting of PEXT and chromogenic reaction takes about 1 h. Thus, this method enabled rapid determination of GM content in soybean powder between 0.1 to 5%. Many countries and areas have specified GMO labeling regulations, and the labeling thresholds for GMOs are 0.9% in EU, 3% in Korea and 5% in Japan[1-3]. The present method can detect these threshold levels of GM soybean (RRS). This method is applicable to a specimen of high RRS contents by adjusting the input amount of DNA.

Ⅳ Acknowledgments

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論　　文

プライマー伸長反応を使用した遺伝子組換え大豆の発色定量法

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キーワード:アレイ化プライマー伸長、DNAマイクロアレイ、遺伝子組換え食品、ラウンドアップレディー大豆

概　要

プライマー伸長反応を利用して遺伝子組換え(GM)大豆の短時間で簡便な発色定量法を開発した。この方法では、GM大豆(ラウンドアップレディー大豆(RRS))の特異的DNA配列に対して、それに対応するオリゴを固定したプレート上で、DNAポリメラーゼにて伸長反応を行い、その際にビオチン11-dUTPを取り込ませ、そのビオチンをアビジン-ビオチン複合体とアルカリフォスファターゼで発色させた。その結果、PCR産物を精製して作成したRRS特異的配列DNA断片を10^9コピーから検出でき、10^{10}-10^{12}コピーの間ではコピー数の対数と発色の濃さの間に高い相関が得られた。更に、0.1-5%GM大豆の抽出DNAからのPCRサンプルで検討した結果、大豆含量の対数と発色の濃さの間に相関を得ることができた。

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Note

Improvement of Polymerase Chain Reaction-Based Bt11 Maize Detection Method by Reduction of Non-Specific Amplification

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The Bt11 maize-specific qualitative detection method based on polymerase chain reaction (PCR) in the JAS analytical test handbook has been widely used for administrative monitoring of GM crops and quality control of commercially distributed grains. In the present investigation, some apparently false-positive detections were observed in assays using the Bt11 maize-specific method, and these erroneous results were proved to have been caused by non-specific DNA amplification. We improved the detection method to reduce non-specific amplification by decreasing the concentration of magnesium ions in the PCR mixture. The subsequent evaluation of analytical performance demonstrated no marked difference between the currently used and the improved methods, except for the reduced non-specific amplification. We conclude that the currently used standard method should be replaced with the improved method for the reliable detection of Bt11 maize.

Key words: polymerase chain reaction (PCR); genetically modified organism (GMO); detection; non-specific amplification

Introduction

The production of genetically modified (GM) crops has expanded in many parts of the world, and large amounts of GM crops and their products have been imported into Japan[1)]. A system for enforcing the safety assessment of GM foods in Japan was introduced in 2001, and only authorized genetically modified organisms (GMOs) are allowed to be imported[2)]. Meanwhile, the utilization of GM foods was controversial among general consumers, and a food labeling system for GMOs was also implemented in 2001 under The Law Concerning Standardization and Proper Labeling of Agricultural and Forestry Products (The Japanese Agricultural Standards Law) to expand consumers' choices. Simultaneously, a labeling system was adopted under The Food Sanitation Law in order to publicize the fact that the product has undergone the safety assessment. According to the implementation of the labeling system, standard GMO detection methods for monitoring the labeling system were developed and published in the Japanese Agricultural Standard (JAS) analytical test handbook from the Food and Agricultural Materials Inspection Center (Saitama, Japan) and "Testing for Foods Produced by Recombinant DNA Techniques" from the Ministry of Health, Labour, and Welfare of Japan[*1, *2] These detection methods have been widely used, not only for monitoring the food labeling system, but also for the quality control of food and feed materials, and commercially distributed seeds.

The Bt11 maize-specific qualitative detection method based on polymerase chain reaction (PCR) is one of the detection methods described in the JAS analytical test handbook. The specificity of PCR detection in a qualitative analytical method is critical, and may directly affect the testing results. However, we confirmed that the Bt11 maize detection method can generate false-positive results owing to non-specific DNA amplification. Thns, we attempted to improve the method by eliminating the potential for false-positives. Furthermore, we evaluated the performance of the improved method.

[*1] Japanese Agricultural Standard (JAS) analytical test handbook: genetically modified food quality, labeling analysis manual for individual products (2002). The Food and Agricultural Materials Inspection Center, Japan. http://www.famic.go.jp/technical_information/jashandbook/index.html

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

Materials and Methods

Cereal materials for analytical samples

We used Bt11, Event176, GA21, MON810, MON863, NK603, T25, and TC1507 maize as representative GM maize events. As a representative GM soy event, we used 40-3-2 soybean (Roundup Ready Soybean, RRS). F1 generation seeds of Bt11 and Event176, and ground F1 generation seeds of GA21 were kindly provided by Syngenta Seeds AG (Basel, Switzerland); F1 generation seeds of MON810, MON863, and NK603 were kindly provided by Monsanto Company (St. Louis, MO, USA); and F1 generation seeds of TC1507 were kindly provided by Pioneer Hi-Bred International (Johnston, IA, USA). F1 generation seeds of T25 and progeny seeds of RRS were imported directly from the USA. The following five seed samples of conventional dent corn were used as non-GM maize. Strike5512 maize was directly imported. Maize variety LG2265 was obtained in Japan. Maize varieties DK537 and RX740 were provided by Monsanto Co. QC9651 maize was obtained from Quality Technology International (Huntley, IL, USA). Dry soybean seeds harvested in Ohio in 1998 were also imported directly and used as a non-GM soy sample. Seeds of the conventional rice variety Kinuhikari (*Oryza sativa*), the conventional wheat variety Haruyutaka (*Triticum aestivum*), and the conventional barley variety Harrington (*Hordeum vulgare*) were obtained in Japan and used as non-GM materials.

Preparation of test samples and DNA extraction

All dry seeds except for non-GM maize seeds were ground with a P-14 speed rotor mill (Fritsch GmbH, Idar-Oberstein, Germany). Ten kernels in each of 5 conventional maize samples were ground with a Multi-beads shocker (Yasui Kikai Co., Osaka, Japan). The ground materials were stored below −20℃ until DNA extraction. For maize, soy, wheat, barley, and rice, DNA extraction was performed using a DNeasy Plant Maxi Kit (Qiagen GmbH, Hilden, Germany) as described previously[3]. The DNA concentrations of solutions were determined by measuring ultraviolet (UV) absorbance with a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). DNA concentration was calculated by taking one optical density unit at 260 nm as equal to 50 ng/μL. All extracted DNAs were diluted to 10 ng/μL with sterile distilled water. For the evaluation of sensitivities of detection methods, a Bt11 maize genome sample (10 ng/μL) was mixed with a non-GM maize genome sample (10 ng/μL) to make three concentrations [0.2% (v/v), 0.1% (v/v), and 0.05% (v/v)].

PCR assay and electrophoresis conditions

We used two PCR assay methods described in the JAS analytical test handbook, i.e., detection methods for Bt 11 maize and starch synthase IIb (*SSIIb*) gene as the maize endogenous reference gene. Twenty-five microliters of reaction mixture contained 25 ng of genomic DNA, 200 μmol/L dNTPs, 0.625 units of AmpliTaq Gold Polymerase (Applied Biosystems, Inc., Foster City, CA, USA), 1.5 mmol/L $MgCl_2$, 2.5 μL of 10×PCR buffer II (Applied Biosystems, Inc.), and 0.5 μmol/L of a primer pair for the respective detection method. The primer pair of Bt11 3-5′ (5′-AAAAGACCACAACAAGCCGC-3′) and Bt11 3-3′ (5′-CAATGCGTTCTCCACCAAGTACT-3′), and the primer pair of *SSIIb* 3-5′ (5′-CCAATCCTTTGACATCTGCTCC-3′) and *SSIIb* 3-3′ (5′-GATCAGCTTTGGGTCCGGA-3′) were used for the Bt11 maize-specific and *SSIIb*-specific detection methods, respectively[3), 4)].

To improve the Bt11 maize-specific method, we modified the concentration of $MgCl_2$ to 1.2 mmol/L. The PCR amplification was carried out on an ABI PRISM 9700 (Applied Biosystems, Inc.) with thermal cycles consisting of 95℃ for 10 min for preincubation, 40 cycles of 95℃ for 30 sec for denaturation, 60℃ for 30 sec for annealing, and 72℃ for 30 sec for extension, and 72℃ for 7 min for final extension. For the experimental positive control in the PCR assays, GM maize detection positive control plasmid (Nippon Gene Co., Ltd., Tokyo, Japan) was used as template DNA. For the analysis of PCR products, agarose gel electrophoresis was carried out with 3% (w/v) LO3 agarose gel (Takara Bio, Inc., Otsu, Japan) in Tris-acetate-ethylenediaminetetraacetate (TAE) buffer with 0.5 μg/mL of ethidium bromide (Sigma Aldrich, St. Louis, MO, USA). Five microliters of each reaction mixture was mixed with 1 μL of 6×loading buffer (Nippon Gene Co., Ltd.), and the samples were subjected to electrophoresis at a constant voltage (100 V) for approximately 20 min in the TAE buffer. After the electrophoresis, the gel was photographed under UV radiation using a Densitograph system (ATTO, Tokyo, Japan).

We previously reported the development of real-time PCR array analysis as a comprehensive detection method for GM crops[5]. In this investigation, the method was utilized to confirm that the analytical samples of conventional maize were genuine non-GM maize. The assay was performed with a real-time PCR array plate including 31 targets, such as GM maize and soy events, recombinant DNA segments, and endogenous reference genes.[5]

Nucleotide sequence analysis

For the nucleotide sequence analyses of the non-specific amplification product, the products were cloned with pGEM-T vector (Promega, Madison, WI, USA) in *Escherichia coli* DH5α. Then, the cloned DNAs were analyzed by a DNA sequencing system, CEQ8000 (Beckman Coulter, Inc.) according to the manufacturer's protocol. The nucleotide sequences were subjected to a homology search using the Basic Local Alignment Search Tool (BLAST) system.*3

*3 Basic Local Alignment Search Tool at the National Center for Biotechnology Information website, http://blast.ncbi.nlm.nih.gov/Blast.cgi

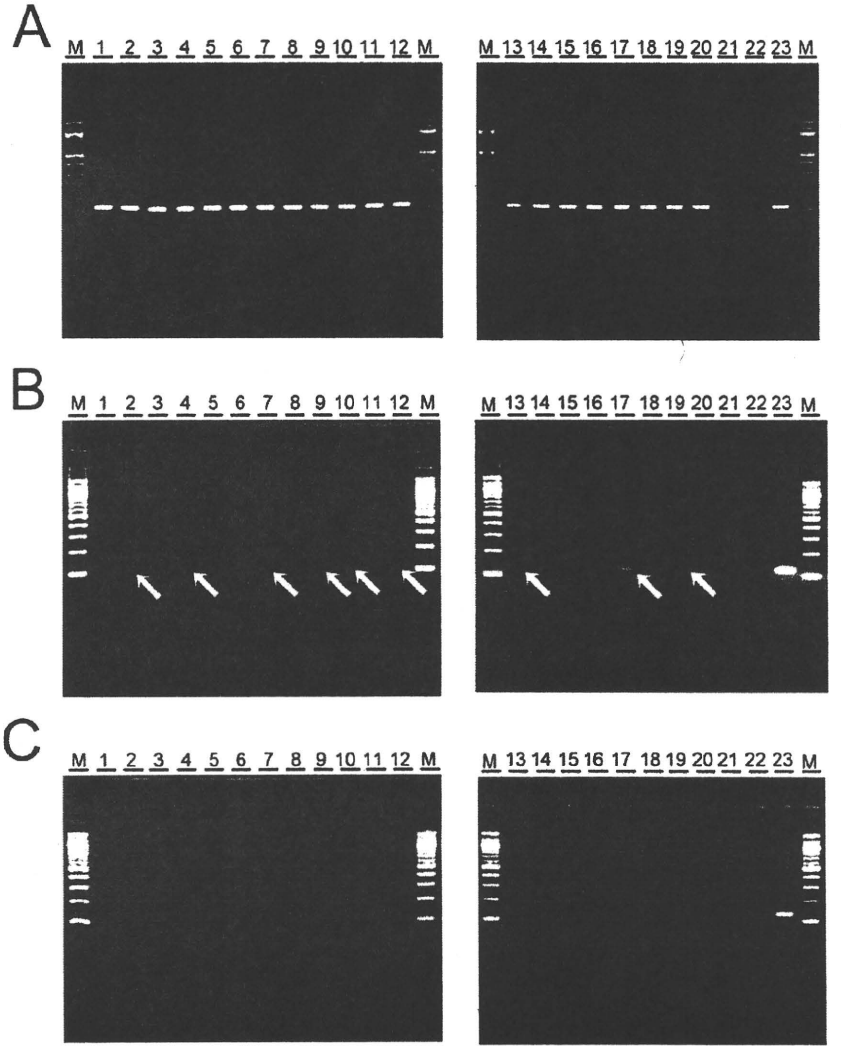

Fig. 1. The PCR assay results for the non-GM maize DNAs

A, the *SSIIb* assays; B, the assays with the currently used Bt11 maize-specific method; C, the assays with the improved Bt11 maize-specific method. Duplicate PCR assays of two DNA extracts were performed for each kind of maize materials. Lanes 1–4, the Strike5512 maize; Lanes 5–8, the LG2265 maize; Lanes 9–12, the DK537 maize; Lanes 13–16, the RX740 maize; Lanes 17–20, the QC9651 maize. In lane 21, no primer reaction for the negative control. In lane 22, no template reaction for the negative control. In lane 23, the reaction with positive control plasmid. In B and C, higher intensity of UV radiation was adopted to show the experimental results clearly. The arrows indicate non-specific amplification products.

Results

Non-specific amplification in the currently used standard method

The DNA extraction of ground non-GM maize samples was carried out in duplicate. The DNA extracts were analyzed using the real-time PCR array and were confirmed to be non-GM maize DNAs. The DNA samples were assayed twice in parallel with the *SSIIb* detection method as an experimental control, and DNA amplifications with the expected size in all the reactions were observed in the electrophoresis analyses (Fig. 1A). Samples were then analyzed twice in parallel with the currently used standard Bt11 maize-specific detection method. Faint signals of DNA amplification products of a size similar to that of the original Bt11 maize-specific amplification were observed in some reactions (Fig. 1B).

Nucleotide sequence analyses of non-specific amplification

The nucleotide sequence of the non-specific amplification product from the Strike5512 maize sample was analyzed as described above. In the homology search using the BLAST system, the nucleotide sequence of the non-specific amplification matched a part of the conventional maize genome (GenBank Accession No. CG441379) with more than 90% homology in the internal region between the two primers. The nucleotide sequence alignment of the original Bt11 maize-specific

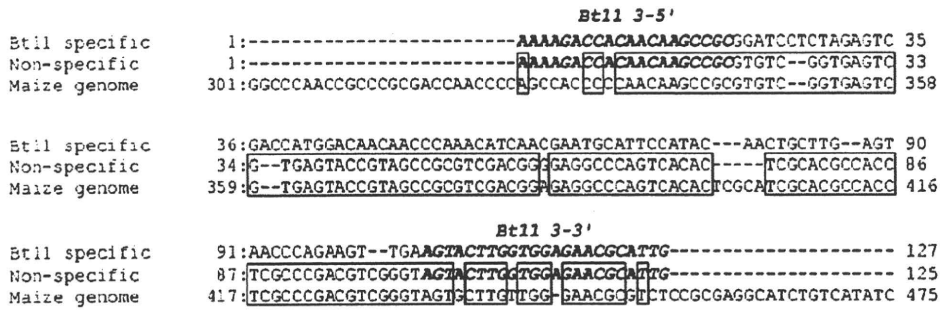

Fig. 2. The nucleotide sequence alignment of the original Bt11 maize-specific amplification product, the non-specific amplification product in the currently used detection method, and a part of a conventional maize genome obtained from the public database

The regions corresponding to the Bt11 primer pair in the the original Bt11 maize-specific amplification product and the non-specific amplification product are shown with bold and italicized face. The homologous nucleotides in the non-specific amplification product and the conventional maize genome are surrounded by boxes.

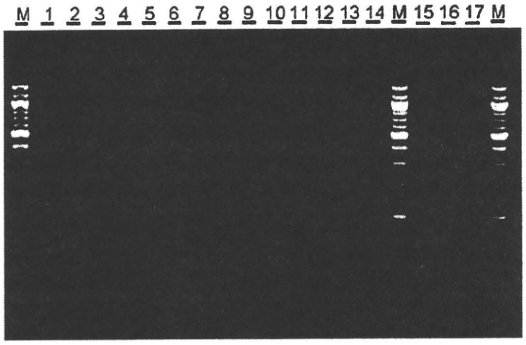

Fig. 3. The results of specificity evaluation for the improved Bt11 maize-specific method

Lanes 1–14, PCR results with each of the template DNAs extracted from Bt11, Event176, GA21, MON810, MON863, NK603, T25, TC1507, non-GM maize, RRS, non-GM soy, non-GM wheat, non-GM barley, and non-GM rice; lane 15, no primer reaction for the negative control; lane 16, no template reaction for the negative control; lane 17, the reaction with positive control plasmid.

amplification product, the non-specific amplification product, and the part of the conventional maize genome obtained from the public nucleotide sequence database are shown in Fig. 2.

Improvement of the Bt11 maize-specific detection method

We modified the concentration of $MgCl_2$ in the PCR reaction mixture from 1.5 to 1.2 mmol/L and evaluated non-specific amplification with the same non-GM maize DNA samples that we had used for the standard method. Non-specific amplification was not observed in any of the reactions (Fig. 1C).

Evaluation of analytical performances

For the specificity evaluation of the improved method, we assayed representative GM maize and soy samples, the non-GM maize sample, the non-GM soy sample, the non-GM wheat sample, the non-GM barley sample, and the non-GM rice sample using the improved method (Fig. 3). The amplification product was observed only in the reaction with Bt11 maize DNA as a template, besides the positive control reaction. For the sensitivity evaluation of both the currently used and the improved methods, simulated DNA samples at various concentrations of Bt11 maize DNA [0.2% (v/v), 0.1% (v/v), and 0.05% (v/v)] were assayed 21 times. The results are shown in Table 1. All the assay results for 0.2% (v/v) and 0.1% (v/v) samples by the both methods were positive and the false-negative rates were 0% in the present examination. The assay results for 0.05% (v/v) samples included several false-negative results and the false-negative rates were 9.5% and 24% for the currently used and the improved methods, respectively.

Table 1. Sensitivity evaluation of the Bt11 maize-specific detection methods

Mixing level	The currently used method	The improved method
0.2% (v/v)	21 (0%)	21 (0%)
0.1% (v/v)	21 (0%)	21 (0%)
0.05% (v/v)	19 (9.5%)	16 (24%)

The numbers of table elements indicate the numbers of positive results in the total of 21 assays.
The percentages in parentheses indicate the false-negative rate in the total of 21 assays.

Discussion

Non-specific amplification was observed in testing with non-GM maize genome DNA as template DNA by the currently used Bt11 maize-specific detection method, as shown in Fig. 1B. The non-specific amplification products could not be distinguished on agarose gel electrophoresis assay, because the size of non-specific products was similar to that of the original products. This result suggested that the non-specific amplification may cause erroneous decisions about the presence or absence of Bt11 maize in analytical samples in practical testing. The nucleotide sequence analyses of the non-specific products and homology search in the

BLAST system proved that the non-specific amplification was caused by miss-annealing of the primer pair to conventional maize genomes. It was reported that the magnesium concentration in the PCR mixture affected primer annealing, strand dissociation temperatures of both the template and PCR products, product specificity, formation of primer-dimer artifacts, and enzyme activity and fidelity[6]. We designed the improved detection method by decreasing the magnesium concentration and succeeded in eliminating non-specific amplification, as shown in Fig. 1C. The specificity evaluation demonstrated that the improved method was highly specific to Bt11 maize. In Europe, the European Network of GMO Laboratories validates analytical performances of official GMO detection methods as described in the report entitled "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing"*4 in agreement with international standards, *i.e.*, the IUPAC Protocol for the Design, Conduct and Interpretation of Method Performance Studies[7] and International Standard ISO 5725*5, ISO 21571*6 and ISO 24276*7. According to the report, the limit of detection (LOD) is defined as the lowest amount or concentration of analyte at which the presence of the analyte should be detected at least 95% of the time, ensuring less than 5% false-negative results. For example, in the in-house validation of the European official Bt10 maize-detection method, simulated DNA-mixtures containing Bt10 maize DNA at low concentrations were assayed 21 times and the LOD was determined based on the examination result in accordance with the LOD definition.*8 We also performed LOD determination based on the results in Table 1 under the criteria, and the LODs of both the currently used and the improved detection methods were determined as 0.1% (v/v). The LOD determined in this investigation was representative of the performances of the methods. However, the LOD determined here may not directly reflect the actual LOD in practical testing, because we evaluated the LOD without taking into accout the bias derived from sample matrices and the dispertion of the target DNA's copy number occurring in the DNA extraction and dilution steps. We did, however, demonstrate that the sensitivity of the improved method was equivalent to that of the currently used method in this study. Thus, we concluded that the improved method has a lower incidence of false-positive detection owing to non-specific amplification, and would be more reliable than the currently used method in terms of analytical performance.

*4 European Network of GMO Laboratories. Definition of Minimum Performance Requirements for analytical methods of GMO Testing, The website of Community Reference Laboratory for GM Food & Feed, http://gmo-crl.jrc.ec.europa.eu/default.htm.

*5 International Standard 5725, Accuracy (trueness and precision) of measurement methods and results. 1994.

*6 International Standard 21571, Foodstuffs—Method of Analysis for the detection of the genetically modified organisms and derived products—nucleic acid extraction. 2005.

*7 International Standard 24276, Foodstuffs—Nucleic acid based methods of analysis for the detection of genetically modified organisms and derived products—General requirements and definitions. 2006.

*8 Mazzara. M., Foti, N., Maretti, M., Savini, C., Van den Eede, G. Report on the In-House Validation of an event-specific Detection Method for Event Bt10 using a qualitative PCR assay and verification by restriction analysis. http://gmo-crl.jrc.ec.europa.eu/summaries/Bt10%20validation%20report%20version2.pdf

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Note

Establishment and Evaluation of Event-Specific Quantitative PCR Method for Genetically Modified Soybean MON89788

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A novel real-time PCR-based analytical method was established for the event-specific quantification of a GM soybean event MON89788. The conversion factor (C_f) which is required to calculate the GMO amount was experimentally determined. The quantitative method was evaluated by a single-laboratory analysis and a blind test in a multi-laboratory trial. The limit of quantitation for the method was estimated to be 0.1% or lower. The trueness and precision were evaluated as the bias and reproducibility of the relative standard deviation (RSD_R), and the determined bias and RSD_R values for the method were both less than 20%. These results suggest that the established method would be suitable for practical detection and quantification of MON 89788.

Key words: MON89788; event-specific; genetically modified (GM); real-time PCR; soybean

Introduction

Glyphosate is a well-known herbicide which is active against a broad spectrum of plant species, and glyphosate tolerance has been genetically engineered into various crops such as canola, cotton, maize, and soybean[1]. A glyphosate-tolerant soybean (GTS), event 40-3-2 [Roundup Ready Soy (RRS)], is one of the most successful genetically modified (GM) crops and has become dominant in several countries where its planting is permitted[1]. Indeed, the GM soybean-planted area made up more than half of the total GM-planted area in 2007[2]. Based on experience of using RRS and the benefits gained from its use, a second-generation GTS, MON89788, has been developed, and is now being commercially cultivated. MON89788 integrates the *cp4 epsps* gene, the chimeric promoter consisting of the enhancer sequence of the Figwort Mosaic Virus (FMV) promoter bound to the *Tsf1* promoter derived from *Arabidopsis thaliana*, and the terminator from pea ribulose 1,5-bisphosphate carboxylase (TRbcS) at a single locus in the soybean genome[3]. It is reported that MON89788 enables the same flexible, dependable weed control as the original RRS, but with higher yield potential resulting from advanced gene mapping and insertion technology*1, and the relative crop yield of MON 89788 is increased from 7 to 11% over RRS*2. Therefore, MON89788 is expected to become widely used.

There are requirements for labeling GM products in crops or derived products in the European Union (EU), Korea, Japan, Australia, and many countries. The threshold for the unintentional commingling level of GM crops was set at 5% in Japan[4],*3. To date, real-time PCR-based detection and quantitative methods for several GM maize events and RRS have already been developed and validated by our international interlaboratory collaborative study[5,6], and adopted as

*1 Monsanto Co., news release http://monsanto.mediaroom.com/index.php?s=43&item=687

*2 Nougyou To Kankyou No. 90, National Institute for Agro-Enviromental Sciences http://www.niaes.affrc.go.jp/magazine/090/mgzn09008.html

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

Japanese standard analytical methods*4, *5. However, in Japan there is no standard method for detecting MON89788. An analytical method to detect MON89788 is needed for practical monitoring because MON89788 cannot be detected even by using target sequences from Cauliflower Mosaic Virus 35S promoter (P35S) and the nopaline synthase terminator regions which are commonly used in many GM crops.

In this report, we describe a new quantitative method for detecting MON89788 with an event-specific PCR strategy, and an evaluation of the method.

Materials and Methods

Plant materials

GM soybean and maize seeds, MON89788, RRS, MON 810, MON863, MON88017, and NK603 were kindly provided by the Monsanto Company (St. Louis, MO, USA), A2704-12 by its developer, Bt11, Event176, GA21, and MIR604 by Syngenta Seeds AG (Basel, Switzerland), and TC1507 and DAS59122 by Pioneer Hi-Bred International (Johnston, IA, USA). T25 was directly imported from the USA. Dry soybean seeds which were harvested in the USA in 2004, were purchased from Ryokokushoji Co., Ltd. (Hiroshima, Japan) and used as a non-GM soy sample.

Oligonucleotide primers and probes

For specific detection of MON89788, the primers (MON89788-F; 5′-TCCCGCTCTAGCGCTTCAAT-3′ and MON89788-R; 5′-TCGAGCAGGACCTGCAGAA-3′) and fluorescent dye-labeled probe (MON89788-P; 5′-CTGAAGGCGGGAAACGACAATCTG-3′) were used for real-time PCR. The sequences of these primers and probe were taken from the report of the European Commission's Joint Research Center (JRC, IRMM, Retieseweg, Geel, Belgium)*6. The soy lectin1 (*Le1*)[7] was used as a soybean-specific endogenous reference DNA for qualitative and quantitative analyses. For specific detection of *Le1*, the primers (Le1n02-5′; 5′-GCCCTCTACTCCACCCCCA-3′ and Le1n02-3′; 5′-GCCCATCTGCAAGCCTTTTT-3′) and fluorescent dye-labeled probe (Le1-Taq; 5′-AGCTTCGCCGCTTCCTTCAACTTCAC-3′) were used. The maize *starch synthase IIb* (*SSIIb*) gene was used as a maize-specific endogenous DNA for qualitative analysis. For specific detection of *SSIIb*, the primers (SSIIb3-5′; 5′-CCAATCCTTTGACATCTGCTCC-3′ and SSIIb3-3′; 5′-GATCAGCTTTGGGTCCGGA-3′)[6] were used. The oligonucleotide primers and TaqMan™ probes were synthesized by Fasmac Co., Ltd. (Kanagawa, Japan) and Life Technologies (Carlsbad, CA, USA), respectively. The oligonucleotide probes were labeled with 6-carboxyfluorescein (FAM) at the 5′ ends and 6-carboxytetramethylrhodamine (TAMRA) at the 3′ ends.

Preparation of calibrant plasmid

The specific sequence fragments from MON89788, endogenous soybean *Le1* gene[8], and P35S[8] were synthesized as a single oligonucleotide in tandem and inserted into the pUC19 vector. The constructed plasmid, designated as pSCS, was purified and diluted with 5 ng/μL ColE1 DNA (Nippon Gene, Tokyo, Japan) solution to 20,125,1500,20,000, and 250,000 copies per 2.5 μL, based on the copy number of the *Le1* segment.

Preparation of test samples

For evaluation of the quantitative method, DNA solutions consisting of different concentrations of extracted MON89788 DNA in non-GM soybean DNA were used. MON89788 and non-GM soybean genomic DNA was extracted from ground seeds using GM Quicker (Nippon Gene) according to the manufacturer's manual. The concentration and quality of the extracted DNA solutions were evaluated by measuring the ultraviolet (UV) absorbance with a spectrophotometer, DU800 (Beckman Coulter, Fullerton, CA, USA) as described previously[6]. Both MON89788 and the non-GM soybean genomic DNA solutions were adjusted to a concentration of 20 ng/μL, and then mixed. Samples containing a mixture of the genomic DNA solutions at concentrations of MON89788 of 0, 0.1, 0.5, 5.0, and 10.0% in volume ratio were prepared. The mixed samples of each concentration were divided into 24 sample tubes, and six tubes were then randomly selected for a single laboratory evaluation and multi-laboratory trial, respectively.

Qualitative PCR

Qualitative PCR using a thermal cycler, GeneAmp PCR system 9700 (Life Technologies) and agarose gel electrophoresis were performed as described by Kuribara *et al.*[8].

Quantitative PCR

TaqMan real-time PCR assays were carried out using an ABI PRISM 7900HT (Life Technologies), in 25 μL final volume, with 50 ng of sample DNA, 12.5 μL Universal Master Mix (Life Technologies), 0.5 μM primer pairs and 0.2 μM probe, according to the following step-cycle program: 2 min at 50℃, 10 min at 95℃, 45 cycles, 30 sec at 95℃, and 1 min at 59℃. In the reaction plate, each sample was quantified in triplicate.

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

*5 Japanese Agricultural Standard (JAS) analytical test handbook: genetically modified food quality, labeling analysis manual for individual products (2002) The Food and Agricultural Materials Inspection Center, Japan. http: / / www. famic. go. jp / technical _ information / jashandbook/index.html

*6 Event-specific method for the quantification of soybean line MON89788 using real-time PCR. http://gmo-crl. jrc. ec. europa. eu / summaries / MON 89788 _ validated _ Method.pdf

Multi-laboratory trial

A multi-laboratory trial was performed at 3 laboratories and consisted of 2 separate stages, a stage to measure the C_f value and a blind test. All experimental protocols were provided by the National Food Research Institute (NFRI). Quantitative real-time PCR was performed with primers, probes, Universal Master Mix and DNA solutions supplied by NFRI.

The C_f value is experimentally determined as the ratio of the copy number of recombinant DNA (r-DNA) to the taxon-specific sequence in the GM plant genome. To calculate the C_f value for MON89788, the genomic DNA was extracted from genuine GM seeds, and the copy numbers of r-DNA and taxon-specific sequences were measured. The measurement was done twice at 3 laboratories, and the C_f value was determined as the mean of the 6 values obtained.

A blind test was carried out as described previously[6]. Blind samples which were designed as blind duplicates of the 5 different concentrations of MON89788 DNA, consisting of 0, 0.1, 0.5, 5.0, and 10.0%, were sent to the participants.

All participants were requested to submit their data from the real-time PCR analyses.

Results and Discussion

Specificity of the PCR system for MON89788

For specific detection of MON89788, an event-specific segment was amplified at the junction site between the native soybean genomic DNA and r-DNA (Fig. 1). The specificity of the primer set was confirmed by a qualitative PCR method. No unexpected PCR products were detected using genomic DNA solutions from non-GM soybean, GM soybean RRS and A2704-12, non-GM maize, or 11 lines of GM maize, including glyphosate-tolerant events such as GA21 and NK603 (Fig. 2).

Determination of C_f value for MON89788

To determine the experimental C_f value for MON 89788, the copy numbers of *Le1* and MON89788 in the extracted DNA from the MON89788 seeds were measured.

In Japan, one of the features of standard quantitative methods is utilization of standard plasmid DNA containing both r-DNA and endogenous sequences as reference molecules and calibrators. To harmonize with the Japanese standard method, we developed one novel standard plasmid. The pSCS plasmid includes the specific sequences of MON89788 (Fig. 3), the *Le1* segment, and the P35S segment. The measurements were repeated twice, and the C_f value was determined as the mean from three laboratories. The defined C_f value for MON 89788 was 1.21 (Table 1), which was used in the following quantifications.

Single laboratory evaluation of the PCR quantification

We performed single-laboratory and multi-laboratory evaluations of the proposed quantitative method using samples prepared by mixing extracted DNAs of GM and

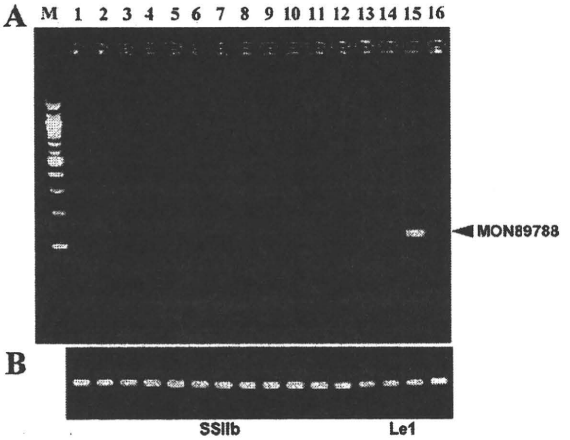

Fig. 2. Specificity test of the designed primer. Agarose gel (3.0%) electrophoretogram of the amplified PCR products corresponding to the 139 bp of MON89788 (A), 114 bp of *SSIIb* and 118 bp of *Le1* in lanes 1–12 and 13–16 of (B), respectively. Lanes 1–11, GM maize events, NK603, Event176, T25, GA21, MON810, TC1507, Bt11, MIR604, MON88017, DAS59122, and MON863, respectively; 12 and 13, non-GM maize and non-GM soy, respectively; 14–16, GM soybean events, RRS, MON89788, and A2704-12, respectively. M, molecular weight marker.

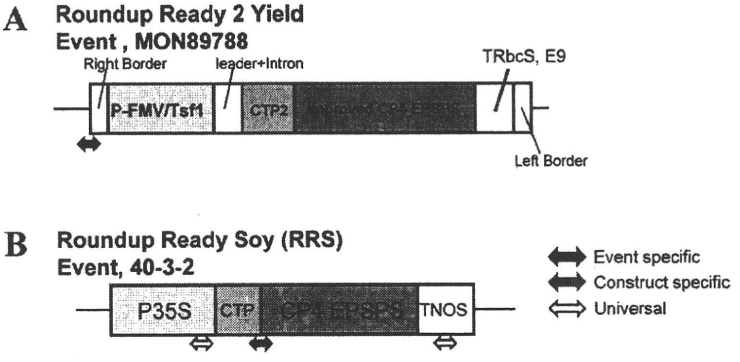

Fig. 1. Schematic diagrams of the recombinant DNA of MON89788 and RRS. Arrows indicate the target positions in MON89788 (A) and RRS (B). Event-specific target sequence of MON89788 was designed for the 5′-flanking region between the exogenous insert and host soybean DNA.

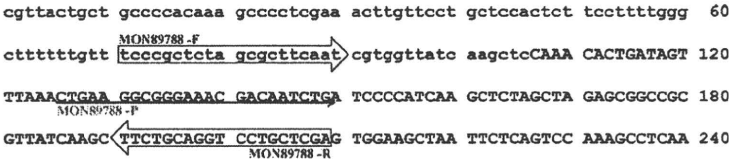

Fig. 3. Sequence between the 5′ exogenous insertion and the soybean genome of MON89788. Lowercase letters represent the flanking soybean genomic sequence, and capital letters show the 5′ transgenic insert containing the right border and partial FMV promoter sequences. The primers and probe for MON89788 are boxed and underlined, respectively.

non-GM DNA solutions. We decided that using DNA solution-based mixing samples would be easy and snitable for evaluation of the method.

In the single-laboratory evaluation, the GMO amount (%) of each sample was analyzed in six repetitions, and statistical analyses were carried out. The mean, bias (mean value, %), and relative standard deviation (RSD) at each mixing level were calculated (Table 2). The largest value of determined bias from the true value was 13.3, and the highest value of the RSD was 14.4% for the 0.1% mixing sample.

Blind test

We next evaluated the proposed quantitative method with a blind test involving 3 laboratories. All participants received primers, probes, and the test samples consisting of 5 different concentrations of MON89788, and the measurement was performed twice. The trueness and precision were determined as previously described[5), 6)]. The bias, repeatability RSD (RSD_r), and reproducibility RSD (RSD_R) of the blind samples were measured (Table 3). The determined bias, RSD_r, and RSD_R ranged from −11.7 to 5.00%, from 2.70 to 19.4%, and from 5.92 to 19.4%, respectively. In Europe, the acceptance criteria for full validation of an analytical method are defined by the European Network of GMO Laboratories (ENGL)*[7]. All of the obtained bias values were below the limit of the trueness acceptable level in

Table 1. Experimental conversion factor for MON89788

7900HT		
Mean	SD	RSD
1.21	0.0866	7.14

SD; Standard deviation
RSD; Relative standard deviation

Table 2. Single-laboratory evaluation

% (v/v)	n	Means	Bias	RSD[a], %	Detection limit
		GMO amount, %	True value, %		Below 20 copies[b]
0.00	6	0.00	0.00	nd	6/6
0.10	6	0.113	13.3	14.4	0/6
0.50	6	0.532	6.34	4.96	0/6
5.0	6	5.00	−0.06	9.03	0/6
10.0	6	9.37	−6.35	8.40	0/6

[a] RSD: Relative standard deviation
[b] Below 20 copies are expressed as the ratio of the number of retained data below 20 copies/the total number of retained data.

Table 3. Trueness and precision

% (v/v)	Retained labs	Trueness		Precision		Detection limit
		Means	Bias			
		GMO amount, %	True value, %	RSD_r[a], %	RSD_R[b], %	Below 20 copies[c]
0.00	3	0.00	0.00	nd	nd	6/6
0.10	3	0.105	5.00	19.4	19.4	0/6
0.50	3	0.442	−11.7	16.8	16.8	0/6
5.0	3	4.85	−3.00	2.70	5.92	0/6
10.0	3	9.18	−8.20	5.19	6.32	0/6

[a] RSD_r: Repeatability relative standard deviation
[b] RSD_R: Reproducibility relative standard deviation
[c] Below 20 copies are expressed as the ratio of the number of retained data below 20 copies/the total number of retained data.
When $RSD_r > RSD_R$, RSD_R was considered to have the same value of RSD_r[9)].

*[7] Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing, European Network of GMO Laboratories (ENGL)
http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf

ENGL (The bias value should be within ±25%). The obtained RSD_r and RSD_R here were similar to, or within a narrower range than, those in previously reported GMO events[5),6)]. ISO 24276*[8] specifies that the RSD_R is 25% or less at the limit of quantitation (LOQ) in GMO analysis, and all the obtained RSD_R values were within the criterion, even for 0.10% samples. The data below 20 copies were extrapolated based on the standard curve in our method because there was no calibrant below 20 copies. In Table 3, all the measured copy numbers of 0.10% samples were over 20 copies. Therefore, it was estimated that the LOQ for MON89788 is 0.10% in this method.

In conclusion, the newly developed method is applicable for the detection and quantification of MON89788 to monitor the validity of the labeling. Several GM soybean events such as RRS, MON89788, and gluphosinate-tolerant soybeans have been approved in Japan. The number of approved GM soybean events is expected to steadily increase. Specific analytical methods for novel GM events or a screening method for GM soybean events will be required.

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*[8] International Standard 24276, Foodstuffs—Nucleic acid based of analysis for the detection of genetically modified organisms and derived products—General requirements and definitions

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Evaluation of Quantitative PCR Methods for Genetically Modified Maize (MON863, NK603, TC1507 and T25)

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Novel real-time PCR-based quantitative methods were developed for three GM maize events; MON863, NK603 and TC1507. The quantitative methods were designed to amplify an event-specific segment for MON863 and NK603, and a construct-specific segment for TC1507. We also developed an event-specific quantitative method for T25. The conversion factor (Cf), which is required for calculating the GMO amount, was determined using three types of real-time PCR equipment; the ABI PRISM 7700, 7900HT and 7500. The quantitative methods were evaluated by blind testing in an interlaboratory study using the ABI PRISM 7700 and 7900HT, and in a multilaboratory trial using the ABI PRISM 7500. The trueness, precision, and limit of quantitation were determined. Although the biases expressing the trueness for MON863, TC1507, and T25 were slightly high, all the data suggested that the developed methods were suitable for identification and quantification of these GM maize events.

Keywords: genetically modified (GM), interlaboratory study, maize (*Zea mays*), real-time PCR

Introduction

In many countries, there is widespread use of genetically modified (GM) crops for food and feed. Although these GM organisms (GMOs) have been assessed and authorized by administrative bodies over the past two decades, acceptance of GMOs and the foods containing them remains controversial in many places, including Europe, Korea and Japan. Because of this, many countries require the labeling of all foods containing or derived from authorized GMOs. To develop a labeling system, it is necessary to define an effective threshold level for the unintentional commingling of GMOs. This threshold was set at 5% in Japan (Department of Food Safety, Ministry of Health, Labour and Welfare (MHLW) of Japan, 2001; Hino, 2002; Ministry of Agriculture, Forestry and Fisheries of Japan, 2000). Protein and DNA-based methods are the two main approaches used for detecting and quantifying the mixing level of GMOs (Ahmed, 2002). Protein-based methods are generally immunotechnologies with specific antibodies that recognize the proteins produced by the introduced trait genes, such as Western blot, enzyme-linked immunosorbent assay (ELISA) and lateral flow strips (Fantozzi *et al.*, 2007; Lipton *et al.*, 2000; Rogan *et al.*, 1999). For DNA-based methods, PCR-based analyses are commonly used to detect the sequences of introduced recombinant DNAs (r-DNAs). Various PCR-based methods have been developed for the detection and quantification of GMOs (Matsuoka *et al.*, 2002; Onishi *et al.*, 2005; Randhawa *et al.*, 2009). In particular, real-time PCR systems using TaqMan® chemistry are a sensitive and specific analytical technique for obtaining precise numerical information (Akiyama *et al.*, 2005; Holst-Jensen *et al.*, 2003; Kuribara *et al.*, 2002; Mano

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et al., 2009; Oguchi *et al.*, 2007).

GM technology has been applied to maize, a major agricultural crop, and various types of GM maize are currently distributed worldwide. The principal traits of GM maize are herbicide tolerance (T25, NK603, GA21, etc.), insect resistance (MON863, MON810, etc.), and combinations of these traits (Bt176, Bt11, TC1507, etc.).

In Japan, detection and quantitative methods using real-time PCR of five types of GM maize (Bt11, Bt176, GA21, MON810 and T25) and Roundup Ready Soy (RRS) have been developed and validated in an international interlaboratory collaborative study (Kodama *et al.*, 2009; Shindo *et al.*, 2002), and adopted as Japanese standard analytical methods (Department of Food Safety, MHLW of Japan, 2001; Japanese Agricultural Standard analytical test handbook, 2002). One of the features of these methods is the utilization of plasmid DNAs containing both r-DNA and endogenous sequences as calibrators. In response to the increased number of approved GM maize events, the MHLW announced a combinational method for quantification of the Cauliflower Mosaic Virus (CaMV) 35S promoter (P35S) region and a construct-specific quantification for GA21 maize, which has been officially used as a screening method for GM maize (Department of Food Safety, MHLW of Japan, 2001). Recently, a duplex real-time PCR method that simultaneously detects P35S and an event-specific segment of GA21 has been developed (Oguchi *et al.*, 2009). This screening method makes it possible to simultaneously detect GA21 and several GM maize events that contain the P35S region. Screening analysis is a useful and practical method, but cannot be used to identify each individual GMO event. Furthermore, the screening method is associated with the possibility of overestimating GM amounts because several GM events have plural copies of P35S regions, and each GM event has an individual Cf value (Kodama *et al.*, 2009; Kuribara *et al.*, 2002; Shindo *et al.*, 2002). To overcome this, individual GM specific analytical methods are indispensable for the identification of GM events and for correct estimation (Holst-Jensen *et al.*, 2003; Yang *et al.*, 2009).

We developed analytical methods for three GM maize (MON863, NK603 and TC1507) events and an improved method for T25. We also determined the Cf values for each GM event and evaluated them by conducting a blind test in an interlaboratory collaborative study. The studies were conducted in Japan, Korea, Taiwan, Europe and the United States with two types of real-time PCR instrument (ABI PRISM 7700 and 7900HT). In addition, measurement of the Cf values and blind testing were also conducted in 3 laboratories using another real-time PCR instrument, the ABI PRISM 7500.

Materials and Methods

Maize seeds We used four lines of F1 generation seeds of GM maize. MON863 and NK603 were kindly provided by the Monsanto Company (St. Louis, MO, USA), TC1507 by Dow AgroSciences LLC (Indianapolis, IN, USA), and T25 was provided by its developer.

Oligonucleotide primers and probes The specific primers and fluorescent dye-labeled probes used for real-time PCR are listed in Table 1. The oligonucleotide primers and TaqMan® probes were synthesized by FASMAC Co. Ltd. (Kanagawa, Japan) and Applied Biosystems (Foster City, CA, USA), respectively. The oligonucleotide probes were labeled with 6-carboxyfluorescein (FAM) at the 5' ends and 6-carboxytetramethylrhodamin (TAMRA) at the 3' ends.

Preparation of reference molecules One novel plasmid designated pIIMul4ver2.1 was constructed and used as a calibrator for quantification of MON863, NK603, TC1507 and T25. The specific sequence fragments from four GM events and the endogenous maize *starch synthase IIb* (*SSIIb*) gene (Harn *et al.*, 1998) were connected by the overlapping extension PCR method. The specific sequences of *SSIIb*, TC1507, NK603, T25 and MON863 were amplified using the following primer pairs: 5'-CTCCCAATCCTTTGACATCTGC-3' (primer A) and 5'-tgcgactaacatTCGATTTCTCTCTTGGTGACAGGA-3', and 5'-gagagaaatcgaATGTTAGTCGCAACGAAACCG-3' and 5'-ggcttgctggccTGAGTTGATTCCAGTTACTGCCA-3' (primer B), and 5'-ggaatcaactcaGGCCAGCAAGCCTTGTAGC-3' (primer C) and 5'-attcgagctcacATCCCGACTCTCTTCTCAAGCATA-3', and 5'-gagagtcgggatGTGAGCTCGAATGTTGTTCTTC-3' and 5'-acctacaaatgcCATGAGACAATAACCCTGATAAATGCT-3' (primer D), and 5'-tattgtctcatgGCATTTGTAGGTGCCACCTTC-3' and 5'-TGACCCTACTTGTTCGGATGG-3' (primer E), respectively. The resulting PCR products for SSIIb and TC1507 were mixed and used as a template for PCR with primer pairs A and B. The resulting PCR products were designated amplicon X, *SSIIb*+TC1507. Similarly, the PCR products for NK603 and T25 were mixed and used as a template for PCR with primers C and D. The resulting PCR products were designated amplicon Y, NK603+T25. Amplicon Y and PCR products for MON863 were mixed and used as a template for PCR with primers C and E. The resulting PCR products were designated amplicon Z, NK603+T25+MON863. Finally, amplicons X and Z were mixed and used as a template for PCR with primers A and E. The resulting PCR products were then inserted into the pUC19 vector. The constructed plasmid was purified and diluted with 5 ng/μL ColE1 DNA (NIPPON GENE, Tokyo, Japan) solution to 20, 125, 1500, 20,000, and 250,000 copies per 2.5 μL, based on the copy number of the

Table 1. List of primers and Taqman probes.

Target	Name	Sequence	Specificity	Length, bp
zSSIIb	SSIIb 3-5'	CCA ATC CTT TGA CAT CTG CTC C	z*SSIIb*/sense primer	114
	SSIIb 3-3'	GAT CAG CTT TGG GTC CGG A	z*SSIIb*/antisense primer	
	SSIIb-Taq	FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA	z*SSIIb*/sense probe	
MON863	MON863 2-5'	TGA CCC TAC TTG TTC GGA TGG	maize genome/sense primer	111
	MON863 2-3'	GCA TTT GTA GGT GCC ACC TTC	P35S/antisense primer	
	MON863-1-Taq	FAM-CAC CCC AAA GTG TAC CAA GCT TTC CGA-TAMRA	Junction[a]/antisense probe	
NK603	NK603 1-5'	GGC CAG CAA GCC TTG TAG C	maize genome/sense primer	113
	NK603 1-3'	ATC CCG ACT CTC TTC TCA AGC ATA	pOsγ-actin1/antisense primer	
	NK603-1-Taq	FAM-ATG ACC TCG AGT AAG CTT GTT AAC GCG GC-TAMRA	pOsγ-actin1/antisense probe	
TC1507	TC1507-5'	TGA GTT GAT TCC AGT TAC TGC CA	cry1F/sense primer	111
	TC1507-3'	ATG TTA GTC GCA ACG AAA CCG	ORF25/antisense primer	
	1507t-2-Taq	FAM-ACT CGA GTA AGG ATC CGT CGA CCT GCA G-TAMRA	Junction/sense probe	
T25	T25 1-5'	CAT GAG ACA ATA ACC CTG ATA AAT GCT	Vector/sense primer	111
	T25 1-3'	GTG AGC TCG AAT GTT GTT CTT CC	maize genome/antisense primer	
	T25-1-Taq	FAM-AGG GTG GAA TCG ACG ACG TCT TCC C-TAMRA	Junction/sense probe	

[a] Junction; Junction region between maize genomic DNA and r-DNA

SSIIb segment.

Preparation of test samples Five mixing levels of test samples containing 0.25, 0.50, 1.0, 5.0 and 10.0 % of each GM line (MON863, NK603, TC1507 and T25) were prepared by mixing dried powders of non-GM and GM maize in the manner described previously by Kuribara *et al.* (2002). Briefly, washed maize seeds were frozen and ground with a speed rotor mill (Fritsch GmbH, Idar-Oberstein, Germany), and were freeze-dried in a freeze dryer (FDU-1100; Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Simulated mixed samples were then prepared. Plant genomic DNA was extracted from those ground materials using a DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany), with small modifications as described elsewhere (Kuribara *et al.*, 2002; Shindo *et al.*, 2002), and the concentrations of extracted DNA solutions were determined by measuring the ultraviolet (UV) absorbance with a spectrophotometer, the DU7400 (Beckman Coulter, Fullerton, CA). The quality of extracted maize genomic DNA was evaluated by the absorbance ratio at 260/280 nm and 260/230 nm. DNA solutions whose absorption ratios were between 1.7 and 2.0 at 260/280 nm and were > 1.7 at 260/230 nm were used.

Qualitative PCR Amplifications were carried out in a 25-μL reaction volume, with 50 ng of sample DNA, 200 μM dNTP, 1.5 mM $MgCl_2$, 0.625 U AmpliTaq Gold polymerase (Applied Biosystems), and 0.25 μM of each primer. Reactions were buffered with the PCR buffer II (Applied Biosystems) and amplified using a thermal cycler, GeneAmp PCR system 9700 (Applied Biosystems) with the following step-cycle program: 10 min at 95°C, and 40 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C, followed by a final extension at 72°C for 7 min. PCR products were electrophoresed on 3.0% agarose gel in TAE buffer and stained with ethidium bromide.

Quantitative PCR TaqMan real-time PCR assays were carried out using the ABI PRISM 7500, 7700 and 7900HT (Applied Biosystems), in 25-μL reaction volumes, with 50 ng of sample DNA, 12.5 μL Universal Master Mix (Applied Biosystems), 0.5 μM primer pairs and 0.2 μM probe (for detection of MON 863 and NK603, 0.1 μM probe was used). Real-time PCR with the ABI PRISM 7900HT and 7500 was performed using the following step-cycle program: 2 min at 50°C, 10 min at 95°C, and 45 cycles of 30 s at 95°C, and 1 min at 59°C. On the ABI PRISM 7700, the cycle number was set at 40 instead of 45. Standard curves were calibrated with the copy number of RMs. In the reaction plate, each sample was quantified in triplicate.

Measurement of Cf value The GM contents of the samples were calculated using the following formula:

$$\text{GM content (\%)} = \frac{\text{Copy number of GM-specific sequence}}{\text{Copy number of taxon-specific sequence}} \times \frac{1}{\text{Cf}} \times 100$$

Cf is experimentally determined as the ratio of the copy number of r-DNA to the taxon-specific sequence in the GM plant genome. To calculate Cf values, genomic DNA was extracted from each GM seed, and the copy number of r-DNA and taxon-specific sequences were measured from the extracted genomic DNA. Cf values were determined from the results of 5 laboratories for ABI PRISM 7700 and 7900HT, and 3 laboratories for ABI PRISM 7500. The specific sequence in z*SSIIb* was selected for the maize taxon-specific sequence.

Homogeneity test Test samples of each GM mixing