Studies on Novel Detection Methods of Genetically Modified Crops Using Genetic Engineering Techniques

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CONTENTS

GENERAL INTRODUCTION

Genetic modification is a technique that involves extracting DNA from cells, altering their genetic composition and order, and expressing a functional gene in the same or different organism [1]. Genetically modified (GM) crops refer to crops or their descendants produced in such a manner, and these have widely used and harvested in agriculture. The area for the plantation of GM crops has rapidly increased in recent years; in 2012, the area was approximately 100 times larger (170.3 million hectares) compared to 1996, when full-scale commercial growing was initiated. In 2012, a total of 28 countries, including 20 and 8 developing and developed countries, respectively, were growing GM crops. Although GM crops require a high degree of production techniques and were grown only in developed countries during the early stages, the popularization of these particular plants has recently, led to rapid increase in its production in developing countries. The rate of increase in the area for plantation was 1.6 million hectares or 3% in developed countries versus 8.7 million hectares or 11% in developing countries. As a result, this was the first time the distribution ratio of global plantation area skewed towards developing countries, with 52% occurring in developing countries versus 48% for developed countries. In particular, Brazil has undergone four consecutive years of increases, including a 21% (6.3 million hectares) increase in 2011 for a total of 36.6 million hectares of plantations. The global increase in plantation area for GM crops in 2012 (10.3 million hectares) was at least two-fold higher than that of Japan's total cultivated area (4.54 million hectares) and continues to grow. Furthermore, Japan's largest importer of grains, the United States of America, is also the world's largest grower of GM crops, with a plantation area of 69.5 million hectares in 2012 (representing 41% of the world's total plantation area for GM crops) [2]. Japan relies on the import of 65% of its soy beans

and 75% of its maize from the United States, increasing the likelihood that GM crops would be integrated into the food industry [3].

International guidelines for establishing the safety of GM crops in the food industry are determined by the CODEX Commission. In Japan, a safety assessment system of GM crops was initiated under various laws and regulations. Safety inspections are currently in progress for foodstuffs under the Food Sanitation Act and feed under the Feed Safety Act (laws and regulations related to the improvement of quality and maintenance and safety of feed). Additionally, the environmental impact of using GM crops is being assessed under the Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Cartagena Protocol). In general, only those GM crops that have received approval under these safety acts can be distributed. However, of the feeds that have been assessed as safe under inspection systems as strict as Japan's (conducted by the Ministry of Agriculture, Forestry and Fisheries), approximately 1% is comprised of GM crops [4]. Moreover, the formulation of a product labeling system has been investigated, with the objective of supporting consumer choice as GM crops become available. In April 2001, the Act on Standardization and Proper Quality Labeling of Agricultural and Forestry Products (JAS Law) was established. Furthermore, a similar labeling requirement was established under the Food Sanitation Act, with the aim of displaying the presence or absence of GM crops in food items. Under this act, if a food item contains soy beans (including soy beans in a pod and bean sprouts), maize, potato, canola, cottonseed, alfalfa, sugar beet, and papaya that are distributed as GM crops and is one of the first three ingredients by weight or if the component weight is greater than 5% of the total, then such GM crops and their derivatives must be identified in the product label [5]. On the other hand, the use of non-GM crops should still be included in the product label; however,

to include such a label, the food product must use appropriately separated ingredients and have a certificate for each of the following steps: growing, harvest, storage, and export [6]. Accordingly, non-GM crops that have been appropriately classified and distributed are being actively procured by domestic markets in the food industry to cater to consumer trends towards non-GM foods. Oftentimes, distribution centers do not use non-GM crops-specific silos and tankers due to economic reasons, and the same equipment is cleaned for subsequent use in handling both types of products. For this reason, GM crops can remain in silos and tankers after storage or transportation, and there is a possibility of unintentional mixing of GM and non-GM crops, especially in distribution centers of countries that process large volumes of GM crops such as the United States. Accordingly, industries handling grains and seeds conduct independent scientific investigations in addition to securing administrative guarantees through certificates of compliance and quality control. Recently, a number of food fraud issues have arisen, thus increasing the interest of consumers for food labeling. A confirmed mislabel can have large negative impacts on the industry's brand image and the resulting economic consequences could be significantly damaging. Based on this information, a simple investigative test for GM crops was required by the administrative and industry organizations in Japan, and a variety of methods have been developed and investigated.

There are two types of analyzing techniques developed for GM crops: that analyzes recombinant 1) proteins and 2) DNA.

1) Existence of recombinant proteins in GM crops can be detected directly, thus allowing the development of a simple analyzing system. Those are enzyme-linked immunosorbent assay (ELISA) and immunochromatography are representative analyzing techniques for GM crops, and assay kits based on these principles are currently available [7, 8]. In particular,

immunochromatography only requires a test strip to be immersed in the sample, simplifying the procedure and rapidly giving results without the need for setting up special equipment. Due to its simplicity, this technique is most often used as a corroborative test for distribution processing in identity preserved (IP) handling, primarily in countries producing GM crops. However, the method for detecting recombinant proteins can only confirm the presence of the target protein, whereas GM events containing the same recombinant DNA cannot be distinguished. Moreover, this method is capable of detecting contamination of up to 0.1%, although some proteins are unstable and often undergo denaturation in processed foods, making protein detection extremely difficult and highly sensitive [9].

2) In contrast, recombinant DNA can be detected after amplification, avoiding the issues associated with protein detection. Methods for DNA amplification include polymerase chain reaction (PCR) [10, 11] and loop-mediated isothermal amplification (LAMP) [12]. In these methods, the region of amplification can be selected, allowing GM event-specific amplification and detection of DNA sequences, as well as guaranteeing traceability. Moreover, DNA is generally more stable at high temperatures compared to proteins, so some level of detectability can be maintained depending on the region of amplification even when the DNA has undergone fragmentation during the processing stage. For this reason, most of the GM crop detection methods in Japan and the European Union rely on DNA detection, using the technologically popularized PCR method. The specificity and sensitivity of PCR is dependent on the purity of template DNA, reaction temperature, composition of the reaction buffer, and primers; therefore, the development of a reliable method of detection will require careful consideration of these parameters. In Japan, qualitative PCR has been used to detect the presence of GM crops, whereas quantitative PCR has been used to measure the rate of contamination by GM crops. In qualitative

PCR, the target DNA is PCR amplified using primers and products are separated by agarose gel electrophoresis. GM events are confirmed if a band matching the size of the target DNA is detected. Additionally, the specific tests for GM events must include PCR of endogenous genes (i.e., soybean: soybean lectin gene; maize: starch synthases IIb gene) as a control reaction for the method of examination (sample breakdown, DNA extraction, and PCR) [13]. On the other hand, GM detection by quantitative PCR involves measuring the copy number of the endogenous gene and recombinant gene using fluorescent probes and real-time PCR. In the EU, the ratio of the copy number for the endogenous gene to the recombinant gene is used as the GM contamination rate. In Japan, a conversion factor is used to convert the copy number ratio to a weighted ratio of contamination rate, which has been published in the ISO standard for detecting GM crops: ISO21570-2005 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Quantitative nucleic acid based methods. The GM contamination rate is calculated by substituting the conversion factor and measured value of the unknown sample into the following equation [14, 15]:

$$\text{GM contamination rate (\%)} = \frac{\frac{Recombinant\ DNA\ copy\ number}{Endogenous\ gene\ copy\ number} \times 1}{Conversion\ factor} \times 100$$

Furthermore, because approval for GM crops differs according to country, some GM crops may be approved by the import partner country, but not by Japan. A standard analytical method for unapproved GM crops can be found in the notice, “Investigative methods for unapproved recombinant DNA-applicable foodstuffs”, published by the Ministry of Health, Labour and Welfare. The development and addition of methods for the detection of new, unapproved GM crops and their events will be required when the possibility of contamination becomes evident.

In this study, we focused on the following four points in detecting recombinant DNA in

GM crops:

1. False positive results due to contamination of the positive control;
2. Complexity of methods of DNA extraction from processed foods;
3. Investigation of GM canola RT73 *Brassica rapa* (RT73 *B. rapa*), where species identification and recombinant DNA detection must be performed from a single seed; and
4. Determination of distribution conditions of GM maize from the United States, including its methodology.

We resolved these issues using genetic engineering techniques to further improve the accuracy of GM crop detection technology. The following is a detailed explanation of each method.

1. Generally, positive and negative control reactions are indispensable in DNA detection analysis to ensure that there are no contaminations, switching, or mixing of samples during the assay. Because PCR can amplify DNA up to 10^7 copies, adequate consideration must be given to DNA contamination due to a variety of reasons. For example, sample grinding, DNA extraction, preparation of PCR solutions, and electrophoresis should all be performed in separate rooms, with each room maintained in a clean state. However, in the fields where PCR is routinely performed, the positive control was not always included in the analysis, thus leading to issues where the absence of GM crops could not be confirmed. The objective of this study was to actively promote the use of control reactions and increase the precision of detection methods. By improving the plasmid DNA sequence used for positive control, we developed a new method for easily distinguishing between false positives due to contamination and true positives derived from genomic DNA.
2. DNA extraction and purification from samples is important to detect recombinant DNA in processed foods. However, several points such as incomplete extraction, leading to insufficient

amounts of DNA required for GM detection, and insufficient purification, leading to contamination from impurities that impede the PCR reaction, lead to inaccurate results. In GM detection, the methods for DNA extraction from plants include using cetyltrimethylammonium bromide (CTAB), a silica spin column, or an anion exchange resin. These methods of DNA extraction were developed and used depending on the crop species or degree of food processing. On the other hand, DNA in processed foods is often fragmented chemically, physically, or biologically, and normal methods of DNA extraction are ineffective in isolating the target DNA. Therefore, the development of a method of DNA extraction and refining with superior operability and less variability has been the focus of research interest. In this study, we established and optimized a method of DNA extraction, as well as conditions for refining processed foods that contain soybeans and maize allowing to isolate an adequate amount of quality DNA for use in GM detection.

3. The distribution of unapproved GM crops across the market place is due to human error at the research stage, specifically during seed management or production. This has caused concerns regarding for food safety. There have been several cases of documented contamination of unapproved GM crops, including inappropriate shipping of GM maize Bt10 (March 2005) [16], cultivation of insect-resistant GM rice in China (Hubei) (April 2005) [17], contamination of LL RICE601 (Liberty Link Rice 601) in the United States (August 2006) [18], contamination of GM maize DAS59132 in the United States (February 2008) [19], contamination of GM flax FP967 (October 2009) [20], contamination of GM papaya PRSV-YK in Taiwan (February 2011) [21], and contamination of PRSV-SC in Thailand (July 2013), etc. As mentioned before, in these cases, the development of a detection method appropriate for each GM event is required. The case of contamination of GM canola RT73 *B. rapa* reported

by the government of Canada in December 2005 signifies that the methods developed thus far are inappropriate. GM canola RT73 *B. rapa* is a hybrid strain between herbicide resistant RT73 *Brassica napus* (RT73 *B. napus*), which was already determined to be safe, and the non-GM canola (*B. rapa*). Planting had ceased in Canada in 2000, thus safety tests were not conducted in Japan based on the assumption that the grain was no longer being distributed. In order to analyze RT73 *B. rapa*, species identification between *B. rapa* and *B. napus*, as well as detection of GM canola-specific regions must be carried out using the same seed. To secure the reliability of the test, it was necessary to test several batches of seeds (numbering between ten and 100) in one lot individually and establish a systematic detection method. Additionally, the seeds of canola are extremely small and full of lipids; therefore, the development of extraction methods must consider these characteristic points. This incident occurred in Canada, Japan's largest import partner for canola, and the inability to establish a method of detection led to continued inability to perform the necessary detection tests required by law. Accordingly, in this study, we developed a new method of DNA extraction and real-time PCR to manage multiple canola samples and combined these to establish a detection method for GM canola RT73 *B. rapa*.

4. According to the GM food label regulation of Japan, inclusion of non-labeled GM crops cannot exceed the weighted ratio of 5%. However, using the quantitative detection methods outlined thus far, a higher GM contamination ratio will be calculated for stacked events containing multiple copies of exogenous genes produced through GM plant hybridization. For example, contamination with a hybrid species between herbicide-resistant GM maize and insect-resistant GM maize could result in a higher contamination ratio if a common sequence (CaMV 35S promoter region) is again used for quantification used in the screening. Therefore,

to avoid this, we modified the DNA extraction method to detect the GM canola RT73 *B. rapa* outlined in 3) for use with a single maize kernel. We then combined it with previously described methods of real-time PCR and multiplex PCR to develop a method capable of identifying and quantifying groups of GM maize that contain stacked events.

CHAPTER 1

Development of pBT63, a positive control plasmid for qualitative detection of genetically modified rice

1. Introduction

Genetically modified (GM) crop production has been steadily increasing globally. As of 2012, the cultivation area for GM crops was 170.3 million hectares, a 100-fold increase from when the first GM crop was commercialized in 1996 [2]. Today, GM crops for food and feed use have been produced in 28 countries worldwide.

International guidelines for assessing the safety of the industrial use of GM organisms (GMOs) have been established [22, 23]. The standards of the Food Sanitation Law have also been revised to restrict distribution of GM foods that have not been assessed for safety in Japan. This law has been implemented according to Notification No. 232 (May 1, 2000) and No. 233 (May 1, 2000), issued by the Ministry of Health, Labour and Welfare (MHLW) of Japan. In addition, since April 2001, safety assessment and labeling of GM foods have been legally required [24, 25]. This situation demanded the development of testing methods for unauthorized GM foods. We have developed PCR-based detection methods for unauthorized GM foods that have been adopted as standard Japanese methods. One is the detection method for GM Shanyou 63 (Bt63) [26-29], which is a GM rice variety developed in China that has been conferred insecticidal activity by the introduction of the *cry1Ac* gene of *Bacillus thuringiensis* (Bt). Our Bt63 detection method was widely used to prevent the importation and distribution of unauthorized Bt63 in the Japanese market [8, 17, 30-33], until replaced by the real-time PCR-based qualitative method described in Notification No. 116-4 (Nov. 16, 2012) issued by the MHLW [34, 35].

For GM product testing laboratories, a reliable reference material is indispensable as a positive control to ensure accurate test results. However, it is quite difficult to obtain enough of genuine unauthorized GM crops or their purified genomic DNA. Plasmid DNA including PCR

target sequences are frequently used instead, because they can be easily replicated within *Escherichia coli* and steadily supplied [36-41]. Especially in Japan, plasmid DNA is required as DNA templates for positive controls in the standard testing methods for authorized GM events (GM maize lines MON810, Bt11, GA21, Event176, and T25; GM soybean lines GTS40-3-2, MON89788, and A2704-12; GM papaya line 55-1; and GM potato lines Bt6, SPBT02-05, RBMT21-129, RBMT21-350, and RBMT22-82) [5, 14, 42-45] and for unauthorized GM events (GM rice lines Shanyou 63 (Bt63), NNBt, CpTI, and LL601; GM maize lines Bt10 and CBH351; GM canola line RT73; and GM papaya lines PRSV-YK and PRSV-SC) [21, 46-48].

In practice, extra precautionary procedures and cleaning have been required in testing laboratories to reduce the risk of contamination [5, 49]. Japanese regulations impose restrictions on the distribution of unauthorized GMOs, and in fact, the test results of qualitative analyses for unauthorized GMOs are negative in most cases. In this situation, contamination from positive control plasmids is a more frequent cause of positive results than contamination by ground seeds, DNA extracts or PCR products in the tests. Consequently, special caution should be implemented to avoid false-positive results when conducting such tests. However, there are currently no practical ways of confirming whether a positive test result was derived from contamination by positive control plasmids or another source.

We have developed a positive control plasmid for the testing of the unauthorized GM rice line Bt63. In order to eliminate false detection caused by control plasmid contamination, we introduced a restriction site in the middle of the PCR target sequence of the positive control plasmid. Then, restriction enzyme treatment allowed us to discriminate between true and false positive detection. To our knowledge, this is the first report of the development of a system to assess whether PCR products are derived from a positive control plasmid or a true GMO sample.

2. Materials and methods

2.1 Sample

For the detection of the rice endogenous sequence *sucrose phosphate synthase* (SPS), we used genomic DNA of the Koshihikari cultivar (*Oryza sativa)* purchased at a local market in Toyama. DNA extracted from Bt63-positive rice vermicelli, obtained by the National Institute of Health Sciences, Japan, was used as a positive control for Bt rice. The plasmid pRICE4, which is commercially available as the GM Rice Detection (IR) Rice Positive Control Plasmid 2 (Nippon Gene, Tokyo, Japan), was used for comparing amplification efficiency with that of the positive control plasmid developed in this study. Unless otherwise noted, all reagents were analytical grade and were used without further purification.

2.2 Plasmid Construction

2.2.1 First PCR

PCR primers for the construction of pBT63 were synthesized by Nippon Gene Material Co., Ltd. (Toyama, Japan). The first PCR, with four PCR primers binding the template to construct pBT63, was performed under the following conditions. A 50 μL reaction solution was prepared consisting of 5.0 μL 10× Gene*Taq* Universal Buffer, 0.2 mmol/L dNTP, 0.625 units Gene*Taq* FP polymerase (Nippon Gene) and 0.2 μmol/L each primer (Bt63r-1F, Bt63r-1R, Bt63r-2F, Bt63r-2R) (Table 1-1). PCR was performed with the GeneAmp PCR System 9700 (Life Technologies, Carlsbad, CA, USA). The reaction conditions were 5 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 60°C, and 2 min at 72°C, followed by a final extension at 72°C for 15 min, then storage at 4°C. The PCR product obtained was separated by electrophoresis in a 3% agarose gel.

2.2.2 Second PCR

The second PCR was carried out in a 50 μL reaction solution consisting of 5.0 μL 10× Gene*Taq* Universal Buffer, 0.2 mmol/L dNTP, 0.625 units Gene*Taq* FP, 0.2 μmol/L each primer (Bt63r-3F and Bt63r-3R) (Table 1-1), and 1.0 μL of the amplified product obtained in the first PCR. Thermal cycling conditions were set for 5 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 60°C, and 2 min at 72°C, followed by a final extension at 72°C for 15 min, then storage at 4°C. The PCR product obtained was separated by electrophoresis in a 3% agarose gel, then excised and purified with a MinElute Gel Extraction Kit (QIAGEN, Hilden, Germany).

Table 1-1 The synthetic oligonucleotide sequences used for construction of pBT63

Name	Sequence (5' → 3')	Length (nt)
Bt63r-1F	TTC ATC GGT AGT TTT TCT TTT CAT CGC CGA GCC CGG TTT AAG GCG TTT CCG TTC GGA TCC GCA GGA GTG ATT ATC GAC AG	80
Bt63r-1R	CCA AAG GAT CCC GCT TTC GGC CTG CAG TTG TCC ATA TAT CCG TTC AGG CGC AAT TTA ACT GTC GAT AAT CAC TCC TGC GGA TCC GAA C	88
Bt63r-2F	CTG CAG GCC GAA AGC GGG ATC CTT TGG CCT CTG CTC ATC CCG AAA AGA TCA ACC GTG TTC GCC TAT GGA ACC TCT TTG AAT CCT G	85
Bt63r-2R	TTC TGT GGT GGG ATT TCG TCA TGG TAC GGA TAC TCG CAC CGA AAG GAT CCA AGA CCG GCA ACA GGA TTC AAA GAG GTT CCA TAG GCG AAC	90
Bt63r-3F	AAA GGC GCC TTC ATC GGT AGT TTT TCT TTT CAT CGC CGA GCC C	43
Bt63r-3R	TTT GGC GCC TTC TGT GGT GGG ATT TCG TCA TGG TAC GG	38

2.2.3 TA Cloning

TA cloning was performed under the following conditions. A total volume of 20 μL was prepared with 0.5 μL pGEM-T Vector (Promega, Madison, WI, USA), 4.5 μL of the insert DNA solution and 10 μL 2× ligation mix from the Ligation-Convenience Kit (Nippon Gene). After incubation at 16°C for 30 min, 10 μL of the solution was added to 100 μL of DH5α competent cells and incubated on ice for 30 min. The cells were heat-shocked for 1 min at 42°C, followed by storage on ice for 3 min. After sufficient cooling, 400 μL Hi-Competence Broth (Nippon Gene)

was added and the cells cultured for 1 h at 37°C. The culture solution was diluted 50 times with Hi-Competence Broth, and 100 μL of the solution was plated on Luria-Bertani (LB) agar medium that included ampicillin [50], 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropylthio-β-galactoside, allowing blue/white screening [51]. After culturing for 16 h at 37°C, white colonies were used for direct colony PCR. The 10 μL reaction solution included the following: 1.0 μL 10× Gene*Taq* Universal Buffer, 0.2 mmol/L dNTP, 0.2 μmol/L each primer pair (Universal 64-F: 5'-CACGACGTTGTAAAACGACGGCCAGTGAATT-3' and Universal 64-R: 5'-GGAAACAGCTATGACCATGATTACGCCAAGC-3'), and 0.625 units Gene*Taq* FP. Reaction conditions were 5 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 60°C, and 2 min at 72°C, followed by a final extension at 72°C for 7 min, then storage at 4°C. The PCR product obtained was separated by electrophoresis in a 3% agarose gel to determine whether the target insert sequence was present. Clones in which the insert sequence was confirmed were subcultured in 5.0 mL liquid LB and shaken for 16 h at 37°C. Cells were then collected by centrifugation, and the plasmid DNA was purified with a QIAprep Spin Miniprep Kit (QIAGEN). The DNA concentration of the plasmid was determined by measuring the UV absorbance at 260 nm with a DU-800 spectrophotometer (Beckman Coulter, Miami, FL, USA).

2.2.4 Coning into pUC19

To cut the insert from the plasmid DNA obtained from TA cloning, we used the restriction enzyme *Nar*I (Nippon Gene) to cut its recognition sequence, which had been placed at both ends of the insert. After incubating with *Nar*I for 4 h at 37°C, the digested DNA was separated by agarose electrophoresis. After confirmation of the length of the insert, the DNA was excised from the gel, purified with a MinElute Gel Extraction Kit, and dissolved in 20 μL Buffer AE.

pUC19 was preliminarily cut with *Nar*I, and purified using phenol/chloroform/isoamyl alcohol (25:24:1) (PCI)[52]. The plasmid was dephosphorylated with bacterial alkaline phosphatase (Nippon Gene) in order to inhibit self-ligation. Ligation, transformation, and plasmid purification were performed under the same conditions described for TA cloning.

2.2.5 Sequence

To confirm the DNA insertion sequence of the plasmid DNA obtained through the procedures described above, a sequencing sample was prepared with the primers used for direct colony PCR (Nar1-F for pUC19: 5'-TTGTACTGAGAGTC-CACCATATGCGGTGG-3' and Nar1-R for pUC19: 5'-CATAATAGCGAAGAGGCCCGCACC-3') according to the manufacturer's instructions for the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Freiburg, Germany). Sequencing was performed with an ABI PRISM 310 Genetic Analyzer (Life Technologies).

2.3 Preparation of positive control plasmid, pBT63

2.3.1 Preparation of pBT63

A clone in which the designed sequence was appropriately inserted was subcultured in 600 mL (300 mL × 2) liquid LB and shaken for 15 h at 37°C. Cells were then collected by centrifugation, and the plasmid DNA was purified with a QIAGEN Plasmid Mega Kit (QIAGEN). DNA concentration was determined by measuring the UV absorbance at 260 nm.

2.3.2 Linearization of pBT63

pBT63 was linearized with *Nde*I and *Pst*I (Nippon Gene), as there were no recognition sites for *Nde*I or *Pst*I in the insert DNA of pBT63. After reacting for 16 h at 37°C, linearization of pBT63 was confirmed by 1.0% agarose gel electrophoresis. PCI purification was carried out

twice. An equal amount of chloroform was then added to the collected upper layer, followed by stirring and centrifugation. A one-tenth volume of 3.0 M sodium acetate solution (pH 5.2) and an equal amount of isopropyl alcohol were added to the collected upper layer. After inverting and centrifuging, the supernatant was discarded. After washing with 70% ethanol, the precipitate was vacuum-dried and dissolved in 100 μL TE (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA).

The DNA concentration was determined, and the linearized pBT63 solution was diluted to 1.0 ng/μL with TE containing ColE1 DNA at a concentration of 5.0 ng/μL, which was then stored at -20°C.

2.4 Evaluation of pBT63

2.4.1 PCR amplification

All primer pairs shown in Table 1-2 were synthesized by Nippon Gene Material Co., Ltd. The 25 μL PCR solution consisted of 2.5 μL 10× PCR Buffer II, 0.16 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 0.6 μmol/L each primer pair, 0.8 units/μL Ampli*Taq* Gold DNA Polymerase (Life Technologies), and 5 μL pBT63.

The PCR conditions for the rice endogenous sequence SPS were 10 min at 94°C, 45 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, followed by a final extension at 72°C for 7 min, then storage at 4°C. The PCR conditions for the primer pairs AC-3F and 3R, and Oscry1Ac-F and OsNOS-R2 were as follows: 10 min at 95°C, 45 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, followed by the final extension at 72°C for 7 min, and stored at 4°C. The PCR conditions for the primer pair of actACS3F and 3R were the same as those for SPS detection, except for the number of cycles, which was 35 instead of 45[17, 46, 47].

A 4 μL aliquot of each PCR product derived from each primer pair was mixed with 1.0

μL 6× loading buffer Orange G (Nippon Gene), then electrophoresed in a 3.0% agarose X (Nippon Gene) gel including 50 μg/100 mL ethidium bromide with TAE (40 mM Tris-acetate, 1 mM EDTA) as a running buffer for 30 min at a constant voltage of 100 V. After electrophoresis, images were acquired using a UV irradiation instrument.

Table 1-2 Primer sequences for GM rice Bt63 detection and length of amplicons

Target segment	Name	Sequence (5' → 3')	Length of amplicon (bp)
sucrose phosphate synthase (SPS)	SPSF	TTG CGC CTG AAC GGA TAT	81
	SPSR	CGG TTG ATC TTT TCG GGA TG	
Cry1Ac	AC-3F	GTT CGC CTA TGG AAC CTC TT	90
	AC-3R	TTC TGT GGT GGG ATT TCG TC	
Bt63-1	Oscry1Ac-F	GCA GGA GTG ATT ATC GAC AG	147
	OsNOS-R2	AAGACCGGCAACAGGATTCA	
Bt63-2	actACS3F	TTC ATC GGT AGT TTT TCT TTT CAT	120
	actACS3R	GGC CTG CAG TTG TCC AT	

2.4.2 Restriction enzyme digestion of pBT63-derived PCR products

We obtained PCR products using pBT63 and rice vermicelli-derived DNA as templates. To 25 μL of each PCR product, 20 units of *Bam*HI was added with incubation at 37°C for 30, 60, and 120 min. Aliquots (5.0 μL) of the reaction solutions were electrophoresed in agarose gels and examined to determine whether pBT63-derived amplified products had been cut as designed.

2.4.3 Comparison of amplification efficiency

We evaluated whether there was any significant impact on amplification efficiency due to differences in template sequence by comparing the amplification curves of pBT63 and pRICE4, which also includes the rice endogenous sequence SPS and was amplified with SPSF and SPSR primers. We compared amplification curves based on real-time PCR using the intercalation method.

A 25 μL reaction solution was prepared with 12.5 μL 2× GeneAce SYBR qPCR Mix α Low ROX (Nippon Gene), 0.5 μM SPSF and SPSR primers, and 2.5 μL pBT63 or pRICE4. pRICE4 was diluted with ColE1/TE to a concentration of 50 copies/μL. Real-time PCR was performed in triplicate with the ABI PRISM 7500 Fast Sequence Detection System (Life Technologies). Reaction conditions were 10 min at 95°C and 45 cycles of 30 s at 95°C, 1 min at 60°C.

3. Results and discussion

3.1 Plasmid Construction

3.1.1 Design of positive control plasmid

In PCR testing processes, although including a positive control test is preferable, the use of a positive control could itself cause contamination. To solve this problem, we developed a system to easily assess whether or not the PCR products obtained in testing for Bt63 rice are derived from the positive control. We selected four target sequences, namely SPS, *Cry1Ac*, Bt63-1, and Bt63-2. SPS is an endogenous reference gene for rice and was used as a positive control to confirm successful DNA extraction. *Cry1Ac* was used for screening for Bt rice events. Bt63-1 and the Bt63-2 were used to identify Bt63 rice. To distinguish between PCR products derived from genomic DNA of Bt63 and those from the positive control plasmid, we inserted a restriction enzyme recognition site into the control plasmid. *Bam*HI was chosen as the most appropriate restriction enzyme because of its adequate enzymatic activity in PCR mixtures, low cost, and absence of a *Bam*HI recognition site in the original PCR target sequences. We changed the internal sequences of the PCR targets into artificial sequences including a *Bam*HI recognition sequence, keeping the primer annealing regions and the length of the amplicon unchanged. Moreover, the *Bam*HI recognition sequence was placed in the center of the amplicon to easily detect the digested PCR products using agarose gel electrophoresis. We designed the sequences of the four PCR target regions to overlap, as shown in Figure 1-1. Consequently, we designed an insert of 257 bp, which is 181 bp shorter than if the four sequences had been connected in tandem.

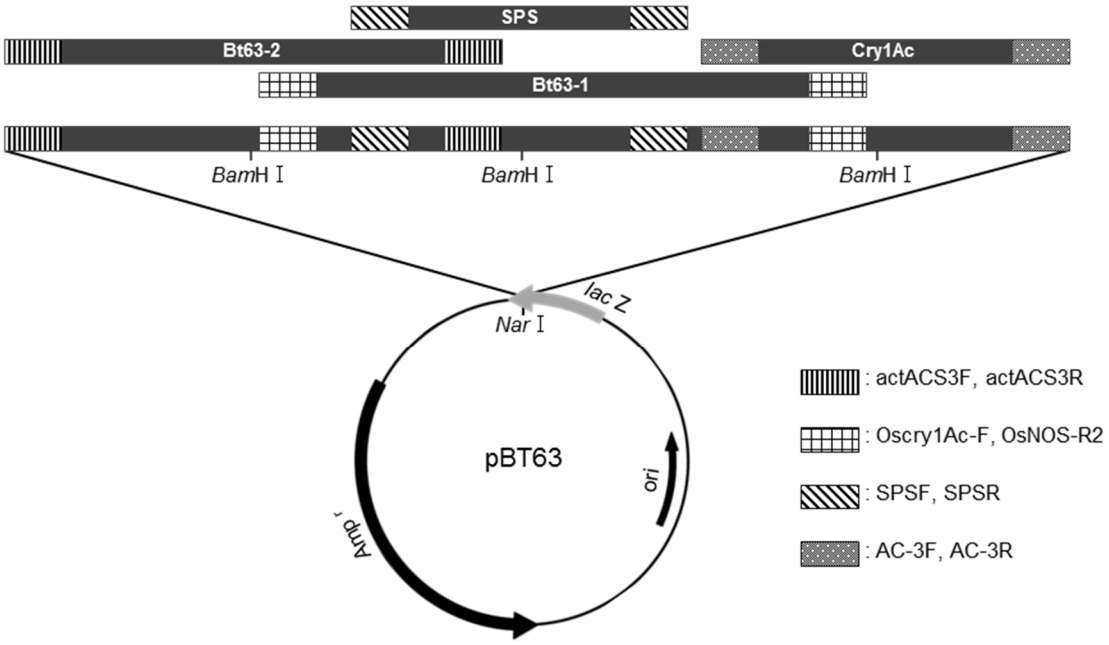

Figure 1-1 Construction of positive control plasmid pBT63
Schematic of pBT63. Except for the primers defined in the legend at the lower bottom of the figure, the primers are mock sequences that are not tandemly arranged and overlap for the purpose of shortening the sequence of the designed insert. SPSF and SPSR are primers that amplify the endogenous rice SPS gene, AC-3F and AC-3R are primers that amplify *Cry1Ac*, Oscry1Ac-F and OsNOS-R2 are primers that specifically amplify the junction region of the *cry1Ac* gene and the NOS terminator in Bt rice, and actACS3F and actACS3R are primers that specifically amplify the junction region of the 5'-intron and the *cry1Ac* gene in Bt rice.

3.1.2 Design of primers and adjustment of insert sequence

As shown in Table 1-1, the insert sequence was covered by four primers (Bt63r-1F, Bt63r-1R, Bt63r-2F, Bt63r-2R) and we designed these primers to overlap approximately 27-30 bp between pairs to allow amplification of the entire sequence by PCR. As shown in Figure 1-2, the insert sequences were prepared in two rounds of PCR. A smeared band was obtained from the first round of PCR products (Figure 1-3A). Many amplified products were detected around 111-147 bp and 242 bp, and were considered to respectively be the DNA amplified by 2 or 4 primers. In the second PCR, the insert sequence was amplified using primers designed at the two ends of

the insert (Bt63r-3F, Bt63r-3R), and the *Nar*I recognition sequence that would be used to join the insert to pUC19 was added using PCR as shown in Figure 1-2. The PCR products obtained were detected as a single band following agarose gel electrophoresis (Figure 1-3B).

To clone the designed sequence, we performed TA cloning of the insert. Multiple (20) positive clones were obtained, and these were sequenced. We chose a clone that had a sequence identical to that designed. An insert sequence with cohesive ends was obtained by digestion with *Nar*I. The insert was cloned into pUC19, and multiple clones were obtained.

The cloned plasmid DNA in pUC19 was sequenced using primers Nar1-F and Nar1-R. As shown in Figure 1-4, the plasmid DNA was entirely consistent with the designed sequence. The plasmid was named pBT63.

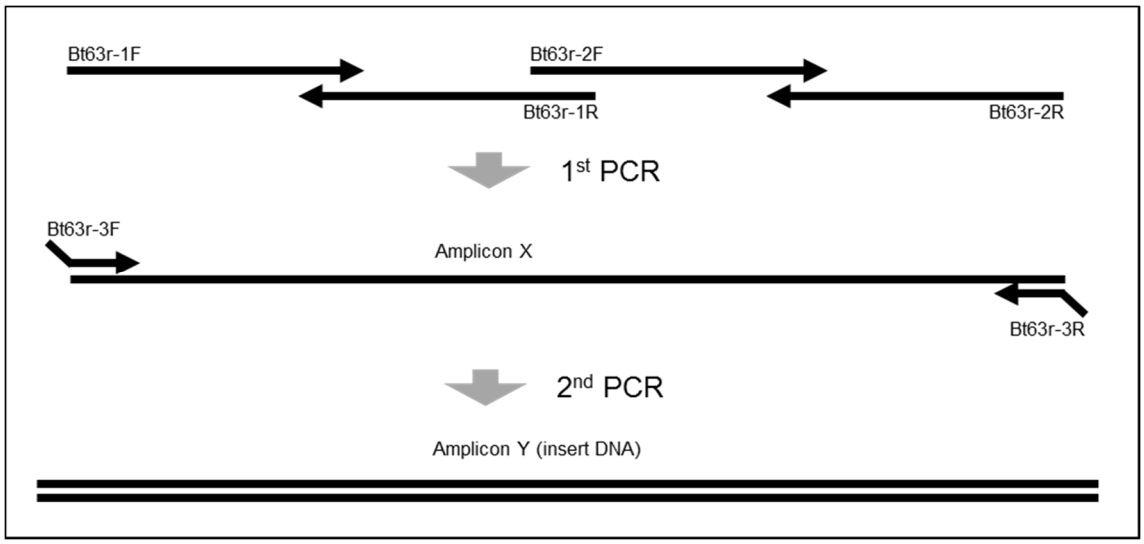

Figure 1-2 Outline of process for construction of insert DNA using PCR

1. Amplicon X was produced in the first PCR amplification using four long synthetic oligonucleotide primers (80-90 mers).
2. Amplicon Y (insert DNA) was produced in the second PCR amplification using tailed primers.

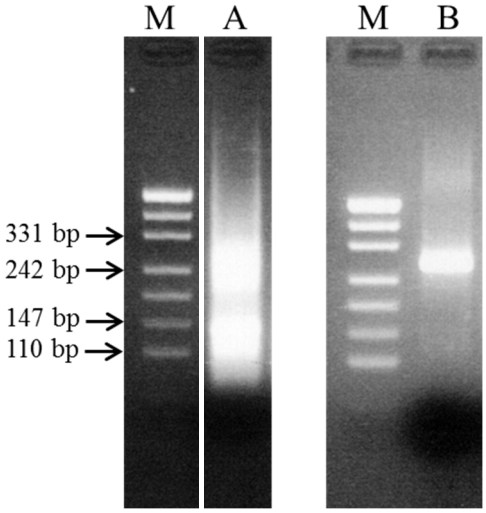

Figure 1-3 Agarose gel electrophoresis of PCR product used for preparing insert DNA of pBT63 Lane M: pUC19/*Msp*I digest; lane A: first PCR products; lane B: second PCR products.

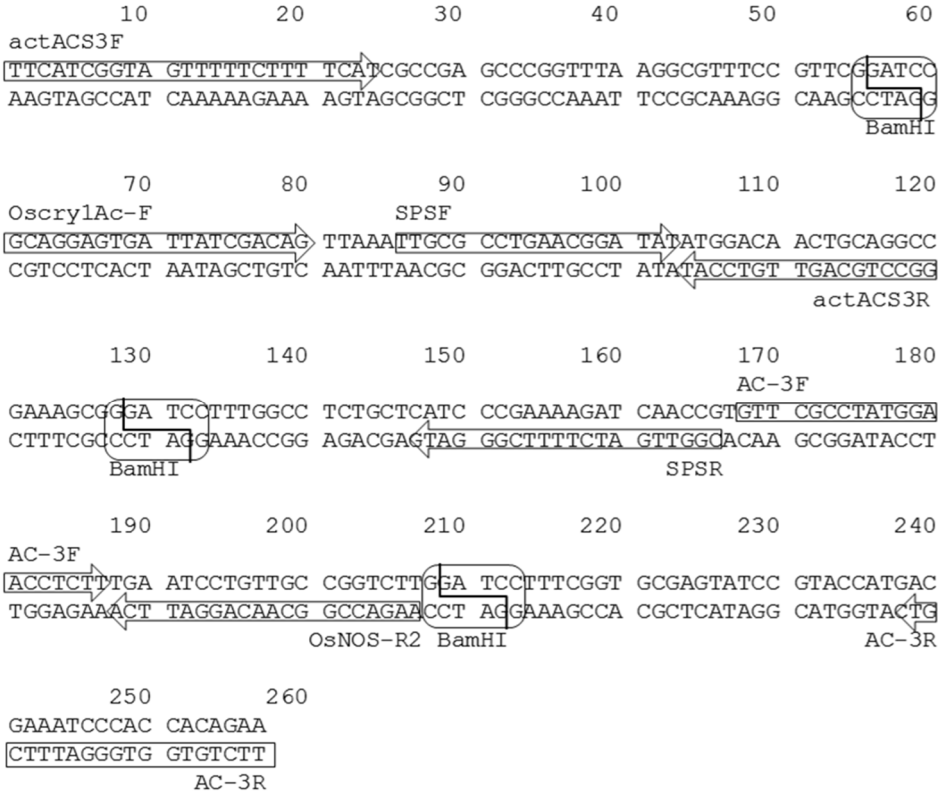

Figure 1-4 pBT63 insert sequence

Position and orientation of primers are indicated. A staggered line through the sequence marks a restriction enzyme site.

3.2 Preparation of positive control plasmid, pBT63

E. coil DH5α transformed with pBT63 was cultivated in 600 mL (300 mL × 2) of liquid LB. After the plasmid DNA was extracted, its concentration was determined using UV absorbance. The DNA yield was 2.19 mg, and the 260/230 nm and 260/280 nm ratios were 2.33 and 1.98, respectively. pBT63 (219 μg) was then digested with *Nde*I and *Pst*I for linearization. The DNA yield was 150 μg, and the 260/230 nm ratio and the 260/280 nm ratios were 2.44 and 1.96, respectively. The linearized pBT63 was diluted to 0.1, 0.01 and 0.001 pg/μL. To prevent nonspecific adsorption of the DNA to the microtube, TE including ColE1 DNA as a carrier was used for dilution. PCR was performed using 5 μL of the linearized pBT63 with each primer pair shown in Table 1-2. Analysis of the PCR products confirmed the absence of nonspecific amplification at 0.5, 0.05 and 0.005 pg (157k, 15.7k and 1.57k copies) of pBT63 with all primer pairs, and the lengths of the amplicons were identical, as designed (data not shown). These results indicated that amplification was appropriate for the evaluated range of plasmid concentrations. To obtain precise results, we thereafter used 0.01 pg/μL of linearized pBT63 in ColE1/TE solution, which was the median plasmid concentration.

3.3 Evaluation of positive control

3.3.1 PCR amplification

To evaluate the validity of the positive control plasmid pBT63, we tested all four primer pairs for detection of Bt63 rice. As shown in Table 1-2, a PCR product with the target length was amplified without producing any nonspecific PCR products, as shown in Figure 1-5A-D. Accordingly, the results showed that pBT63 functions adequately as a positive control to check for preparation of test samples and to act as a size indicator of the target amplicon using

electrophoresis.

3.3.2 *Bam*HI digestion of PCR products derived from positive control plasmid

In this study, we inserted a recognition site for *Bam*HI into nearly the center of the target amplicon sequence to detect false-positive reactions derived from contamination by positive control plasmid. We examined whether *Bam*HI digested PCR products of four types of target sequences after simply adding the restriction enzyme to the PCR buffer and incubating at 37°C, with no optimizing of the buffer for the enzyme. We judged that sufficient digestion occurred following incubation for 30 min (data not shown). Next, we checked whether genomic DNA-derived PCR products were unintentionally cut with *Bam*HI. For the rice endogenous sequence SPS, the genomic DNA of rice variety Koshihikari was used as a positive control for rice genomic DNA. The PCR product derived from genomic DNA of Koshihikari was not digested by incubation with *Bam*HI for 30 min at 37°C (data not shown). Then, we examined whether contamination of a PCR reaction buffer by pBT63 could be detected using each PCR product amplified with the four pairs of PCR primers shown in Table 1-2. As a reference for Bt63-positive results, the genomic DNA of Bt63-positive rice vermicelli was used. As shown in Figure 1-5A-D, all the PCR products derived from pBT63 were digested by the restriction enzyme, while the PCR products derived from Bt63-positive rice vermicelli remained intact after *Bam*HI digestion. The SPS fragment was digested with 43- and 38 bp-fragments, Cry1Ac with 41- and 49 bp-fragments, Bt63-1 with 68- and 79-fragments, and Bt63-2 with 55- and 65 bp-fragments. These results showed that the positive control plasmid pBT63 can confirm whether or not contamination occurred from the positive control using a simple procedure.

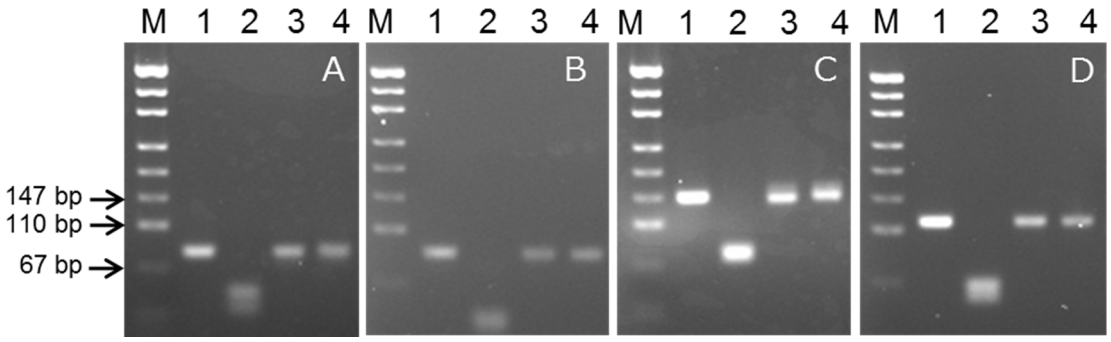

Figure 1-5 Distinguishing true positives from false positives in the detection of Bt rice by digesting PCR products using the restriction enzyme *Bam*HI.
Lanes show the products of each template DNA with the primer pairs indicated in parentheses. A: SPS (SPSF and SPSR); B: *cry1Ac* (AC-3F and AC-3R); C: Bt63-1 (Oscry1Ac-F and OsNOS-R2); D: Bt63-2 (actACS3F and actACS3R). Lane M: pUC19/*Msp*I digest; lane 1: PCR products of positive control; lane 2: PCR products of positive control digested with *Bam*HI; lane 3: PCR products of genomic DNA from Bt rice vermicelli; lane 4: PCR products of genomic DNA from Bt rice vermicelli digested with *Bam*HI.

3.3.3 Amplification efficiency

In pBT63, a 43 bp segment of the 81 bp amplified using the SPS primer pair (53.1% of the full length) is an artificial sequence. On the other hand, in pRICE4, the all sequence of the 81 bp amplified using the SPS primer pair is an endogenous rice sequence.

We compared the difference in amplification efficiency that could be attributed to the differences in base sequence of the two sequences using real-time PCR. As shown in Figure 1-6, similar amplification curves were observed for pBT63 and pRICE4. Although we drastically changed the internal sequences of the respective PCR targets, the amplification efficiency was unchanged. The results strongly suggested a possibility that other target region to be amplified can be changed. Our approach to eliminate false-positive detection would be applicable not only for Bt63 rice testing but also for other PCR-based testing.

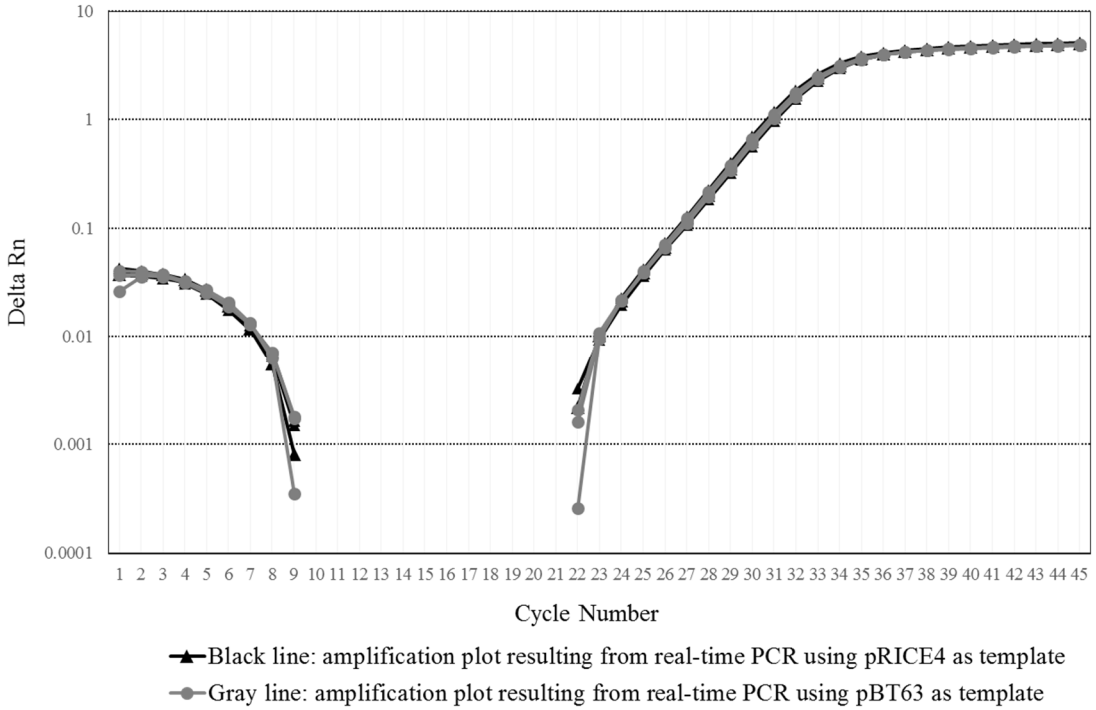

Figure 1-6 Comparison of real-time amplification of mock SPS sequence in pBT63 and real SPS sequence in pRICE4.

Three replicates of each sample were amplified.

CHAPTER 2

Development and evaluation of a novel DNA extraction method suitable for processed foods

1. Introduction

DNA analyses based on molecular biological techniques, such as polymerase chain reaction (PCR), are widely performed for food testing to detect genetically modified organisms (GMOs), allergens, pathogenic microorganisms and so on [14, 53, 54]. PCR analysis is generally comprised of four steps, i.e., sample grinding as pretreatment, DNA extraction, PCR and electrophoresis analysis. Of these steps, the DNA extraction step tends to be the most labor-intensive. An easy and fast DNA extraction method is highly desirable for efficient food testing. So far, methodologies enabling DNA extraction and purification from biological materials have been established. The Cetyltrimethylammonium bromide (CTAB)-based method [55, 56], the anion exchange resin-based method [57], and the silica membrane-based method [58] are practical, and a variety of DNA extraction kits based on these methods are commercially available.

From the viewpoint of consumer protection, it is important to test end products in food supply chain, many of which are processed foods. DNA in processed foods appears to be fragmented or degraded by physical, chemical, and/or biological factors during processing [59]. Additionally, processed foods are composed of numerous materials and/or ingredients. Hence, successful DNA extraction from processed foods is difficult. In fact, the currently existing DNA extraction methods have several drawbacks, including unstable yield, long handling time, complex operation, and/or use of toxic organic solvents such as phenol and chloroform. So, we attempted to develop a DNA extraction method for processed foods that is easier to use and faster.

Previously, we developed a silica membrane-based DNA extraction kit for raw grain materials, named GM quicker 2 kit [18]. The kit is recognized as simple and reliable, and was adopted as part of the Japanese official testing method for GMO in food [60, 61]. In this study,

we developed a DNA extraction method suitable for processed foods based on the GM quicker 2 kit. Comparative evaluation of the developed method and the currently existing DNA extraction methods was also performed.

2. Materials and methods

2.1 Samples

Three kinds of processed soybean products (soy milk, roasted soybean flour, miso) and three kinds of processed maize products (canned whole kernel sweet corn, corn snack, dried soup mix) were purchased at a local market in Toyama, Japan. As the pretreatment for DNA extraction, miso and canned whole kernel sweet corn were combined with an equal volume of distilled water and ground using a knife mill Grindomix GM200 (Retsch, Dusseldorf, Germany). Corn snack was ground using the knife mill Grindomix GM200 directly. Soy milk, roasted soybean flour and dried soup mix were used without pretreatment.

2.2 Optimization of buffer condition for DNA recovery from processed foods

GM quicker 2 kit (NIPPON GENE, Tokyo, Japan) was used as the base technology for developing the new DNA extraction method. One gram of sample was transferred to a 50 ml polypropylene centrifuge tube (Sarstadt, Nuembrecht, Germany), and 1.0 ml (for soy milk) or 4.0 ml (for roasted soybean flour) of GE1 buffer, 20 μl (for soy milk) or 40 μl (for roasted soybean flour) of proteinase K solution (20 mg/ml), 2 μl of α–amylase solution (60 units/μl) and 10 μl of RNase A solution (100 mg/ml) were added and vortexed. The mixture was incubated at 65°C for 30 min, with vortexing every 10 min during incubation. Then, 200 μl (for soy milk) or 400 μl (for roasted soybean flour) of 0.3 or 1.0 M potassium acetate (pH 3.5, 4.5 or 5.5) or 2.0 M potassium acetate (pH 3.7, 4.5 or 5.5) was added to the mixture, and mixed well by inverting. The mixture was centrifuged for 10 min at 4,000 × g using a KUBOTA 3780 (KUBOTA, Tokyo, Japan) and 800 μl of supernatant was transferred to a 2 ml microcentrifuge tube. Six hundred microliters of

8.0 M guanidine hydrochloride (condition A), or 300 μl of 8.0 M guanidine hydrochloride and 300 μl of 2-propanol (condition B) was added to the supernatant and mixed well by inverting. The mixture was centrifuged for 5 min at 10,000 × g, and first, half of the supernatant (about 700 μl) was transferred to the spin column. The spin column was centrifuged for 1 min at 10,000 × g, and the filtrate was removed. The remaining supernatant was transferred to the same spin column, and the spin column was centrifuged for 1 min at 10,000 × g. After removing the filtrate, 600 μl of GW buffer was added to the spin column, and the spin column was centrifuged for 1 min at 10,000 × g. After removing the filtrate, the spin column was re-centrifuged for 1 min at 10,000 × g and placed in a correction tube. The DNA was eluted by the addition of 50 μl of TE (10 mM Tris-HCl, pH8.0, 1 mM ethylenediaminetetraacetate (EDTA)) buffer (pH 8.0), incubation for 3 min at room temperature and centrifugation for 1 min at 10,000 × g. The DNA extraction was subjected to real-time PCR assay as described below.

2.3 Optimization of proteinase K treatment for lysing processed foods

Soy milk and roasted soybean flour samples were employed in determining the optimal amount of proteinase K for DNA extraction from processed foods. One gram of sample was transferred to a 50 ml polypropylene centrifuge tube and 1.0 ml (for soy milk) or 4.0 ml (for roasted soybean flour) of GE1 buffer, 0, 10, 20, 40, 80 μl of proteinase K solution (20 mg/ml), 2 μl of α-amylase solution (60 units/μl) and 10 μl of RNase A solution (100 mg/ml) were added and vortexed vigorously. The mixture was incubated at 65°C for 0, 15, 30, 45, 60 min with vortexing every 15 min during incubation. Then, 200 μl (for soy milk) or 400 μl (for roasted soybean flour) of 2.0 M potassium acetate (pH 3.7) was added to the mixture, and mixed well by inverting. The mixture was centrifuged for 10 min at 4,000 × g using a KUBOTA 3780, and 800

μl of supernatant was transferred to a 2 ml microcentrifuge tube. Six hundred microliters of 8.0 M guanidine hydrochloride was added to the supernatant and mixed well by inverting. The mixture was centrifuged for 5 min at 10,000 × g, and half of the supernatant (about 700 μl) was transferred to the spin column. The spin column was centrifuged for 1 min at 10,000 × g, and the filtrate was removed. The remaining supernatant was transferred to the same spin-column, and the spin column was centrifuged for 1 min at 10,000 × g. After removing the filtrate, 600 μl of GW buffer was added to the spin column, the spin column was centrifuged for 1 min at 10,000 × g. After removing the filtrate, the spin column was re-centrifuged for 1 min at 10,000 × g, and placed in a correction tube. The DNA was eluted by the addition of 50 μl of TE (pH 8.0), incubation for 3 min at room temperature and centrifugation for 1 min at 10,000 × g. The DNA extracts were subjected to real-time PCR assay as described below.

2.4 Development of a novel DNA extraction method for processed foods

2.4.1 DNA extraction from wet processed foods (soy milk, miso, canned whole kernel sweet corn)

One gram of sample (ground and mixed with water) was transferred to a 50 ml polypropylene centrifuge tube, then 1.0 ml of GE1 buffer, 20 μl of proteinase K solution (20 mg/ml), 2 μl of α-amylase solution (60 units/μl) and 10 μl of RNase A solution (100 mg/ml) were added and vortexed vigorously. The mixture was incubated at 65°C for 30 min, with vortexing every 5 min during incubation. Then, 200 μl of 2.0 M potassium acetate (pH 3.7) was added to the mixture and mixed well by inverting. The mixture was centrifuged for 10 min at 4,000 × g using a KUBOTA 3780, and 800 μl of supernatant was transferred to a 2 ml micro centrifuge tube. Six hundred microliters of 8.0 M guanidine hydrochloride was added to the supernatant and

mixed well by inverting. The mixture was centrifuged for 5 min at 10,000 × g, and half of the supernatant (about 700 μl) was transferred to the spin column. The spin column was centrifuged for 1 min at 10,000 × g, and the filtrate was removed. The remaining supernatant was transferred to the same spin-column, and centrifuged for 1 min at 10,000 × g. After removed the filtrate, 600 μl GW buffer was added to the spin column, and the spin column was centrifuged for 1 min at 10,000 × g. After removing the filtrate, the spin column was re-centrifuged for 1 min at 10,000 × g, and placed in a correction tube. The DNA was eluted by the addition of 50 μl of TE (pH 8.0), incubation for 3 min at room temperature and centrifugation for 1 min at 10,000 × g.

2.4.2 DNA extraction from dry processed foods (roasted soybean flour, corn snack, dried soup mix)

One gram of sample was transferred to a 50 ml polypropylene centrifuge tube, and 4.0 ml of GE1 buffer, 20 μl of proteinase K solution (20 mg/ml), 2 μl of α-amylase solution (60 units/μl) and 10 μl of RNase A solution (100 mg/ml) were added and vortexed. The mixture was incubated at 65°C for 30 min, with vortexing every 5 min during incubation. Then, 500 μl of 2.0 M potassium acetate (pH 3.7) was added to the mixture and mixed well by inverting. Afterwards, the same procedure as 4.1 was performed.

2.5 Conventional DNA extraction methods for processed foods

2.5.1 Silica membrane-based method

The silica membrane-based method was performed with the DNeasy® Plant Maxi kit (QIAGEN, Hilden, Germany) as described in the Japanese Agricultural Standard (JAS) analytical test handbook. According to the handbook, protocols A and B were applied to processed food made from soybean and maize, respectively [5].

2.5.2 CTAB-based method

The CTAB-based method was performed as described in the JAS analytical test handbook [5].

2.5.3 CTAB/Silica membrane-based method

The CTAB/Silica membrane-based method was performed with the DNeasy® *mericon*™ Food kit (QIAGEN) as described in the manufacturer's protocol for small DNA fragments.

2.5.4 Anion exchange resin-based method

The anion exchange resin-based method was performed with the Genomic-tip 20/G kit (QIAGEN) as described in the JAS analytical test handbook [5].

2.6 Estimation of amount and quality of extracted DNA

The concentration of DNA solutions was determined by both UV absorption and fluorescence detection of DNA intercalation. The UV absorbance at 260 nm was measured using a DU-800 spectrophotometer (Beckman Coulter, FL, USA). Fluorescence intercalator detection was carried out using the PicoGreen ® dsDNA Quantitation Kit (Molecular Probes, OR, USA) according to the manufacturer's protocol. The fluorescence of DNA sample was measured at 520 nm after excitation at 480 nm using a Infinite™ F200 (Tecan, Mannedorf, Switzerland).

2.7 Agarose gel electrophoresis of extracted DNA

Agarose gel electrophoresis was carried out with 1% (w/v) Agarose S gel (NIPPON GENE) in Tris-acetate-ethylenediaminetetraacetate (TAE) buffer with 0.5 μg/ml of ethidium bromide. Ten microliters of solution including 250 ng of DNA was mixed with 2 μl of 6 × loading buffer, and the samples were subjected to electrophoresis at a constant voltage (100 V) for approximately

30 min in TAE buffer. After the electrophoresis, the gel was photographed under UV radiation using a densitograph system (ATTO, Tokyo, Japan).

2.8 Real-time PCR assay of extracted DNA

For processed soy foods, the copy number of the taxon-specific gene encoding the soy lectin1 (*Le1*) was analyzed by real-time PCR. For processed maize foods, the taxon-specific sequence encoding the maize starch synthase IIb gene (*SSIIb*) was analyzed. The PCR amplification was carried out in 25 μl total reaction volume containing 2.5 μl of a DNA extract, 12.5 μl of TaqMan® Universal PCR Master Mix (Life Technologies, CA, USA), 0.5 μM of the primer pair and 0.2 μM of double dye-labeled probe. PCR amplifications were performed using the following program: preincubation at 50°C for 2 min and 95°C for 10 min, 45 cycles of denaturation at 95°C for 30 sec, and annealing and extension at 59°C for 1 min. The GM maize detection SSIIb03 (for endogenous gene) oligonucleotide set and the GM soybean detection Le1 (for endogenous gene) oligonucleotide set (NIPPON GENE) were used as primers and probes. The oligonucleotide probes were labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethyl-rhodamine (TAMRA) at the 5' and 3' ends, respectively. The ABI PRISM™ 7500 Fast Sequence Detection System (Life Technologies) was used as the real-time PCR instrument.

3. Results and discussion

3.1 Development of new DNA extraction method for processed foods

3.1.1 Optimization of buffer condition for DNA recovery from processed foods

To mix a sample sufficiently and recover supernatant, adequate volumes of GE1 buffer were different for dry and wet processed foods. Therefore, processed foods were fell into two categories, dry and wet materials, based on its water content. We chose roasted soybean flour and soy milk as representative samples of dry and wet materials, respectively, in order to determine the optimal condition for efficient DNA extraction and purification from both kinds of processed food. First, several different buffer conditions were compared concerning binding efficiency of DNA to the silica membrane (Figure 2-1). Copy numbers of *Le1* measured using real-time PCR were used as an indicator of DNA yield. For extraction of proteins and saccharides, potassium acetate buffer of various concentrations and pH were prepared. To boost DNA binding to the silica membrane, guanidine hydrochloride was used as a chaotropic agent. For condition A, 8.0 M of guanidine hydrochloride was used in accordance with the original report of the silica membrane-based method by Boom R. et al [58]. For condition B, a mixture of equal volumes of 8.0 M of guanidine hydrochloride and 2-propanol was used, the same as for the GM quicker 2 kit. The results indicated that extracted DNA bound to the silica membrane most efficiently under condition A with using 2.0 M potassium acetate buffer (pH 3.7). Thus 2.0 M potassium acetate (pH 3.7) and guanidine hydrochloride (condition A) were selected as buffers for the developed method. Adoption of condition A, which does not require 2-propanol, is expected to simplify the DNA extraction process.

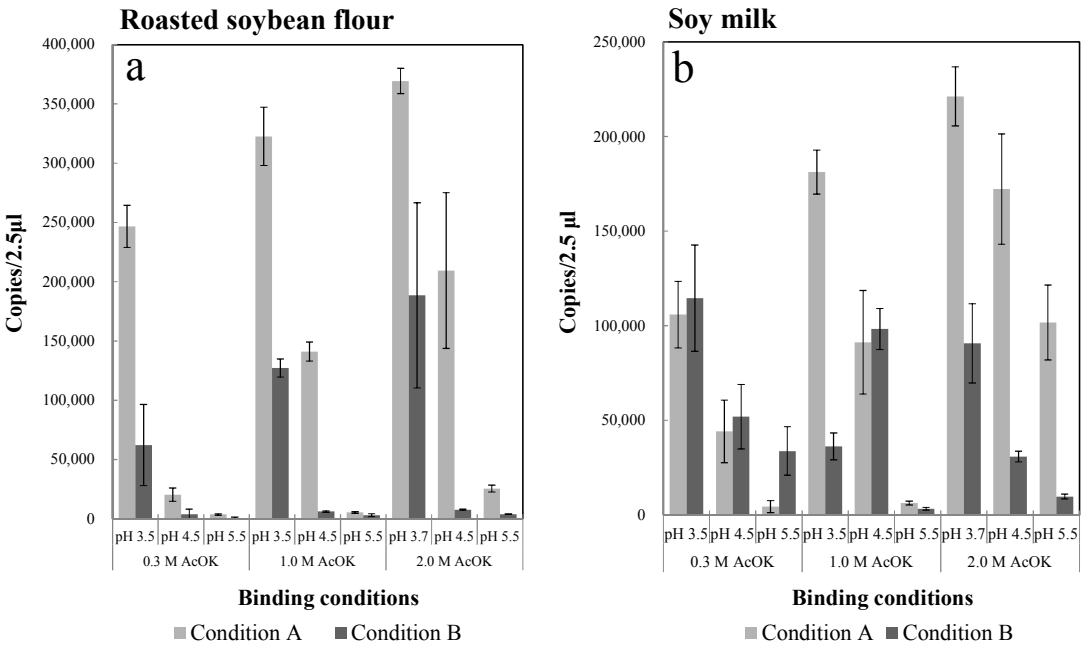

Figure 2-1 Real-time PCR analysis of DNA extracts obtained under the various buffer conditions. A, roasted soybean; B, soy milk. Error bars indicate the standard deviation for three replicates. AcOK means potassium acetate.

3.1.2 Optimization of proteinase K-treatment condition

Optimal volume of proteinase K and incubation time were examined (Figure 2-2). Figure 2-2 shows that DNA can be extracted efficiently from roasted soybean flour using more than 10 μl of proteinase K for a longer than 30 min-digestion, and from soy milk using more than 10 μl of proteinase K for a longer than 15 min-digestion. In Figure 2-2, the copies numbers at 0 min were increased depending on the amount of proteinase K. We speculated that the proteinase K reacted during a short time until the reaction stop by the acidic potassium acetate buffer. To make the developed method applicable for a variety of foods that differ in protein content, a 20 μl volume of proteinase K and 30 min digestion time were selected.

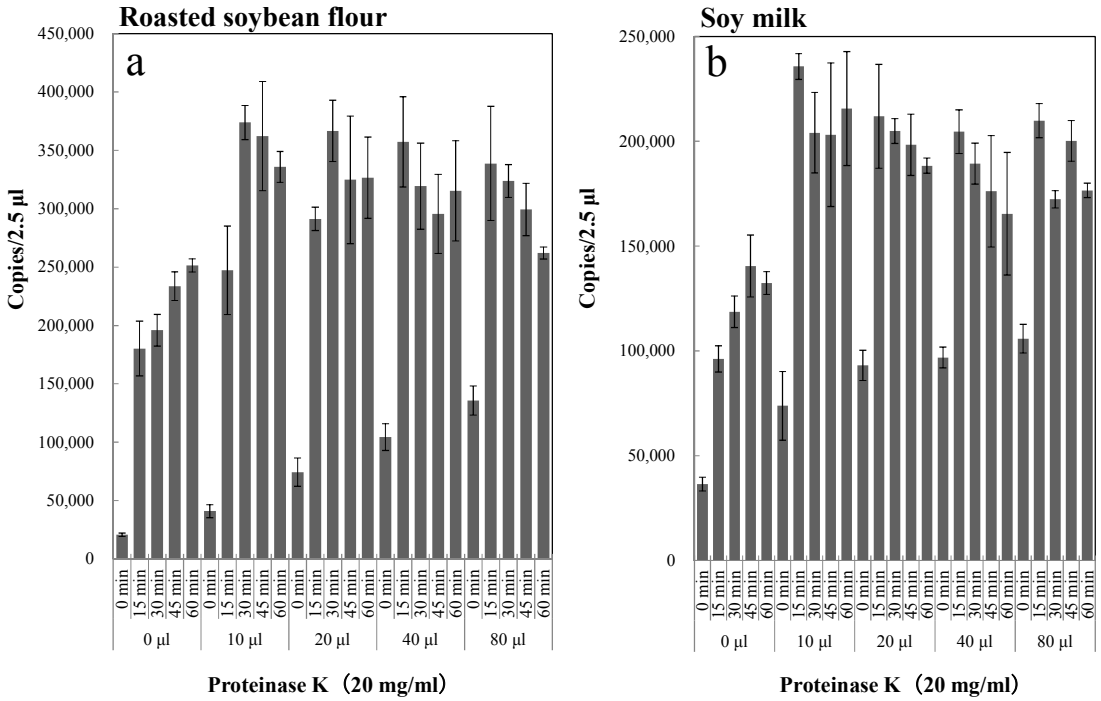

Figure 2-2 Real-time PCR analysis of DNA extracts obtained under the various proteinase K digestion conditions. A, roasted soybean; B, soy milk. Error bars indicate the standard deviation for three replicates.

3.2 Evaluation of extracted DNA from processed foods by agarose-gel electrophoresis

Extracted DNA from roasted soybean flour and soy milk generated by the developed method were analyzed by agarose gel electrophoresis (Figure 2-3). For DNA extracted from soy milk, smears of DNA fragments were observed in the range between 0.42 kb and 7.74 kb. On the other hand, only DNA fragments shorter than 0.42 kb were obtained from DNA extracted from roasted soybean flour. These results show that various length of DNA fragments, from short to long, can be recovered by the developed method.

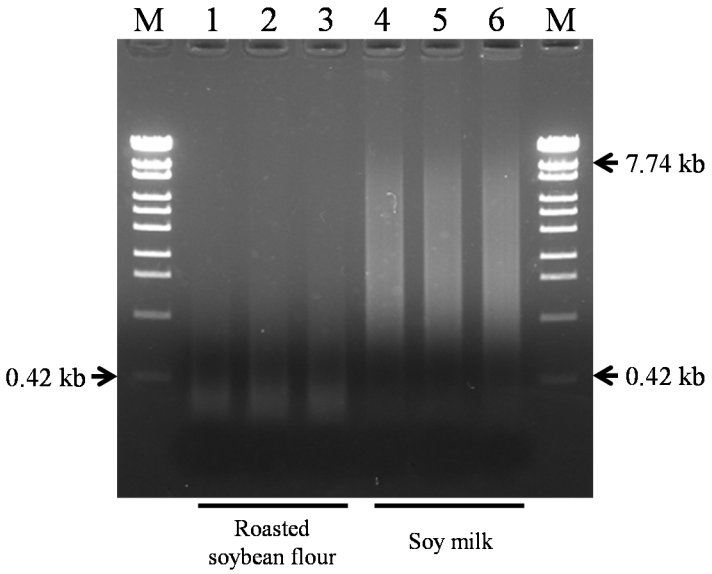

Figure 2-3 Analysis of DNA fragmentation using ethidium bromide-stained agarose gel electrophoresis. Lane M, λ/*Sty* I digest (OneSTEP Marker 6, NIPPON GENE); Lane 1 to 3, DNA extracts from roasted soybean flour; Lanes 4 to 6, DNA extracts from soy milk.

3.3 Evaluation of extracted DNA from processed foods by absorbance measurements and PicoGreen® assay

UV absorbance at 260 nm is the most commonly used for DNA quantitation method; however, it is possible that the proteins or saccharides remaining in the DNA extracts may result in inaccurate readings. Meanwhile, although the PicoGreen® assay takes longer operation time and higher cost, it can detect double stranded DNA with high specificity. We measured DNA concentration using these two different methodologies. The DNA samples were extracted from processed foods (soy milk, roasted soybean flour, miso, canned whole kernel sweet corn, corn snack and dried soup mix) using the newly developed method and four other methods (Silica membrane-based, CTAB-based, CTAB/Silica membrane-based and anion exchange resin-based method). Then, the amounts of DNA were analyzed in triplicate (Table 2-1). In the official GMO testing methods[12], the use of DNA diluted to 10 ng/μl is recommended. The developed method

and anion exchange resin-based method gave us above 10 ng/μl of DNA, while the other 3 methods showed insufficient DNA yield from several samples. Meanwhile, the anion exchange resin-based method showed large differences in the results of roasted soybean flour and dried soup mix, suggesting that impurities remained in the DNA extracts. These results support that the developed method is a better choice in terms of stable DNA yield and purity.

Table 2-1 Comparison of DNA yields by five DNA extraction methods.

Sample name	Extraction method	Absorbance measurements DNA Conc. (ng/μl)		PicoGreen® assay DNA Conc. (ng/μl)	
		Means	S.D.(i)	Means	S.D.(i)
Roasted soybean flour	New silica membrane-based method	105.62	7.05	119.86	4.98
	Silica membrane-based method	119.15	20.59	88.04	6.11
	CTAB-based method	240.08	56.00	46.01	9.54
	CTAB/Silica membrane-based method	42.30	1.86	25.39	0.84
	Anion exchange resin-based method	2218.50	43.49	535.92	11.68
Soy milk	New silica membrane-based method	80.18	6.46	49.90	4.86
	Silica membrane-based method	8.57	6.74	3.97	3.80
	CTAB-based method	18.50	1.37	6.79	0.42
	CTAB/Silica membrane-based method	35.88	1.10	14.04	1.19
	Anion exchange resin-based method	272.90	15.77	160.91	6.28
Miso	New silica membrane-based method	44.77	1.24	92.36	4.42
	Silica membrane-based method	7.62	2.14	9.17	3.32
	CTAB-based method	7.38	0.51	15.93	1.13
	CTAB/Silica membrane-based method	5.53	0.53	5.76	0.55
	Anion exchange resin-based method	171.28	7.67	337.07	1.50
Canned whole kernel sweet corn	New silica membrane-based method	71.42	7.46	89.19	13.32
	Silica membrane-based method	636.70	198.36	20.12	0.40
	CTAB-based method	127.23	13.72	17.17	2.01
	CTAB/Silica membrane-based method	6.02	0.53	4.65	0.70
	Anion exchange resin-based method	604.83	21.88	401.44	10.61
Corn snack	New silica membrane-based method	50.25	2.81	19.88	3.50
	Silica membrane-based method	121.07	13.70	14.31	1.36
	CTAB-based method	22.23	5.06	6.73	0.88
	CTAB/Silica membrane-based method	6.43	0.32	1.71	0.04
	Anion exchange resin-based method	238.68	20.21	113.37	8.99
Dried soup mix	New silica membrane-based method	224.80	29.20	165.36	6.76
	Silica membrane-based method	173.97	47.15	133.09	36.55
	CTAB-based method	64.10	4.45	38.17	4.31
	CTAB/Silica membrane-based method	27.08	3.35	18.02	1.86
	Anion exchange resin-based method	1661.33	119.05	405.99	6.86

(i) S.D. means standard deviation. n = 3.

3.4 Evaluation of extracted DNA from processed foods by Real-time PCR analysis

DNA extracted from processed soy and maize foods were analyzed by real-time PCR without adjustment of DNA concentration (Tables 2-2 and 2-3). Although the anion exchange resin-based method showed high DNA concentration (Table 2-1), the DNA sample from roasted soybean flour did not show DNA amplification in the real-time PCR analysis. We speculated that the failed DNA amplification was attributed to PCR inhibition. To evaluated PCR inhibition in real-time PCR analyses, therefore, the DNA samples which were diluted 10-fold with TE buffer were also analyzed by real-time PCR and then, the obtained copy numbers were multiplied by 10 for comparison with the measurement results of the undiluted samples (Table 2-2 and 2-3). In case of the DNA samples from canned whole kernel sweet corn and dried soup mix, the calculated copy numbers from diluted samples were more than 100-fold higher, suggesting that undiluted samples caused PCR inhibition. Meanwhile, all the DNA extracts obtained by the developed method were not subject to PCR inhibition even in the undiluted state.

To compare the DNA extraction methods in terms of DNA extraction efficiency, total DNA copies obtained from 1 gram of the respective initial samples were calculated (Tables 2-2 and 2-3). Regarding the DNA samples which showed PCR inhibition i.e., DNA samples from roasted soybean flour, canned whole kernel sweet corn and dried soup mix by the anion exchange resin-based method, the copy numbers obtained from diluted samples were adopted for the calculation. The measured copy number in 2.5 μl of DNA sample was multiplied by the elution volume and divided by the weight of the initial sample. The calculated total copy numbers by the developed method was the highest for miso, the second highest for the canned whole kernel sweet corn and corn snack and the third highest for the other three processed food samples. We concluded that

the developed method was comparatively good in terms of the DNA yield from a certain amount of starting material.

3.5 Comparison of DNA extraction methods in terms of handling

To compare the usability of the DNA extraction methods, handling time, the number of pipette operations and use of toxic organic solvent were assessed and are summarized in Table 2-4. This survey revealed that the newly developed silica membrane-based method permitted the shortest handling time and the smallest number of pipette operations. Additionally, the method did not require toxic organic solvents. It is clear that our new silica membrane-based method is superior in terms of user-friendliness.

Table 2-2 Real-time PCR analysis of DNA extracts from processed soy foods.

Sample name	Extraction method	Le1 copy number/2.5 μl		Sample weight (g)	Elution volume (μl)	Total $\times 10^3$ copies /1 g sample
		Means	S.D.[(i)]			
Roasted soybean flour	New silica membrane-based method	49325.63	1951.63	1.0	50	986.51
		50006.84 [(ii)]	3323.63 [(ii)]			
	Silica membrane-based method	11821.04	1951.04	1.0	100	472.84
		21518.22 [(ii)]	2967.09 [(ii)]			
	CTAB-based method	9109.98	2042.37	0.2	100	1822.00
		8082.47 [(ii)]	1921.79 [(ii)]			
	CTAB/Silica membrane-based method	6674.50	577.10	2.0	100	133.49
		3707.23 [(ii)]	170.70 [(ii)]			
	Anion exchange resin-based method	N.D. [(iii)]		2.0	50	3011.89 [(ii)]
		301189.00 [(ii)]	18924.38 [(ii)]			
Soy milk	New silica membrane-based method	135828.44	16608.37	1.0	50	2716.57
		138207.22 [(ii)]	4649.92 [(ii)]			
	Silica membrane-based method	13472.48	10837.53	1.0	100	538.90
		8586.11 [(ii)]	10052.22 [(ii)]			
	CTAB-based method	15667.50	1156.56	0.2	100	3133.50
		10901.33 [(ii)]	813.29 [(ii)]			
	CTAB/Silica membrane-based method	10106.03	846.67	2.0	100	202.12
		4618.56 [(ii)]	332.49 [(ii)]			
	Anion exchange resin-based method	418532.00	21997.07	2.0	50	4185.32
		347826.67 [(ii)]	54309.70 [(ii)]			
Miso	New silica membrane-based method	2792.68	230.39	1.0	50	55.85
		2432.71 [(ii)]	109.09 [(ii)]			
	Silica membrane-based method	228.12	71.28	1.0	100	9.12
		202.85 [(ii)]	117.24 [(ii)]			
	CTAB-based method	262.10	16.58	0.2	100	52.42
		172.20 [(ii)]	42.04 [(ii)]			
	CTAB/Silica membrane-based method	149.88	3.15	2.0	100	3.00
		79.30 [(ii)]	22.21 [(ii)]			
	Anion exchange resin-based method	5361.06	907.34	2.0	50	53.61
		5217.98 [(ii)]	238.50 [(ii)]			

(i) S.D. means standard deviation. (ii) DNA samples were diluted 10-fold with TE buffer, and the obtained quantitative value was multiplied by 10.
(iii) N.D. means not detected.

Table 2-3 Real-time PCR analysis of DNA extracts from processed maize foods.

Sample name	Extraction method	SSIIb copy number/2.5 μl		Sample weight (g)	Elution volume (μl)	Total $\times 10^3$ copies /1 g sample
		Means	S.D.[(i)]			
Canned whole kernel sweet corn	New silica membrane-based method	8526.71 5108.83 [(ii)]	508.66 992.13 [(ii)]	1.0	50	170.53
	Silica membrane-based method	1536.81 1257.50 [(ii)]	118.15 87.62 [(ii)]	1.0	100	61.47
	CTAB-based method	628.06 519.58 [(ii)]	15.31 29.78 [(ii)]	0.2	100	125.61
	CTAB/Silica membrane-based method	318.56 117.32 [(ii)]	72.60 58.00 [(ii)]	2.0	100	6.37
	Anion exchange resin-based method	4.17 19887.52 [(ii)]	4.53 1642.11 [(ii)]	2.0	50	198.88 [(ii)]
Corn snack	New silica membrane-based method	749.86 603.87 [(ii)]	82.81 82.00 [(ii)]	1.0	50	15.00
	Silica membrane-based method	209.30 200.51 [(ii)]	102.54 64.59 [(ii)]	1.0	100	8.37
	CTAB-based method	69.95 89.82 [(ii)]	9.47 18.95 [(ii)]	0.2	100	13.99
	CTAB/Silica membrane-based method	71.32 49.21 [(ii)]	10.87 13.37 [(ii)]	2.0	100	1.43
	Anion exchange resin-based method	2046.13 1786.51 [(ii)]	122.63 259.51 [(ii)]	2.0	50	20.46
Dried soup mix	New silica membrane-based method	205311.00 194405.44 [(ii)]	12155.67 24353.90 [(ii)]	1.0	50	4106.22
	Silica membrane-based method	129172.68 119840.50 [(ii)]	30538.63 34993.75 [(ii)]	1.0	100	5166.91
	CTAB-based method	16415.04 13302.74 [(ii)]	1886.49 721.43 [(ii)]	0.2	100	3283.01
	CTAB/Silica membrane-based method	21875.12 11439.08 [(ii)]	2858.30 1149.47 [(ii)]	2.0	100	437.50
	Anion exchange resin-based method	3232.16 1121762.22 [(ii)]	1493.46 45616.94 [(ii)]	2.0	50	11217.62 [(ii)]

(i) S.D. means standard deviation. (ii) DNA samples were diluted 10-fold with TE buffer, and the obtained quantitative value was multiplied by 10.

Table 2-4 Summary of operation procedures of the DNA extraction methods.

DNA extraction method		Handling time[(i)]	The number of pipette operations[(iii)]	Toxic organic solvent[(iv)]
Method name	Commercially available kit			
New silica membrane-based method	GM quicker 2 kit	60 min	11	-
Silica membrane-based method (protocol A, for soybean)	DNeasy® Plant Maxi kit	190 min (+12-24 hours)[(ii)]	26	-
Silica membrane-based method (protocol B, for maize)	DNeasy® Plant Maxi kit	160 min (+12-24 hours)[(ii)]	19	-
CTAB-based method	-	130 min	19	Phenol and chloroform
CTAB/Silica membrane-based method	DNeasy® *mericon*™ Food kit	70 min	11	Chloroform
Anion exchange resin-based method	Genomic-tip 20/G	130 min (+12-24 hours)[(ii)]	40	-

(i) Approximate time needed for DNA extraction from one sample. (ii) DNA precipitant was suspended for 12-24 hours. (iii) When using micropipettes up to 5 ml. (iv) Toxic organic solvents as designated by the Poisonous and Deleterious Substances Control Act of Japan.

CHAPTER 3

A novel detection system for the genetically modified canola (*Brassica rapa*) line RT73

1. Introduction

In recent years, great advances have been made in food biotechnology including transgenic crop breeding and genetic modification for food production. In some countries, however, the acceptance of genetically modified (GM) food by consumers remains controversial, and concerns about safety persist among the public. GM products have been approved for use in animal feed and human food in some countries, based on individual criteria for safety assessment. In the European Union (EU), the approval and use of GM food and feed are stipulated by the provisions of European Community (EC) regulations 1829/2003 and 1830/2003 [62, 63]. Japan has also announced a mandatory safety assessment of GM foods and processed foods containing GM ingredients. Since April 1, 2001, any GM food that has not been approved is prohibited from import or sale in Japan. Methods for the qualitative detection of regulated and unapproved GM foods are therefore required. We previously reported techniques for the qualitative detection of GM maize, potatoes, papayas, rice, and flax, which included polymerase chain reaction (PCR) methods and a histochemical assay [17, 19, 20, 64-73].

Canola is the source of 13% of the world's oilseed, and is second only to soybean in terms of its contribution to global oilseed production [74]. GM canola was reported to be one of the four principal GM crops worldwide, and to occupy 6.4 million hectares representing 5% of the global crop area in 2009 [75]. Other members of the *Brassica* genus include a wide variety of commercially cultivated species, including broccoli, cauliflower, cabbages, kale, Brussels sprouts, wong-bok, turnips, kohlrabi, swedes, rutabagas, and mustards.

The GM Roundup Ready canola (*B. napus*) line RT73 (also known as GT73), which is tolerant to the herbicide glyphosate, was developed by the Monsanto Company (St. Louis, MO).

To produce RT73 *B. napus*, one exogenous recombinant DNA molecule that contained two exogenous herbicide-tolerance gene cassettes, the CP4 5-enolpyruvylshikimate-3-phosphate synthase gene (*CP4-EPSPS*) and the glyphosate oxidoreductase gene from *Achromobacter* sp. strain LBAA (*GOX v247*) was transformed into the rapeseed genome [76]. These genes confer novel tolerance to glyphosate, which can control or suppress economically important weeds in canola production. RT73 *B. napus* has been approved for environmental release, and for use in feed and food worldwide. However, seed companies have developed three *B. rapa* canola (RT73 *B. rapa*) lines (ZSR500, ZSR502, and ZSR503) derived from interspecific crosses with RT73 *B. napus*, which have not been approved in Japan as the effects of crossing species are unclear (http://www.inspection.gc.ca/english/plaveg/bio/dd/dd9821e.shtml).

On December 21, 2005, the Japanese government reported the possibility of importing unapproved Canadian GM canola into Japan. The RT73 *B. rapa* lines have been approved in Canada, and were cultivated there in 2004 and 2005. According to online information, this GM canola accounted for 0.009% of the total planted acreage, and it was deleted from the seed registry in 2003 (http://www5d.biglobe.ne.jp/ ~ cbic/english/2006/journal0602.html). Although GM canola is unlikely to be cultivated in the future, the possibility of contamination by pollen and seed spillage remains. The potential for unapproved GM canola to be exported into Japan is therefore likely to exist for some time.

To detect and identify RT73 *B. rapa* contamination in imported canola samples, it is necessary both to distinguish *B. rapa* and *B. napus* and, simultaneously, to detect an RT73-specific sequence in individual kernels. The present study developed a novel system for detecting contamination by RT73 *B. rapa* in canola grain samples by single-kernel analysis, which comprised DNA extraction from individual canola kernels, the discrimination of *B. napus* and *B.*

rapa, and the identification of an RT73-specific sequence.

2. Materials and methods

2.1 Plant Materials

The GM canola line RT73 (RT73 *B. napus*) and non-GM *B. napus* (Eagle) were kindly supplied by the Monsanto Company, St. Louis, MO. The following non-GM *Brassica* lines were purchased from the National Institute of Agrobiological Sciences Genebank (Tsukuba, Ibaraki, Japan): the Wase syokuyou aburana, Maruba natane, Gokuwase natanena, Wakana, Niida aona, Nozawana, Shin kuro mizuna, Shinobu fuyuna, and Narusawana lines of *B. rapa*; the Westar, Nugget, and N-404 lines of *B. napus*; the Awasa selection line of *B. carinata*; the Kaichoy line of *B. juncea*; the Debre zeit local line of *B. nigra*; and the Badger ball head year line of *B. oleracea*. The *B. rapa* lines C147, C148, C149, C150, and C155, which were originally grown in Canada, were kindly supplied by the Tohoku University *Brassica* Seed Bank (Sendai, Miyagi, Japan).

2.2 Grinding of Individual Canola Kernels.

Canola kernels were ground using a Multibeads shocker (MB601NIHS) with a 96 deepwell plate (PTD-96), a cap mat for a 96 deep-well plate (CPD-96), and disruption medium (MC-96415R; all from Yasui Kikai Co., Osaka, Japan) at 1500 rpm for 20 s [77, 78]. To remove contamination by powder or broken seeds, the kernels were washed with 5% (w/v) sodium dodecyl sulfate (SDS), rinsed with distilled water (DW), and dried at 65 °C for 1 h in the incubation box before being ground.

2.3 Oligonucleotide Primers and Probes for Duplex Real-Time PCR.

To analyze the acetyl-CoA carboxylase (*ACCg8*) genes of *B. rapa* and *B. napus*, we

designed and generated the primers *Brassica ACCg8F*, GGCGACACGGTTACCAAAAGAT, and *Brassica ACCg8R*, TAGGCCCTGAAAAAGAAGAATAGA, and to analyze the cruciferin genes of *B. rapa* and *B. napus*, we designed and generated the primers *BC1F*, GGCTCGGCTCTCATCTCTTCTCT and *BC1R*, CGCATCCAGGGACCACTCTC.

A *B. rapa ACCg8* gene (*BrACCg8*) detection set (*B. rapa-ACCg8F* and *B. rapa-ACCg8R* with a *B. rapa-ACCg8* probe) and a *B. napus* cruciferin gene (*BnC1*) detection set (*B. napus BnC1-969F* and *B. napus BnC1-1043R* with a *B. napus BnC1-994T* probe) were used as primers with 3′-minor groove binder (MGB) probes to detect *BrACCg8* and *BnC1* sequences, respectively, in duplex real-time PCR to discriminate *B. rapa* and B. *napus*. The *B. rapa-ACCg8* probe was labeled with the reporter fluorescent dye 6-carboxyfluorescein (FAM) at the 5′ end and an MGB molecule at the 3′ end. The *B. napus BnC1-994T* probe was labeled with the fluorescent dye VIC and an MGB molecule at the 5′ and 3′ ends, respectively. By contrast, an RT73 detection set (RT73 primer 1 and RT73 primer 2 with an RT73 probe) and a *FatA* detection set (*FatA* primer 1 and *FatA* primer 2 with a *FatA* probe) were used in duplex real-time PCR to identify the RT73-specific sequence [79]. The *FatA* probe was labeled with VIC and the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 5′ and 3′ ends, respectively. The RT73 probe was labeled with FAM and TAMRA at the 5′ and 3′ ends, respectively. All primer pairs were purchased from FASMAC Co., Ltd. (Kanagawa, Japan). The *B. rapa-ACCg8* probe, *B. napus BnC1-994T* probe, *FatA* probe, and RT73 probe were synthesized by Life Technologies (St. Louis, MO).

2.4 DNA Extraction from Canola Seed Samples and Individual Kernels.

The canola and RT73 canola seed samples were ground separately in an ultracentrifugal mill (ZM100; Retsch GmbH, Haan, Germany) using a 0.5 mm sieve ring. DNA extraction and

purification were carried out using the GM quicker 2 kit (Nippon Gene, Toyama, Japan) according to the manufacturer's manual.

Individual kernel samples were ground using a Multibeads shocker. DNA extraction and purification were carried out using the GM quicker 96 kit (Nippon Gene) using the method developed herein; genomic DNA extraction from the ground powder of individual kernels was performed according to a modification of this procedure. GE1 buffer, Proteinase K solution (20 mg/mL), and RNase A solution (100 mg/mL) were mixed to make a working solution at respective volumes of 500 μL, 20 μL, and 10 μL.

A 530 μL sample of the working solution was added to each well containing the ground canola powder. The plate was then vigorously shaken for 15 s, capped, and incubated for 15 min at 65 °C. An 85 μL aliquot of GE2-K buffer solution was then added to each solution, the plate was sealed to avoid leakage, and it was vigorously shaken for 15 s. The plate was centrifuged for 5 min at 1400*g* using a Metalfuge (MBG100; Yasui Kikai Co.). A 400 μL aliquot of each supernatant was carefully transferred to a filter plate, which was centrifuged for 5 min at 1400*g*. A 300 μL aliquot of GB3 buffer/isopropanol was added to each filtrate. A 700 μL aliquot of each sample was then carefully transferred to the 96-well column plate, which was centrifuged for 5 min at 1400*g*. After removing the filtrate, 650 μL GW buffer was added to each sample. The 96-well column plate was centrifuged for 5 min at 1400*g*. After removing the filtrate, the 96-well column plate was recentrifuged for 20 min at 1036*g*, placed in a correction plate, and 50 μL DW was added to each sample. The plate was incubated for 3 min at room temperature, and then centrifuged for 5 min at 1400*g*. For the DNA-extraction method from individual canola kernels, we used a glass-fiber silica-plate base sheet (EPM 2000; GE Healthcare, Windsor, CT) and a polystyrene-column plate (Unifilter; GE Healthcare).

2.5 Duplex Real-Time PCR Conditions.

To simultaneously detect the two DNA sequences from individual canola kernels to discriminate *B. rapa* and B. *napus* and to identify the RT73-specific sequence, two duplex real-time PCR analyses were performed, using a modified version of a method reported previously [77, 78]. The amplification curves of the target sequence were monitored using the corresponding fluorescent dye, which was used to label the designed oligonucleotide probes with the ABI PRISM 7900HT sequence detection system (Life Technologies). The 25 μL duplex real-time PCR reaction volume to discriminate *B. rapa* and B. *napus* contained 2.5 μL of sample genomic DNA solution (average DNA amount, 55.5 ng), 12.5 μL Universal Master Mix (Life Technologies), 0.25 μL *B. rapa-ACCg8F* (50 μM), 0.25 μL *B. rapa-ACCg8R* (50 μM), 0.5 μL *B. rapa-ACCg8* probe (10 μM), 0.25 μL *B. napus BnC1-969F* (50 μM), 0.25 μL *B. napus BnC1-1043R* (50 μM), and 0.5 μL *B. napus BnC1-994T* probe (10 μM). The 25 μL duplex real-time PCR reaction volume to identify the RT73-specific sequence contained 2.5 μL of sample genomic DNA solution (average DNA amount, 55.5 ng), 12.5 μL Universal Master Mix (Life Technologies), 0.25 μL RT73 primer 1 (50 μM), 0.25 μL RT73 primer 2 (50 μM), 0.5 μL RT73 probe (10 μM), 0.25 μL *FatA* primer 1 (50 μM), 0.25 μL *FatA* primer 2 (50 μM), 0.5 μL *FatA* probe (10 μM). The PCR step-cycle program was as follows: 2 min at 50 °C, 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C and 90 s at 60 °C.

To discriminate *B. rapa* and *B. napus*, we analyzed the scatter plots of end-point analyses for the duplex real-time PCR method to detect *BrACCg8* and *BnC1*. In the scatter plots, the *ACCg8* fluorescence intensity (FI) was represented as a horizontal line and the *BnC1* FI as a vertical line. If the amplification curves for RT73 detection could be clearly observed after 15 cycles with the amplification curves of *FatA* detection, we considered the sample to be positive

for the RT73-specific sequence, based on the findings of a previous study [77, 78]. For both methods, the ColE1 plasmid was used as a negative non-template control (NTC). On the reaction plate, real-time PCR was performed using two reaction vessels for the NTC. The other reaction vessels were used for genomic DNA samples extracted from ground canola samples or single canola kernels.

3. Results

3.1 Discrimination of *B. rapa* and *B. napus* in Canola Samples.

Schmidt and Rott previously reported on the development of a *B. napus*-specific real-time PCR assay [80]. We analyzed the partial *ACCg8* gene sequences of both *B. rapa* and *B. napus* using the designed and generated primers of Schmidt and Rott, and GenBank information (accession number DQ173671). From the partial *ACCg8* gene sequence and the amplification products of *B. rapa* seeds, we designed primers and probes for the *B. rapa ACCg8* (*BrACCg8*) sequence (Figure 3-1A) and attempted to amplify specific fragments of *B. rapa* DNA from ground seed samples. However, DNA fragments from some *B. napus* varieties were also slightly amplified (Figure 3-2A).

We therefore searched for the amplification fragments of another endogenous canola gene, in order to clearly discriminate *B. rapa* and *B. napus* using a combination of two different gene sequences [79, 81-85]. We analyzed the partial cruciferin gene sequences of both *B. rapa* and *B. napus* using the designed and generated primers based on GenBank information (accession number X59294). As shown in Figure 3-1B, we found a slight difference between the two cruciferin genes, so designed primers and probes (Table 3-1) based on the *B. napus* cruciferin gene (*BnC1*) sequence for detection using real-time PCR (Figure 3-2B). We developed a duplex real-time PCR method using two sets of primers and probes to discriminate *B. napus* and *B. rapa* DNA (Figure 3-2). Figure 3-2C shows the scatter plots of end-point analyses for the duplex real-time PCR assay. As expected, *B. napus* and *B. rapa* could be clearly discriminated.

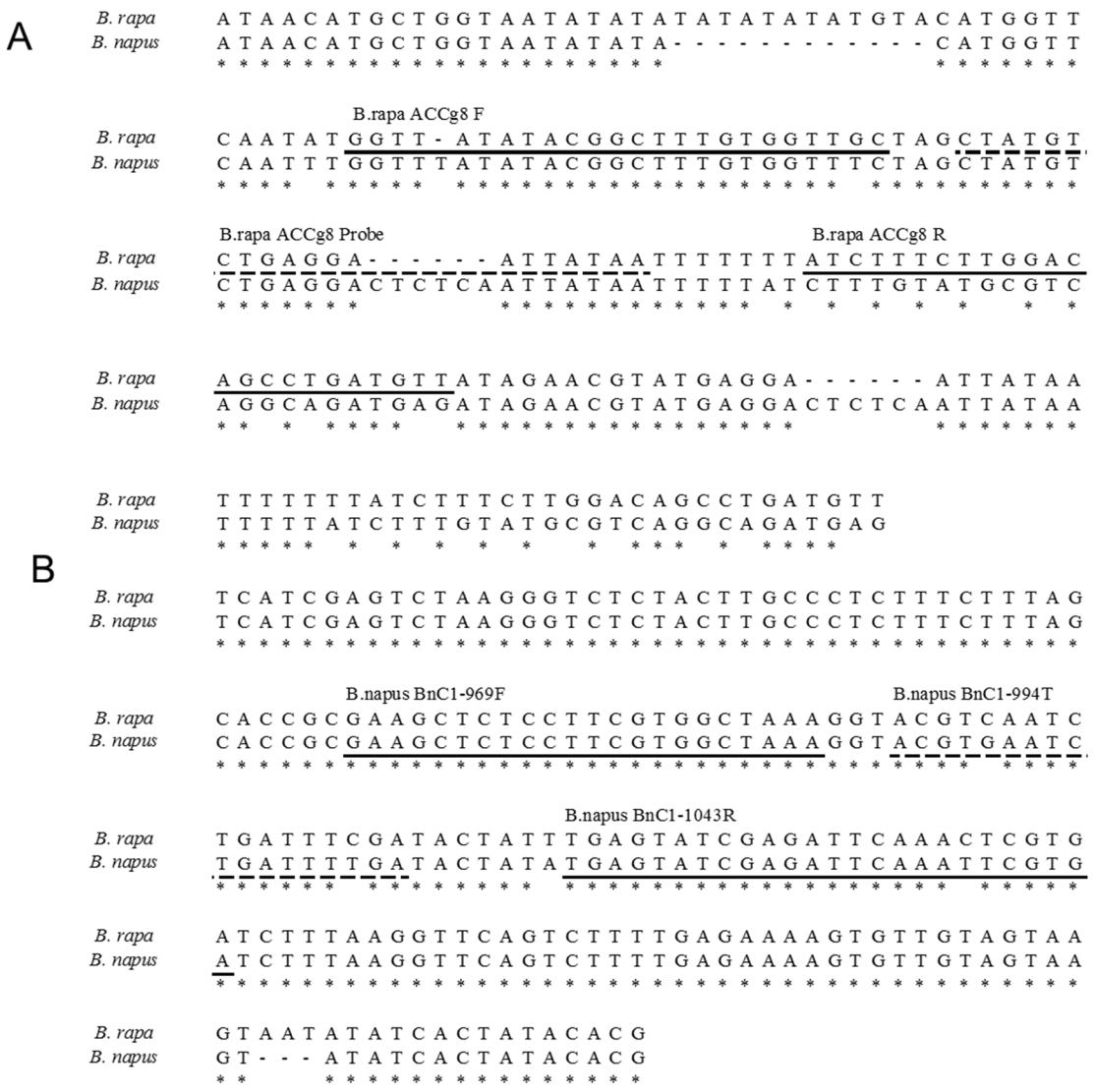

Figure 3-1 Nucleotide sequence alignments and design of the duplex real-time PCR assay for the discrimination of *B. rapa* and *B. napus*. (A) Fragment of the *ACCg8* gene sequence with primer sites underlined and the TaqMan probe site designated by a dotted line. Asterisks indicate the similarity between the *B. rapa* and *B. napus ACCg8* genes. (B) Fragment of the cruciferin gene sequence with primer sites underlined and the TaqMan probe site designated by a dotted line. Asterisks indicate the similarity between the *B. rapa* and *B. napus* cruciferin genes.

Table 3-1 Primer and Probe Sequences for Duplex Real-time PCR

Duplex real-time PCR method for discrimination of B. rapa and B.napus	*B. rapa* ACCg8 gene (BrACCg8) detection	Brapa-ACCg8F	GGTTATATACGGCTTTGTGGTTGC
		Brapa-ACCg8R	AACATCAGGCTGTCCAAGAAAGAT
		Brapa-ACCg8	VIC-CTATGTCTGAGGAATTATAA-NFQ-MGB
	B. napus cruciferin gene (BnC1) detection	B.napus BnC1-969F	GAAGCTCTCCTTCGTGGCTAAA
		B.napus BnC1-1043R	TCACGAATTTGAATCTCGATACTCA
		B.napus BnC1-994T	FAM-ACGTGAATCTGATTTTGA-NFQ-MGB
Duplex real-time PCR method for the identification of RT73	RT73 sepcific seqeuce	RT73 primer1	CCATATTGACCATCATACTCATTGCT
		RT73 primer2	GCTTATACGAAGGCAAGAAAAGGA
		RT73 probe	FAM-TTCCCGGACATGAAGATCATCCTCCTT-TAMRA
	FatA detection	FatA primer 1	GGTCTCTCAGCAAGTGGGTGAT
		FatA primer 2	TCGTCCCGAACTTCATCTGTAA
		FatA probe	VIC-ATGAACCAAGACACAAGGCGGCTTCA-TAMRA

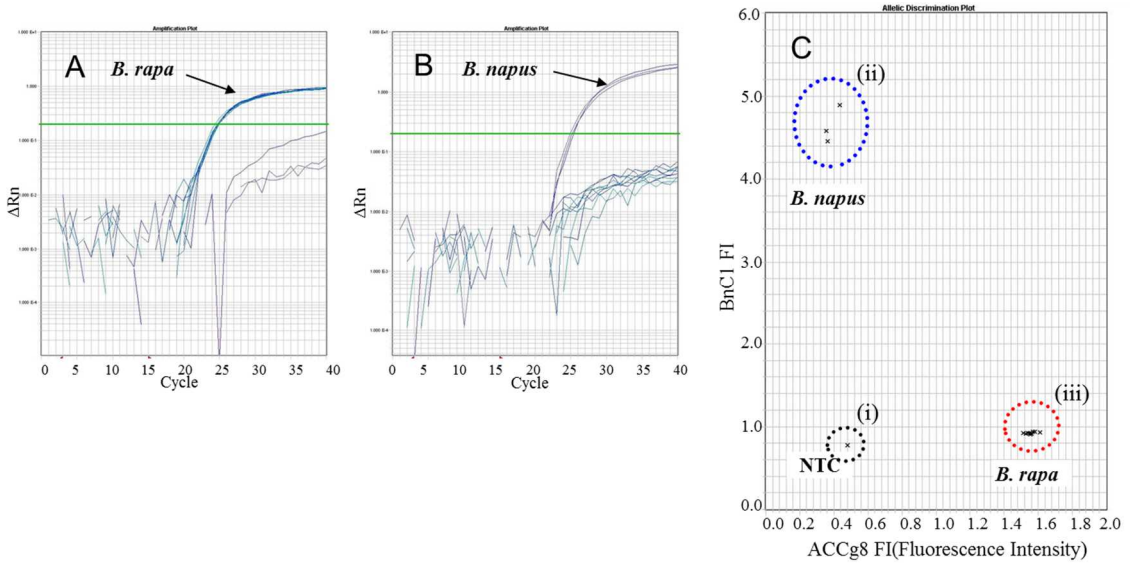

Figure 3-2 Typical amplification curves and plots of end-point analyses for the duplex real-time PCR assay to discriminate between *B. rapa* and *B. napus*. (A) Typical amplification curves for *BrACCg8* detection in *B. rapa* and *B. napus* seeds. (B) Typical amplification curves for *BnC1* detection in *B. rapa* and *B. napus* seeds. (C) Scatter plots of end-point analyses for the duplex real-time PCR assay to discriminate between B. rapa and B. napus. (i) NTC as a negative control. (ii) Amplification of genomic DNA from *B. napus* seeds. (iii) Amplification of genomic DNA from *B. rapa* seeds.

We also used the proposed method to detect other *Brassica* species. Figure 3-3 shows the scatter plots of the end-point analyses of other *Brassica* species DNA using the proposed detection system [86]. For the *BrACCg8* sequence, the DNAs of both *B. rapa* and *B. juncea* showed similar amplification patterns, whereas those of *B. napus*, *B. carinata*, *B. oleracea*, and *B. nigra* were only slightly amplified and their end-point FIs were relatively low (Figure 3-4). By contrast, for the *BnC1* sequence, DNAs from *B. napus*, *B. carinata*, and *B. oleracea* but not *B. rapa* and *B. juncea* could be amplified (Figure 3-4). These results suggest that it would be difficult to differentiate clearly among *B. napus* (2*n*=38, AACC), *B. carinata* (2*n*=34, BBCC), and *B. oleracea* (2*n*=18, CC), and between *B. rapa* (2*n*=20, AA) and *B. juncea* (2*n*=36, AABB) [87] using only the proposed method with scatter plots of the end-point analyses (Figure 3-3).

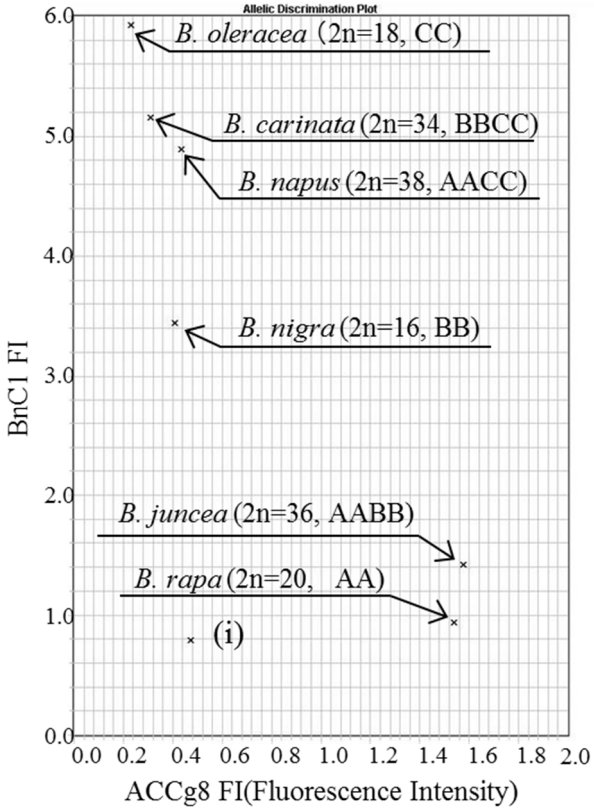

Figure 3-3 Scatter plots of end-point analyses for the duplex real-time PCR assay to discriminate between B. rapa and B. napus among seeds from various Brassica species. (i) NTC as a negative control. Points representing six Brassica species are arrowed.

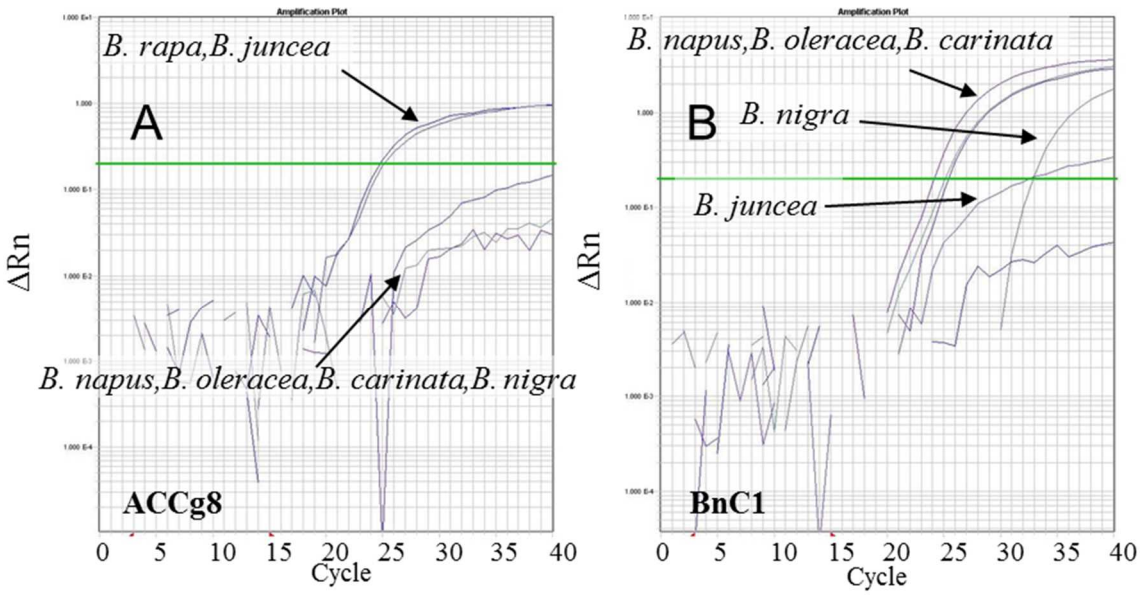

Figure 3-4 Amplification curves of duplex real-time PCR using other *Brassica* species for the discrimination of *B. rapa* and *B. napus*. (A) *BrACCg8* detection (FAM). (B) *BnC1* detection (VIC). Amplification curves of six *Brassica* species are arrowed.

We investigated the detection patterns of DNAs from various *B. rapa* lines in a solution diluted with *B. napus* DNA using scatter plots of the end-point analyses. As the concentration of *B. rapa* DNA increased in the solution, the FI of the *BnC1* gene sequence decreased while that of the *BrACCg8* sequence increased (Figure 3-5). The relationship between the *B. rapa* DNA content and the FI of the *BrACCg8* sequence was expressed by the following formula: $y = -0.1574\,x^4 + 0.0407x^3 + 0.7397x^2 + 1.0051x + 1.4996$, ($R^2$=1).

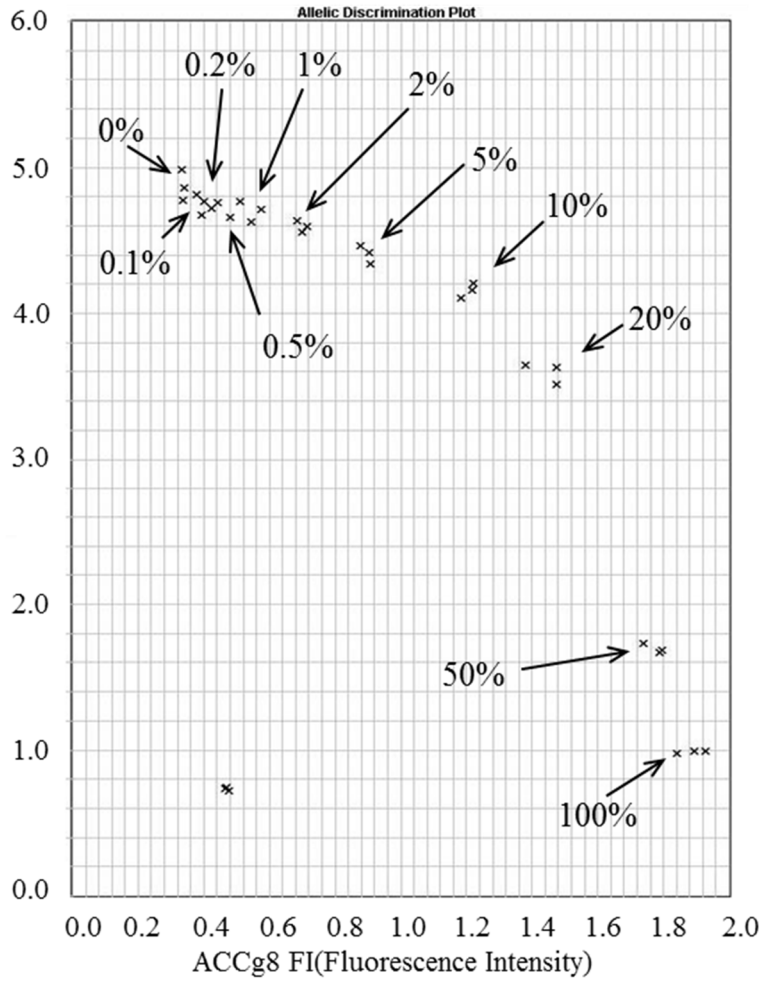

Figure 3-5 Scatter plots of end-point analyses of DNA solutions of various *B. rapa* lines diluted with *B. napus* DNA by the duplex real-time PCR assay. (i) NTC (ColE1 plasmid) as a negative control. Samples representating each percentage of the *B. rapa* DNA content are indicated by arrows.

3.2 Simultaneous Detection of RT73-Specific Sequence and FatA Gene Using Real-Time PCR.

Using both sets of primers and probes, we successfully used the duplex real-time PCR assay to simultaneously detect the RT73-specific sequence and the endogenous *FatA* gene, according to aMonsanto Company report (Figure 3-6) [79].

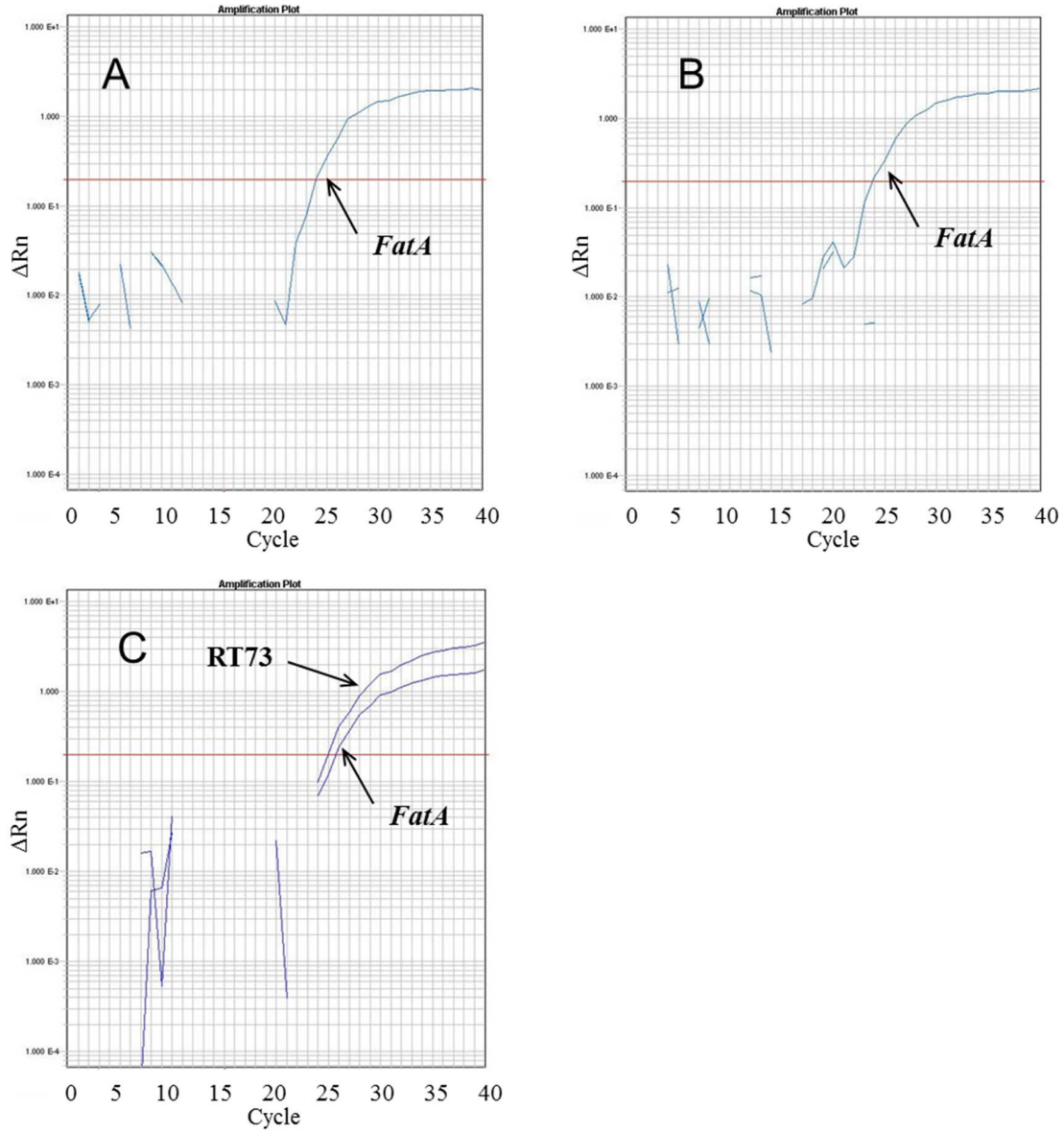

Figure 3-6 Amplification curves of the duplex real-time PCR assay for the simultaneous detection of the RT73-specific sequence and *FatA* gene. (A) *FatA* detection of *B. rapa.* (B) *FatA* detection of *B. napus*. (C) *FatA* detection and RT73-specific detection of RT73 *B. napus*.

3.3 Grinding and DNA Extraction from Individual Canola Kernels.

We next developed a method for DNA extraction from individual canola kernels using 96-well silica-membrane plates. Among the various plate bases that we examined, the glass-fiber silica-plate base (EPM 2000) gave the best recoveries for DNA extraction from a canola kernel using the conventional reagent provided in the GM quicker kit. Generally, a conventional, commercial 96-well silica-membrane plate contains a one-plate sheet, such as a glass-fiber silica base or a polystyrene base. We clipped layers of multiple glass-fiber silica-plate base sheets onto a polystyrene column plate in order to increase the DNA yield from a canola kernel. The number of plate sheets required to make the layer was examined based on the DNA yield from canola kernels and the elution efficiency of the working solution. A layer of three glass-fiber silica-plate base sheets was found to give sufficient recoveries from DNA extraction of individual canola kernels. We then modified the volume of various extraction buffers and the procedure attached for the GM quicker 2 kit, as described in the Experimental Section.

To investigate the yield and quality, we evaluated the concentration and purity of genomic DNA extracted from 12 individual canola kernels. As shown in Table 3-2, the yields from individual canola kernels for all products ranged from 9.60 ng/μL to 54.11 ng/μL, with an average of 22.21 ng/μL. For all *B. rapa*, *B. napus*, and RT73 kernels, the ratios of absorbance at wavelengths of 260 and 280 nm (*A*260/*A*280) were between 1.6 and 1.9.

Table 3-2 Yield and Quality of Genomic DNA for Three Kinds of Canola Seed Kernels[a]

	B. rapa		*B. napus*		RT73	
	mean	RSD (%)	mean	RSD (%)	mean	RSD (%)
DNA conc.(ng/mL)	14.73	0.21	24.30	0.18	27.61	0.37
260/280 ratio	1.66	0.07	1.78	0.05	1.81	0.07

[a] Each value represents the mean and relative standard deviation (RSD(%)) of 12 kernel determinants. The UV adsorption of the 260nm/280nm ratio represents A_{260}/A_{280} of genomic DNA individually extracted from canola line.

3.4 Application of the Developed Method to Canola Kernel Samples.

The proposed detection methods were applied to 60 kernels of three varieties of *B. napus* including RT73 (12 kernels of each of two *B. napus* varieties, and 36 kernels of RT73 *B. napus*), to 36 kernels of nine varieties of *B. rapa* (four kernels of each of the 9 *B. rapa* varieties), and to 94 kernels of an imported canola sample. All DNA extracted from the *B. rapa* kernels showed clearly amplified *BrACCg8* sequence products and the FI values were in the range of 3.8-5.8, whereas no *BnC1* sequence products from *B. napus* were amplified (Figure 3-7, 3-8). By contrast, all DNA extracted from kernels of *B. napus*, including RT73 *B. napus*, showed clearly amplified *BnC1* sequence products with FI values in the range of 1.0-1.6, whereas no *BrACCg8* sequence products were amplified (Figure 3-7, 3-8). For the imported canola sample, all DNA extracted from the individual kernels showed amplified *BnC1* sequence products from *B. napus* but no *BrACCg8* sequence products from *B. rapa*, with the exception of one kernel. However, the end-point FI of the *BrACCg8* sequence products was significantly lower than that for *B. rapa* (Figure 3-9, 3-10). The number of RT73 kernels detected was 44, so the contamination rate was calculated as 46.8% (Figure 3-9, 3-10). We therefore concluded that all tested kernel samples were *B. napus* and that 46.8% of those were RT73 kernels. As expected, no RT73 *B. rapa* kernels were detected.

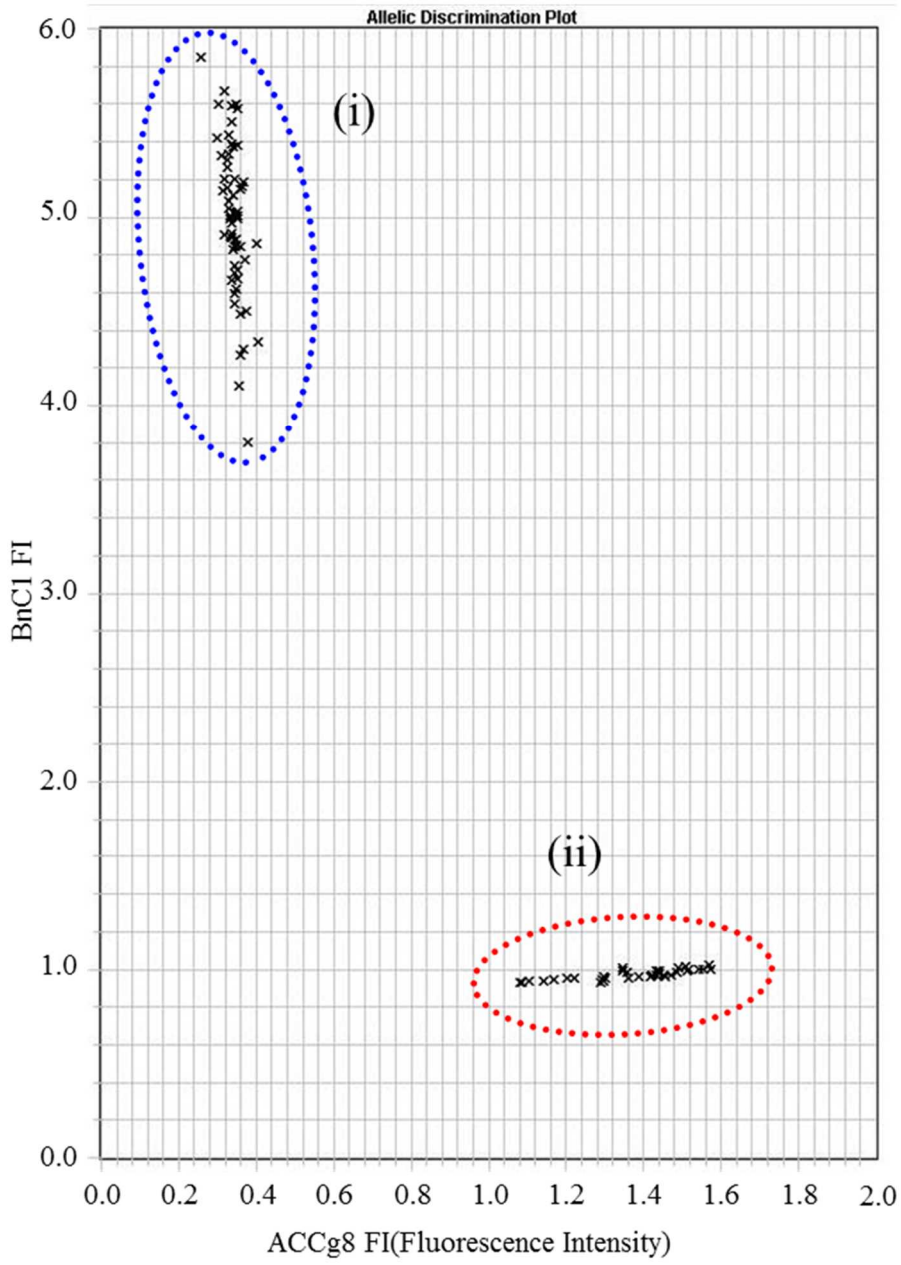

Figure 3-7 Scatter plots of end-point analyses of kernels of nine varieties of *B. rapa* and three varieties of *B. napus* including RT73 in the duplex real-time PCR assay. (i) Sixty kernels of three varieties (Westar, Eagle, and RT73) of *B. napus* (two kernels of the first two varieties and 36 kernels of the third). (ii) Thirty-six kernels of nine varieties (Wase syokuyou aburana, Maruba natane, Gokuwase natanena, Wakana, Niida aona, Nozawana, Shin kuro mizuna, Shinobu fuyuna, and Narusawana) of *B. rapa* (four kernels of each variety).

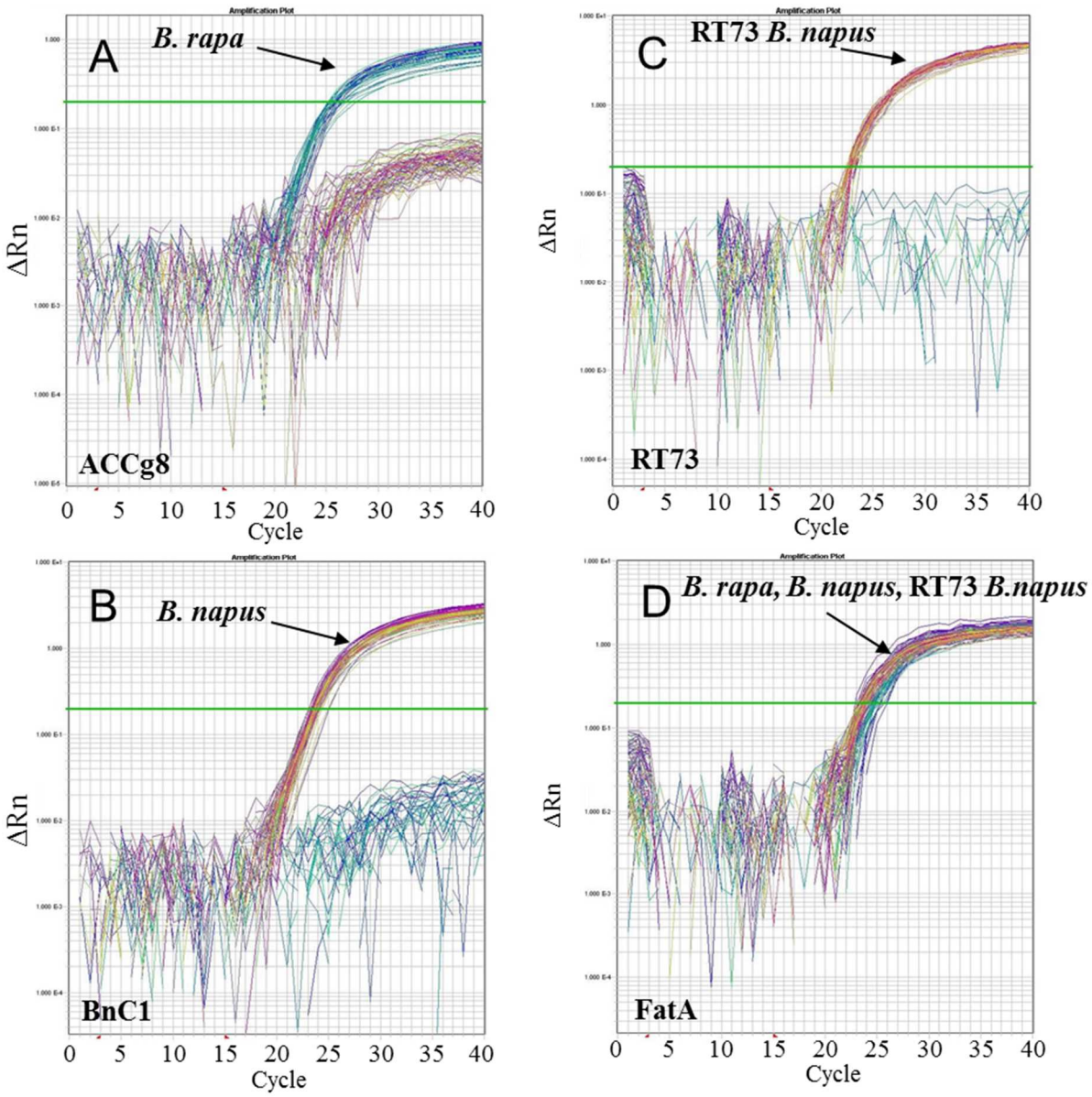

Figure 3-8 Amplification curves of the duplex real-time PCR methods using 60 kernels of three varieties of *B. napus* including RT73 and 36 kernels of nine varieties of *B. rapa*. (A) *BrACCg8* detection (FAM) by duplex real-time PCR for the discrimination of *B. rapa* and *B. napus*. (B) *BnC1* detection (VIC) by duplex real-time PCR for the discrimination of *B. rapa* and *B. napus*. (C) RT73 detection (FAM) by duplex real-time PCR for the identification of the RT73-specific sequence. (D) *FatA* detection (VIC) by duplex real-time PCR for the identification of the RT73-specific sequence.

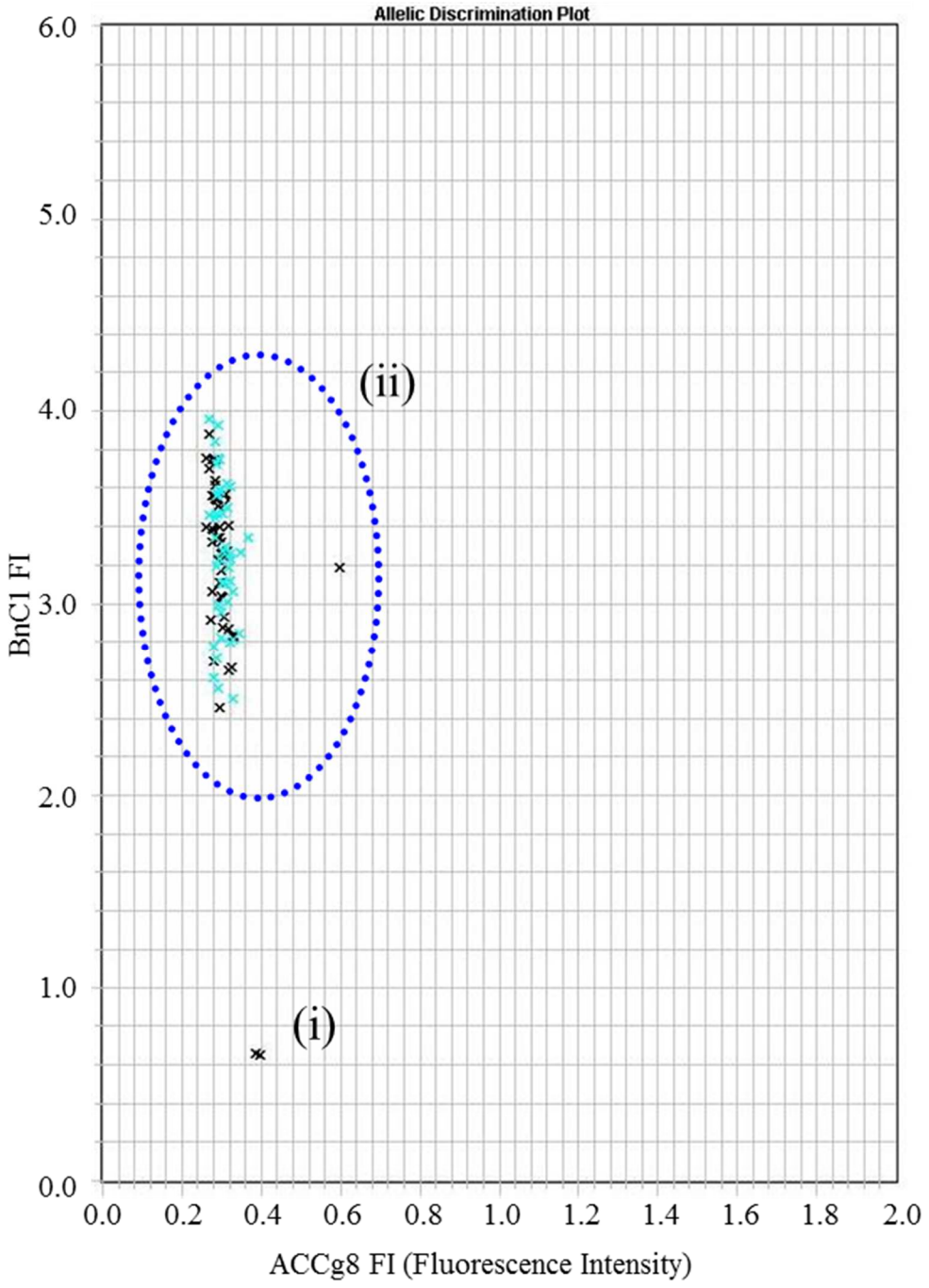

Figure 3-9 Scatter plots of end-point analyses of 94 kernels of an imported canola sample in duplex real-time PCR assays. (i) NTC (ColE1 plasmid) as a negative control. (ii) Amplification of genomic DNA extracted from kernels of the imported canola sample. Blue plots represent the kernels detected RT73-specific sequence.

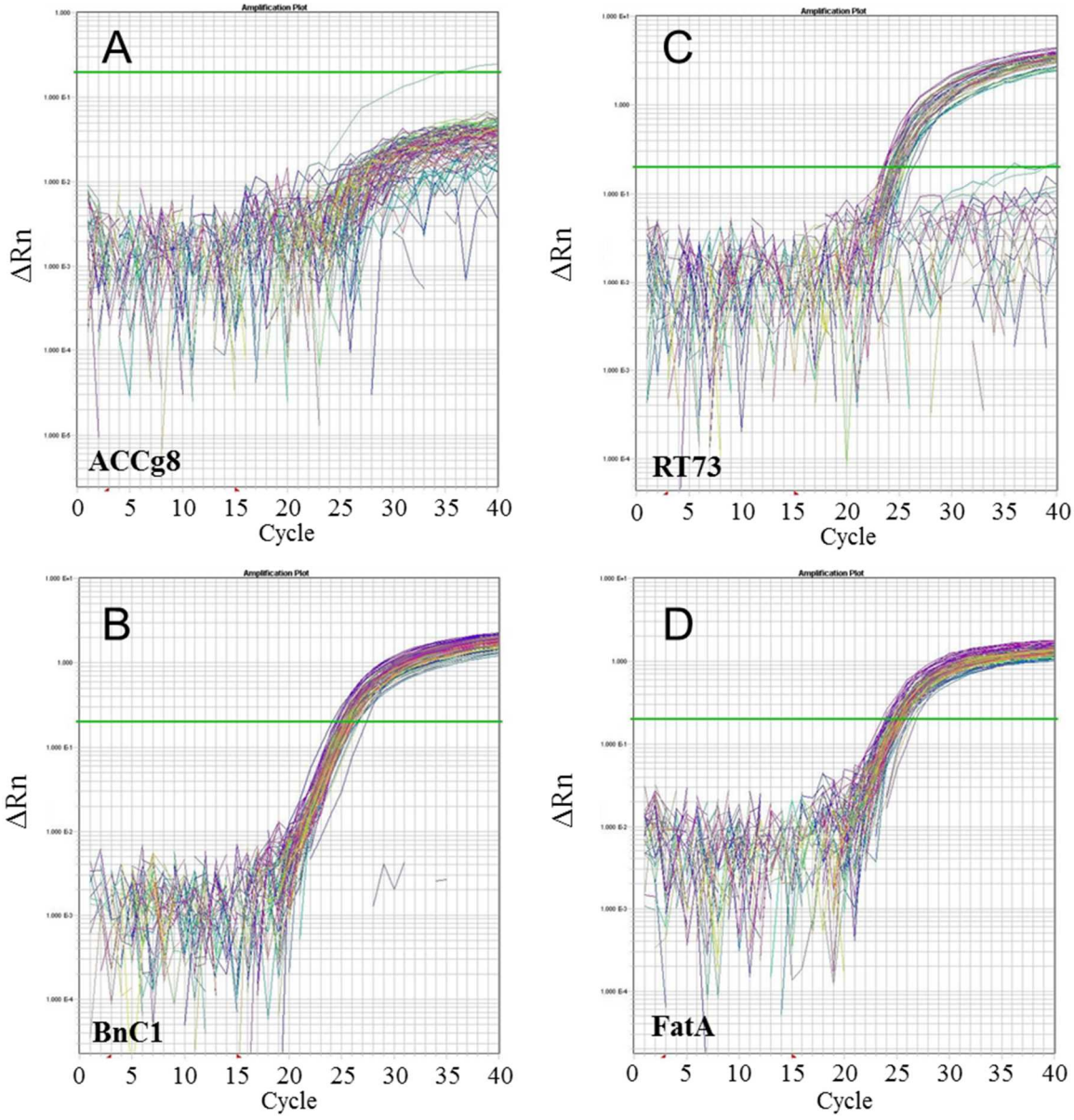

Figure 3-10 Amplification curves of the duplex real-time PCR methods using 94 kernel samples from imported canola. (A) *BrACCg8* detection (FAM) by duplex real-time PCR for the discrimination of *B. rapa* and *B. napus*. (B) *BnC1* detection (VIC) by duplex real-time PCR for the discrimination of *B. rapa* and *B. napus*. (C) RT73 detection (FAM) by duplex real-time PCR for the identification of the RT73-specific sequence. (D) *FatA* detection (VIC) by duplex real-time PCR for the identification of the RT73-specific sequence.

4. Discussion

RT73 *B. rapa* canola is derived from crossing RT73 *B. napus* and *B. rapa*, which are different species with different genome sizes. As it is unclear how this cross-breeding induces changes in genomic DNA and protein expression, RT73 *B. rapa* canola is not permitted to be imported into Japan and should be regulated with zero tolerance. Hence, there is a need to qualitatively detect contamination by RT73 *B. rapa* canola in grain samples. To achieve this, it is necessary both to distinguish *B. napus* and *B. rapa*, and to identify RT73 by individual canola kernel analysis.

Schmidt and Rott developed a *B. napus*-specific PCR assay that detects the *BnACCg8* gene sequence, in order to discriminate *B. napus* and *B. rapa* [80]. From the partial *ACCg8* gene sequence of *B. rapa* seeds, we designed primers and probes for the *B. rapa ACCg8* (*BrACCg8*) sequence and attempted to amplify specific fragments of *B. rapa* DNA from ground seed samples. Although the method of Schmidt and Rott gave good amplification curves for *B. rapa* DNA, some varieties of *B. napus* DNA also appeared to be slightly amplified when using ground samples. We therefore attempted to discriminate clearly between *B. napus* and *B. rapa* by combining the detection of the *BrACCg8* sequence with that of another endogenous canola gene.

Previously, some researchers reported on the detection of endogenous canola genes using PCR assays targeting sequences such as the *ACCg8* gene [84], the high-mobility-group protein *I/Y*(*HMG-I/Y*) gene [82], the phosphoenolpyruvate carboxylase (*pep*) gene [83], the acyl-acyl carrier protein (ACP) thioesterase *FatA* gene [79], the cruciferin gene [80, 81], and the S-locus receptor kinase (*SRK*) gene [88]. Among the candidate endogenous genes that we examined, combining the detection of *B. rapa BrACCg8* and *B. napus BnC1* proved to be the best way to

clearly discriminate *B. napus* and *B. rapa.* We found few differences between *B. rapa* and *B. napus* in terms of the *FatA* gene and *HMG-I/Y* gene (data not shown). In the case of *BnC1* detection, we found that *B. napus*, *B. carinata*, and *B. oleracea* showed similar amplification patterns, whereas *B. rapa* and *B. juncea* showed no amplification. These results suggest that the *BnC1* sequence might be located on the C genome of *B. napus* (2*n*=38, AACC), *B. carinata* (2*n*=34, BBCC), and *B. oleracea* (2*n*=18, CC) [87].

In addition, we developed a duplex real-time PCR method for the detection of an RT73-specific sequence and the *FatA* endogenous canola gene, based on a Monsanto Company report [79]. Our method could detect the RT73-specific sequence and simultaneously evaluate the quality of the extracted genomic DNA for PCR in one run.

We also established a DNA-extraction method for use with single, ground canola kernel samples. The amounts and *A*260/*A*280 ratios of the DNA extracted from individual canola kernels using *B. rapa*, *B. napus*, and RT73 ranged from 14.73-27.61 ng/mL and 1.66-1.81, respectively. These results suggest that the extraction method produced a sufficient content and quality of extracted genomic DNA for PCR (Table 3-2).

According to Japanese regulations, if even a trace of RT73 *B. rapa* is detected in imported canola samples, they have to be disposed of. Therefore, we have to monitor the samples very carefully. However, we consider that individual canola kernel testing should be applied only when contamination by both RT73 canola and *B. rapa* has been simultaneously detected using ground canola samples and this system, since individual canola kernel testing of multiple samples is time-consuming. According to Figure 3-5, the limit of detection (LOD) for *B. rapa* in a screening test of ground canola sample is approximately 0.2-0.5%, even though contamination by RT73 *B. rapa* is unproven at this point.

EU countries have discussed relaxing the zero-tolerance policy for unapproved GM crops to an alternative tolerance threshold with the aim of preventing the import decline and consequential problems for industry. In Switzerland, traces of unapproved GM material of up to 0.5% may be tolerated in food if the respective GM crop is already authorized in another country where comparable procedures are followed, or if a danger to human health can be ruled out after scientific evaluation by responsible authorities and if detection methods and reference materials are made available [89]. Accordingly, in considering the feasibility of performing tests for multiple samples, PCR detection of RT73 and *B. rapa* contamination as an initial screening step, followed by individual canola kernel testing was shown to be a practical approach (Figure 3-11). It remains necessary to take a statistical approach to clarifying the acceptable level of uncertainty in terms of risk and method applicability, in order to determine how many canola grains will need to be inspected.

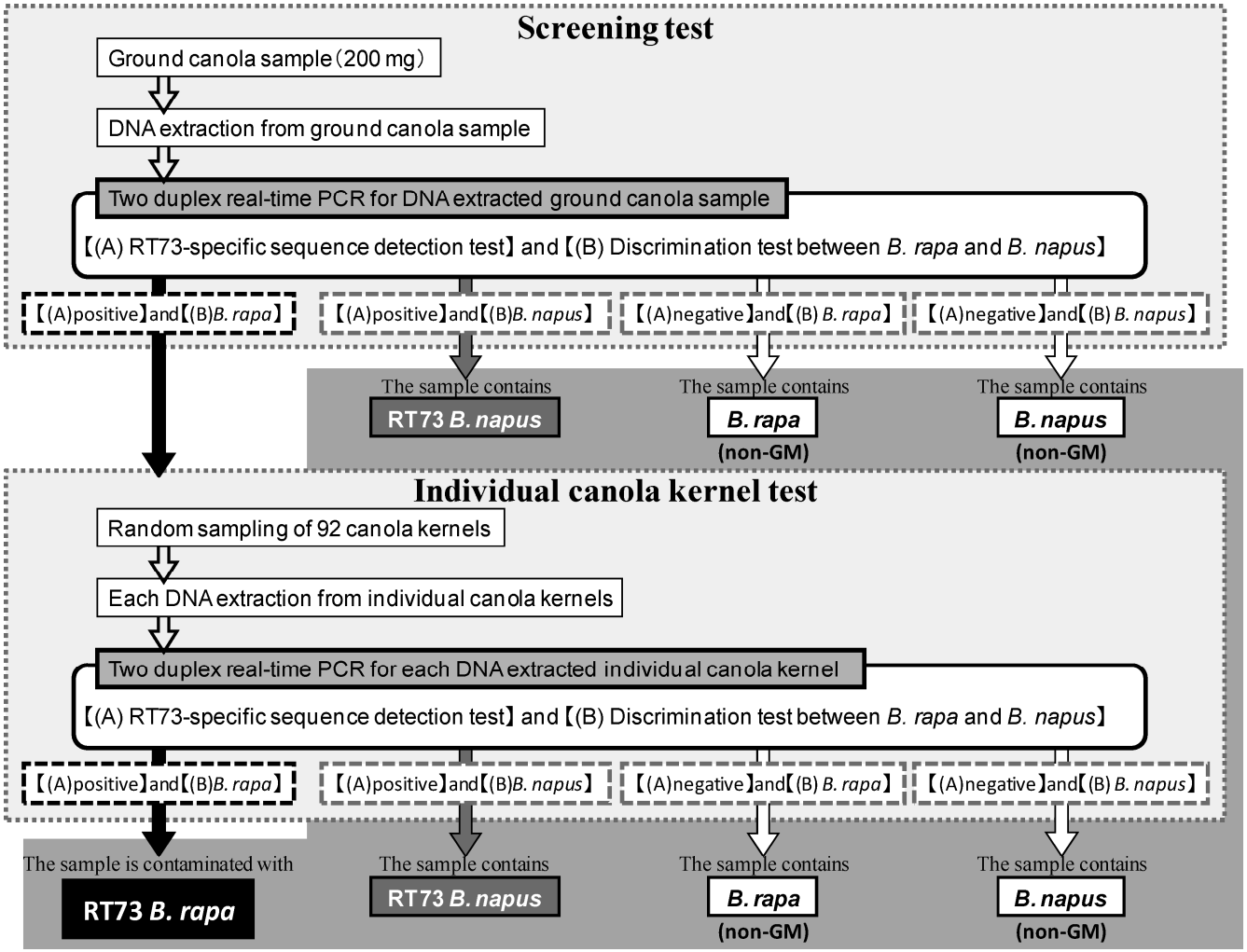

Figure 3-11 Proposal testing scheme for detecting RT73 *B. rapa.*

CHAPTER 4

Quantification and identification of genetically modified maize events in non-identity preserved maize samples in 2009 using and individual kernel detection system

1. Introduction

Genetically modified (GM) crops are currently cultivated widely as sources of food and feed in many countries [75]. GM crops generally have been assessed and authorized for food use by administrative authorities. In some countries, the labeling of grains, feed and foodstuffs is mandatory if the GM crop content exceeds a certain level of the approved GM varieties. For instance, the European Union, Japan and Korea have set threshold values of 0.9%, 5%, and 3%, respectively, of GM organism material in a non-GM background as the basis for labeling [90-96].

In Japan, non-GM crops are segregated as non-GM material and imported from the United States using an identity preserved (IP) handling system that requires documentary certification from US farms to Japanese processing traders. Recently, the production of stacked GM maize grains, in which two or more characteristic events have been inserted, has increased in the United States due to enhanced production efficiency [75]. Although the levels of adventitious commingling of GM maize in non-GM maize according to the labeling system refer to GM maize as a weight per weight (w/w) percentage, conventional applicable detection methods, such as quantitative real-time PCR, do not directly measure the w/w percentage of GM maize, but rather provide relative copy numbers between a specific DNA sequence and a taxon-specific DNA sequence, and these values are converted into a w/w percentage using appropriate reference materials. The GM maize content in a maize sample containing stacked GM maize grains, as determined by current quantitative real-time PCR methods, is likely to be overestimated compared to the actual w/w percentage of GM maize in the sample because the relative copy numbers are calculated on a haploid basis. To solve this problem, we have developed an individual kernel detection system that consists of grinding individual maize kernels, DNA extraction from

each individual ground maize kernel, multiplex real-time PCR using the extracted DNA samples from individual ground maize kernels for GM detection, and multiplex qualitative PCR using the extracted DNA for GM event detection to analyze the precise commingling level and varieties of GM maize [77, 97, 98]. The detection system has already been implemented in Japan as an official GM maize detection method [99].

It is important to investigate the content of GM maize commingled in actual maize samples that contain many GM maize grains, such as non-IP maize samples, in order to determine the main current GM maize and stacked GM maize events and to predict which events are likely to be commingled with IP maize samples. However, there has been little information on the determination of stacked GM maize in non-IP maize samples, because no method is available, except for detection in individual kernels. We previously investigated GM maize content on a kernel basis and determined the varieties of the GM kernels in non-IP maize samples imported from the USA in 2005 [78] using an individual kernel analysis system including a multiplex real-time PCR method [77, 97], and coupled it to a multiplex qualitative PCR method [98] followed by analysis using multi-channel capillary gel electrophoresis [78].

The present study was designed to clarify the GM maize content of non-IP maize samples that contain GM maize produced in 2009, to investigate the content of GM maize grains, and to determine how many stacked GM maize grains are contained therein and which GM maize and stacked GM maize events frequently appeared in 2009 by using the multiplex real-time PCR method [77, 97], two multiplex qualitative PCR detection methods [98, 100] both coupled to the microchip electrophoresis and partially real-time PCR array analysis [72].

2. Materials and methods

2.1 Maize samples

The non-IP maize samples produced in 2009 were purchased from a trading company in Japan. Bt11, GA21 and MIR604 seeds were kindly provided by Syngenta. TC1507 and DAS 59122 seeds were kindly provided by Pioneer Hi-Bred International, Inc. Seeds of MON88017, MON810, MON863, NK603 and stacked maize were kindly provided by Monsanto Co. T25 maize seeds were imported directly from the USA as positive controls of GM maize.

2.2 Oligonucleotide primers and probes for multiplex real-time PCR method

Sets of primer pairs and TaqMan® probes for construct-specific and universal GM quantification were described in our previous papers [77, 97]. The SSIIb-3 system (SSIIb 3–5′ and SSIIb 3–3′ with SSIIb-TaqV) was used for the primers and probe for the detection of the taxon specific gene encoding the maize starch synthase IIb (SSIIb) in the multiplex real-time PCR method, while the p35S-1 system (P35S 1–5′ and P35S 1–3′ with P35S-Taq) and GA21–3 system (GA21 3–5′ and GA21 3–3′ with GA21-Taq) were used in the multiplex real-time PCR method. All sets of primer pairs and TaqMan probe p35S-Taq for the detection of the cauliflower mosaic virus (CaMV) 35S promoter sequence (p35S) and GA21-Taq GA21 for the detection of specific sequence were purchased from Fasmac Co., Ltd. (Kanagawa, Japan). SSIIb-TaqV, which is labeled with VIC® at the 5′ and TAMRATM at the 3′ ends, was synthesized by Life Technologies (Carlsbad, CA, USA) and used as a probe for the detection of SSIIb. The target sequence used by the p35S-1 system to detect the 35S promoter region derived from CaMV is widely found in the recombinant DNA of almost all GM events with the exception of GA21. The GA21–3 system

was designed to detect the construct specific sequence of GM maize event GA21 [77, 97].

2.3 Grinding and DNA extraction of individual maize kernels

The grinding of individual maize kernels were performed according to previous reports [9, 10]. DNA extraction and purification were carried out with the GM quicker 96 kit (Nippon Gene Co., Ltd., Tokyo, Japan) using the method described here; genomic DNA extraction from the ground powder of individual kernels was performed according to the kit procedure. GE1 buffer and RNase A solution (100 mg/mL) were mixed to make a working solution at respective volumes of 1.5 mL and 5 μL. A 1.5 mL aliquot of the working solution was added to each sample tube containing the ground maize powder and metal corn. Twenty-four sample tubes were arrayed in the tube holder. The maize powder and working solution were mixed by vigorously shaking the tubes and the metal corn in a multi-bead shocker at 2,000 rpm for 15 s and incubated for 10 min at room temperature. A 180 μL aliquot of GE2-K buffer solution was then added to each solution. The sample tube was capped to avoid leakage, and vigorously shaken for 15 s in the multi-bead shocker. The tube holder was centrifuged for 10 min at 1,400 ×*g* using a Metalfuge centrifuge (MBG100; Yasui Kikai Co., Ltd., Osaka, Japan). A 400 μL aliquot of each supernatant was carefully transferred to a 96-well plate. A 250 μL aliquot of GB3 buffer–isopropanol (1:1, v/v) was added. A 650 μL aliquot of each sample was then carefully transferred to the 96-well column plate, which was centrifuged for 20 min at 1,400×*g*. After removal of the filtrate, 650 μL of GW buffer was added to each well. The 96-well column plate was centrifuged for 10 min at 1,400 ×*g*. After removal of the filtrate, the 96-well column plate was recentrifuged for 20 min at 1,400×*g*. The plate was placed in a collection plate and 50 μL of DW was added to each well. The plate was incubated for 3 min at room temperature, and then centrifuged for 10 min at 1,400×*g*. For

DNA extraction from individual maize kernels, we used a glass-fiber silica-plate base sheet (EPM 2000; GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, England) for the 96-well column plate.

2.4 Multiplex real-time PCR conditions

To simultaneously detect the genomic DNA from individual GM maize kernels and to confirm the validity of PCR amplification of the extracted genomic DNA, multiplex real-time PCR analyses were performed according to previous papers [77, 78, 97]. The amplification curves of the target sequence were monitored using a fluorescent dye, which was used to label the designed oligonucleotide probes, using an ABI PRISM® 7900HT sequence detection system (Life Technologies). The reaction volume of 25 μL contained 2.5 μL of the sample genomic DNA (10 ng/μL), 12.5 μL of Universal Master Mix (Life Technologies), 0.5 μM each primer pair, and 0.2 μM probe (except 0.1 μM for the p35S probe). The PCR step-cycle program was as follows: 2 min at 50°C, and 95°C for 10 min followed by 45 cycles of 30 s at 95°C and 1 min 30 s at 59°C.

If an amplification curve indicating GMO detection could be clearly observed after 15 cycles, we considered the sample as positive for GMOs; otherwise, it was considered negative, because we adopted exponential character of the amplification curve after 15 cycles of real-time PCR as the threshold for discrimination of GM and non-GM maize kernels in previous studies [77, 78, 97]. In this study, the GM Maize Detection Plasmid Set –ColE1/TE– (Nippon Gene Co.) was used as the positive control. This plasmid set contains six concentrations of the reference plasmid pMul5, into which has been inserted the amplification products of p35S, GA21 and SSIIb, diluted with TE buffer (pH 8.0) including 5 ng/μL of the ColE1 plasmid [78, 97]. The ColE1 plasmid contained none of the amplification GM products, and was used as the negative control. The

positive controls were prepared using two concentrations of the plasmid, set at 250,000 and 1,500 copies per plate. In the reaction plate, real-time PCR was performed in duplicate (each two wells) for the negative control, and for one positive control (250,000 copies) and for the other positive control (1,500 copies). The other 90 reaction wells were used for genomic DNA samples extracted from individual maize kernels.

2.5 Two multiplex qualitative PCR methods

To identify which GM event grains are contained in genomic DNA extracted from individual kernels, two multiple qualitative PCR detections were performed according to our previously reported methods [98]. The first method was performed for the detection of MON810, NK603, T25, GA21, TC1507, Event176, Bt11, and MON863 (construct specific) [98]. The reaction mixture for PCR was prepared in a 96-well plate. The reaction volume of 25 μL contained 25 ng genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, and 1.25 units AmpliTaq Gold® DNA polymerase (Life Technologies), and 15 primers at the following concentrations: 0.2 μmol/L for M810 1–5′, NK603 1–5′ M863 1–5′, M863 1–3′, Bt11 1–5′, and CryIA 1–3′; 0.1 μmol/L for T25 2–5′, T25 2–3′, GA21 1–5′, GA21 1–3′, TC1507 1–5′ and TC1507 1–3′; 0.05 μmol/L for Event 176 1–5′; and 0.045 μmol/L for SSIIb 1–5′ and SSIIb 1–3′. The reactions were buffered with PCR buffer II (Life Technologies) and amplified in a Silver 96-well GeneAmp PCR System 9700 (Life Technologies) thermal cycler in max mode, according to the following PCR step-cycle program: pre-incubation at 95°C for 10 min, 10 cycles consisting of denaturation at 95°C for 0.5 min, annealing at 65°C for 1 min, and extension at 72°C for 1 min; 27 cycles consisting of denaturation at 95°C for 0.5 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min; followed by a final extension at 72°C for 7 min.

The second method was performed for the detection of DAS-59122-7, MIR604, MON863 (event-specific) and MON88017 [100]. The reaction mixture for PCR was prepared in a 96-well plate. The reaction volume of 25 μL contained 25 ng of genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, and 0.625 units AmpliTaq Gold DNA polymerase (Life Technologies) and nine sets of primers at the following concentrations: 0.50 μmol/L for MON88017-mF and MON88017-mR; 0.40 μmol/L for MON863-mF and MON863-mR, 0.30 μmol/L for DAS59122-7-rb1R; 0.25 μmol/L for DAS59122-7-rb1F; 0.15 μmol/L for MIR604-mF; 0.06 μmol/L for SSIIb 3–5′ and SSIIb 3–3′. The PCR conditions were the same as for the first method described above. Both methods were followed by microchip electrophoresis analysis.

2.6 Microchip electrophoresis analysis

The PCR products of multiple samples were analyzed using an MCE-202 MultiNA™ microchip electrophoresis system (Shimadzu, Kyoto, Japan). This system uses microchip technology for automated electrophoretic separation at high sample throughput using a 96-well PCR plate and high-sensitivity fluorescence detection. The analysis was run essentially according to the manufacturer's instruction manual using a DNA-1000 reagent kit (Shimadzu), which consists of Separation Buffer, DNA Marker Solution (100–1,000 bp), and DNA-100 Ladder solution. The Separation Buffer was used to dilute SYBR® Gold (Life Technologies) 100-fold to prepare the DNA-1000 Separation Buffer solution for analysis. The DNA-1000 Ladder solution was prepared using φ x 174 DNA/*Hae*III Markers (Promega, WI, USA), which was diluted 100-fold in TE buffer (10 mM Tris–HCl buffer containing 50 mM KCl, 1.5 mM $MgCl_2$). The PCR products and the diluted DNA-100 Ladder solution in a MicroAmp Optical 96-well reaction plate (Life Technologies) were placed into the instrument alongside the reagents. The samples and

reagents were mixed automatically on-chip and run using MultiNA Control and MultiNA Viewer software (Shimadzu).

2.7 Preparation of real-time PCR array, reaction conditions and data analysis

To clarify GM events in genomic DNAs from some GM grains that gave ambiguous results in analyses using the two multiplex qualitative PCR detection methods, a real-time PCR array was employed according to our previously reported method with some modifications [72]. The following detection targets were selected for one analysis: Bt11, E176, GA21, M810, M863, NK603, T25, TC1507, DAS59122, M88017, MIR604 and SSIIb. To prepare the real-time PCR array, 2 μL of a primer and probe mixture for each detection target, containing 2.5 μM primers and 1 μM probe, was added into each well of a 96-well plate, which was sealed with MicroAmp® Optical Adhesive Film (Life Technologies). Array plates containing primer and probe mixtures stored at -20°C until just before use. For assaying sample DNA with the real-time PCR array, the diluted DNA samples described above, TaqMan® Universal PCR Master Mix (Life Technologies) and sterile distilled water were mixed and added to each well at a volume of 8 μL. Finally, 10 μL of the reaction mixture in each well contained 20 ng of genomic DNA, 5 pmol 5′ primer, 5 pmol 3′ primer, 2 pmol probe and 5 μL TaqMan Universal PCR Master Mix. The plates containing the reaction mixtures were sealed with MicroAmp Optical Adhesive Film, and thermally cycled with the ABI 7500 real-time PCR system (Life Technologies) or ABI PRISM 7900HT Sequence Detection System (Life Technologies). The data were analyzed using Sequence Detection Software Version 1.4 for the 7500 system and Version 2.3 for the 7900HT system. The thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, 45 cycles of 15 s at 95°C and 1 min at 60°C under 9600 emulation mode. Data were analyzed using the “Amplification Plot”

feature of the analysis software with detail settings at the “Delta Rn *vs*. Cycle” view with Manual Ct mode (Threshold, 0.256) and Manual baseline mode (start of baseline, 3; end of baseline, 10). Amplification lines that crossed the threshold were determined to be positive.

3. Results

3.1 Determination of GM maize content in non-IP maize samples using multiplex real-time PCR

We randomly sampled 212 to 289 kernels from each of the five non-IP maize samples produced in 2009 and performed single kernel analyses using the multiplex real-time PCR method. The multiplex real-time PCR method allowed us to individually discriminate GM maize from non-GM maize and simultaneously evaluate the quality of the extracted genomic DNA for PCR in one run. As shown in Table 4-1, the GM maize content on a kernel basis in the five non-IP maize samples was 84.1%, 80.6%, 79.7%, 85.5% and 79.0%, respectively, and their average value and standard deviation were 81.9%±2.8%. This result indicates that the average ratio of GM maize in non-IP maize samples in 2009 was higher than in 2005 [78]. In addition, although the GM maize ratios in non-IP maize samples in 2005 varied greatly (28.3–77.2%), the standard deviation (2.8%) of the GM maize ratio in non-IP maize samples in 2009 was small.

Table 4-1 GM maize grain contents on a kernel basis in five non-IP maize samples in 2009

non-IP maize sample lot	Kernel number			GM content (%)
	non-GM	GM	total	
1	46	243	289	84.1
2	46	191	237	80.6
3	43	169	212	79.7
4	32	189	221	85.5
5	48	181	229	79.0
total	215	973	1188	81.9

3.2 GM event analysis using multiplex qualitative PCR

Next, the genomic DNA extracted from positive kernels of five non-IP maize grain samples was individually analyzed using two multiplex qualitative PCR detection methods [98, 100] both coupled to microchip electrophoresis and a partially real-time PCR array method [72], to clarify whether these GM events are present as single or stacked events and which event is present in the genomic DNA from each kernel.

The percentage of the single GM event population and that of the stacked GM event population in each non-IP maize sample are shown in Table 4-2. The values for the single and stacked GM events are also indicated in the pie charts in Figures 4-1 and 4-2, respectively. For the single GM event grains, MON88017 grains and NK603 grains were mainly detected in four samples (No. 1, No. 2, No.4, No. 5), although MON810 grains were mainly detected in sample No. 3. For the stacked GM event grains, mainly MON88017×MON810 grains and TC1507×DAS59122 grains were detected in four samples (No. 1, No. 2, No. 4, No. 5), although MON810×NK603 grains were mainly detected in sample No. 3.

The total populations of the non-GM grains, single GM event grains and stacked GM event grains in the 1188 kernels of non-IP samples tested in 2009 are shown in Table 4-2. The average percentage of GM grains in the samples was 81.9%, and, in the GM grains, the average percentage of the single GM event grains was 46.9% and that of the stacked GM event grains was 35.0%. These results show that the average ratio (35.0%) of the stacked GM event grain population in non-IP maize samples in 2009 was higher than in 2005 [78] (12.0%). The populations of single GM event grains and stacked GM event grains are indicated in the pie chart of Figure 4-3. For single GM events, MON88017 (39.3%) and the NK603 grains (18.5%) were mainly detected, followed by MON810 (16.0%), TC1507 (9.9%) and DAS59122 grains (5.2%). Most of the

detected stacked GM event grains were MON810×MON88017 (47.6%), followed by the TC1507×DAS59122 (14.9%), MON810×NK603 (14.4%), TC1507×MON88017 (6.0%), TC1507×NK603 (3.8%) and MON810×MON863×NK603 (2.9%).

Table 4-2 Results of the analyses of individual kernels in all grains of the non-IP maize samples in 2009

GM trait	non-IP maize sample lot										Total	
	1		2		3		4		5			
	kernel number	content (%)	kernel number	content (%)	kernel number	content (%)	kernel number	content (%)	kernel number	content (%)	kernel number	content (%)
Bt11	3	1.0	2	0.8	5	2.4	8	3.6	2	0.9	20	1.7
TC1507	4	1.4	16	6.8	15	7.1	7	3.2	13	5.7	55	4.6
MON810	11	3.8	8	3.4	40	18.9	19	8.6	11	4.8	89	7.5
MON863	8	2.8	4	1.7	2	0.9	1	0.5	8	3.5	23	1.9
MON88017	97	33.6	28	11.8	8	3.8	26	11.8	60	26.2	219	18.4
GA21	1	0.3	0	0	1	0.5	2	0.9	0	0	4	0.3
NK603	18	6.2	21	8.9	22	10.4	24	10.9	18	7.9	103	8.7
DAS59122	11	3.8	8	3.4	1	0.5	2	0.9	7	3.1	29	2.4
MIR604	6	2.1	1	0.4	1	0.5	1	0.5	1	0.4	10	0.8
T25	2	0.7	0	0	1	0.5	1	0.5	1	0.4	5	0.4
single GM	161	55.7	88	37.1	96	45.3	91	41.2	121	52.8	557	46.9
Bt11 x MIR604	0	0	0	0	0	0	4	1.8	3	1.3	7	0.6
Bt11 x GA21	2	0.7	0	0	1	0.5	2	0.9	0	0	5	0.4
TC1507 x MON88017	2	0.7	8	3.4	9	4.2	2	0.9	4	1.7	25	2.1
TC1507 x NK603	4	1.4	4	1.7	6	2.8	2	0.9	0	0	16	1.3
TC1507 x DAS59122	21	7.3	17	7.2	0	0	8	3.6	16	7.0	62	5.2
MON810 x MON863	1	0.3	4	1.7	0	0	4	1.8	1	0.4	10	0.8
MON810 x MON88017	42	14.5	54	22.8	24	11.3	52	23.5	26	11.4	198	16.7
MON810 x NK603	7	2.4	8	3.4	26	12.3	16	7.2	3	1.3	60	5.1
MON863 x NK603	0	0	0	0	2	0.9	0	0	0	0	2	0.2
MON88017 x DAS59122	0	0	0	0	0	0	0	0	2	0.9	2	0.2
NK603 x DAS59122	0	0	1	0.4	2	0.9	0	0	2	0.9	5	0.4
Bt11 x MIR604 x GA21	0	0	0	0	0	0	2	0.9	0	0	2	0.2
TC1507 x MON810 x NK603	0	0	0	0	0	0	0	0	0	0	0	0
TC1507 x MON88017 x DAS59122	1	0.3	2	0.8	0	0	1	0.5	0	0	4	0.3
TC1507 x NK603 x DAS59122	0	0	1	0.4	2	0.9	3	1.4	0	0	6	0.5
MON810 x MON863 x NK603	2	0.7	4	1.7	1	0.5	2	0.9	3	1.3	12	1.0
stacked GM	82	28.4	103	43.5	73	34.4	98	44.3	60	26.2	416	35.0
GM	243	84.1	191	80.6	169	79.7	189	85.5	181	79.0	973	81.9
Non-GM	46	15.9	46	19.4	43	20.3	32	14.5	48	21.0	215	18.1
Total	289	100	237	100	212	100	221	100	229	100	1188	100

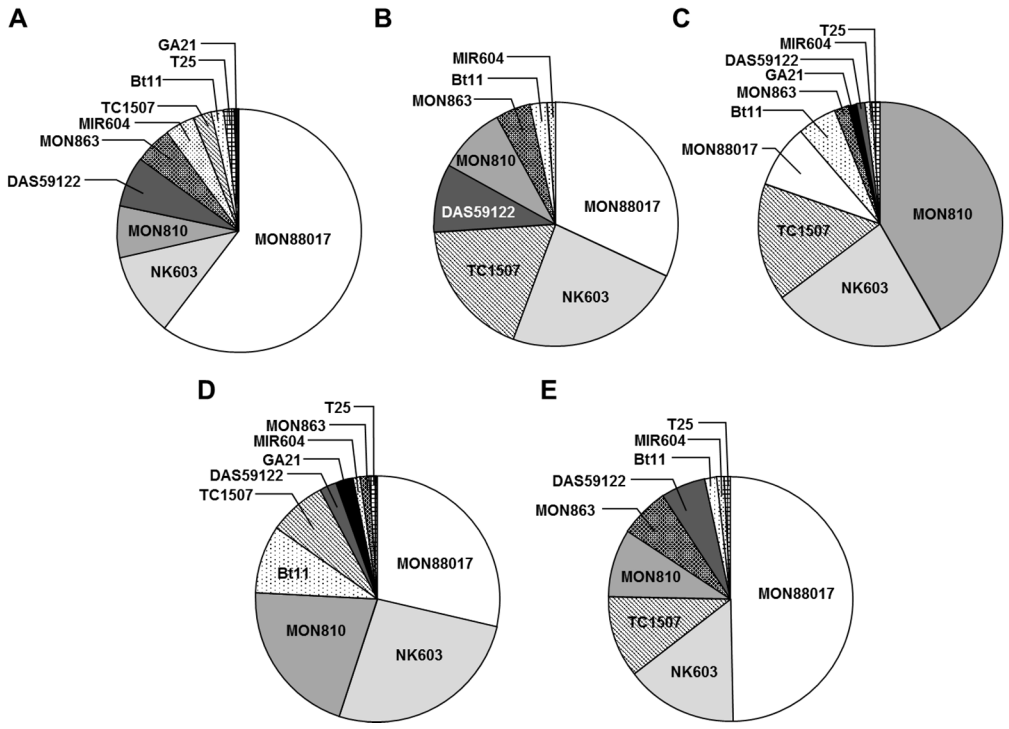

Figure 4-1 Pie chart representing the single GM event grain population in five non-IP maize samples in 2009 A-E show the results for sample, No.1-5, respectively.

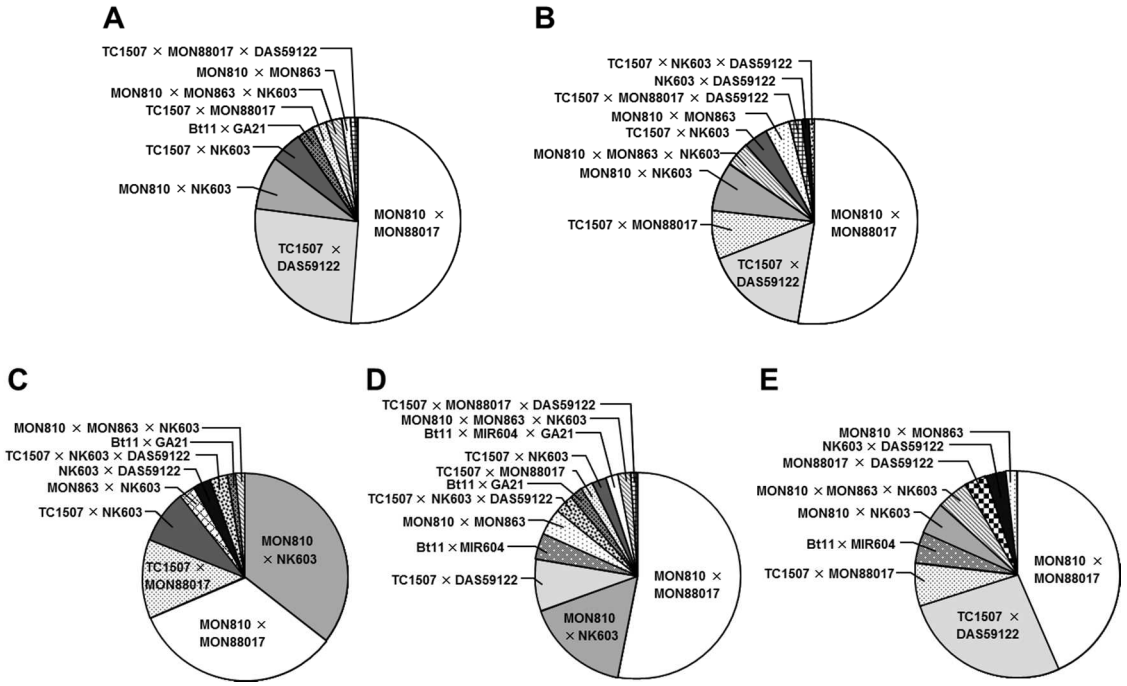

Figure 4-2 Pie chart representing the stacked GM event grain population in five non-IP maize samples in 2009 A-E show the results for sample, No.1-5, respectively.

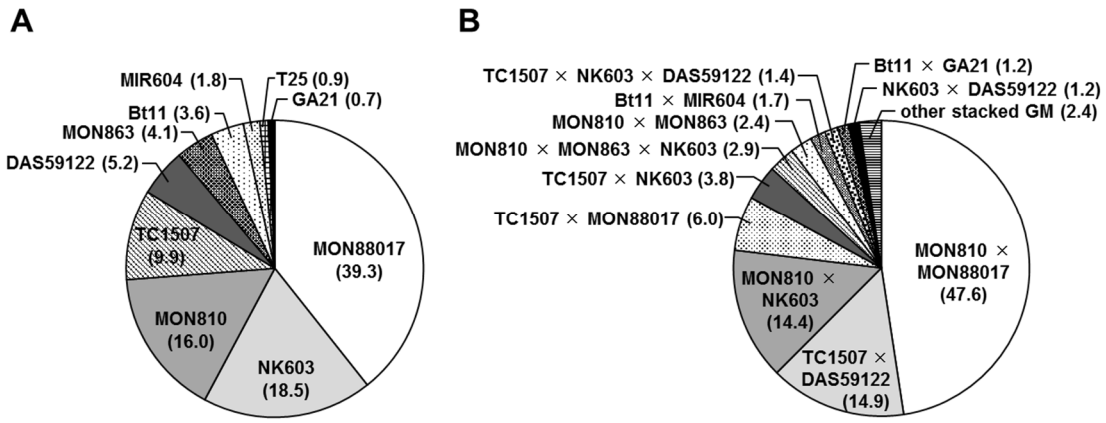

Figure 4-3 Pie chart of average population of single GM event grains (A) and stacked GM event grains (B) in the non-IP maize kernels analyzed. The values in parentheses show the percentage of each population in the single and stacked GM event grains.

4. Discussion

To date, many GM maize events have been authorized for import into Japan. The allowed single GM events include resistance to feeding damage by the European corn borer (ECB) (Event 176 and Bt11 from Syngenta Seeds AG (formerly Novartis Seeds), MON810 from Monsanto Company), resistance to corn rootworm (*e.g.*, MON863 from Monsanto Company), tolerance to the herbicide phosphinothricin (PPT) (*e.g.*, T25 from Bayer Crop Science), resistance to the ECB and tolerance to the herbicide PPT (TC1507 from Pioneer Hi-Bred International, Inc., Mycogen Seeds/Dow AgroSciences LLC), tolerance to the herbicide glyphosate (GA21 and NK603, Monsanto Company), resistance to corn rootworm and tolerance to the herbicide glyphosate (MON88017, Monsanto Company), resistance to corn rootworm and tolerance to the herbicide PPT (DAS59122 from Dow Agro-Sciences LLC/Pioneer Hi-Bred International, Inc.). Furthermore, many stacked event maize varieties (*e.g.,* MON863×NK603, MON810×NK603, MON810×GA21, MON810×T25, MON88017×MON810, TC1507×NK603, MON863×MON810 and MON863×MON810×NK603) have already been authorized in Japan. In this study, we found that GM maize grains of non-IP maize samples produced in 2009 were present at a high level, in the range of 79.7% to 85.5%. In addition, we found that the ratio of the stacked GM event grains in the non-IP maize samples in 2009 was higher than in 2005 [78]. The evidence implies that the cultured area of GM maize in the USA has increased, and in particular, the ratio of the stacked GM maize grains in non-IP maize samples has increased from 2005 to 2009. The National Agricultural Statistics Service (NASS) reported that the percentage of the cultivation area planted with any GM maize events in 2005, 2008 and 2009 in the USA was 52%, 80% and 85%, respectively, and the percentage for stacked GM events in 2005, 2008 and 2009

was 9%, 40% and 46%, respectively [101, 102]. Thus, the percentage of the cultivation area planted with GM maize events has increased substantially from 2005 to 2009. In particular, the area planted with GM maize events in Illinois, Indiana and Ohio has increased more than that in other states. In addition, the percentage of the cultivation area planted with stacked GM maize events has significantly increased from 2005 to 2009. We estimated that our multiplex qualitative PCR analyses can cover almost all the GM maize events cultivated in 2009, since it has been reported that other authorized GM maize events such as DBT41 and DDL25 are not cultivated anymore. Thus, it appears that our results are reasonable and probably reflect the average GM grain mixing in 2009. For the analyses of GM events, we clarified that MON88017 grains (39.3%) and NK603 grains (18.5%) were the major single GM events and that MON88017×MON810 grains were the major stacked GM event in non-IP samples in 2009. We could not follow the MON89034 event because a detection method for the MON89034 event had not yet been developed. However, we considered that MON89034 had not yet been widely cultivated in 2009 because the event was authorized worldwide from 2008 to 2010. This study showed that the major cultured single GM maize event changed from MON810 to MON88017 and the major stacked GM event changed from MON810×NK603 to MON88017×MON810 between 2005 [78] and 2009. The MON88017 event maize has both resistance to corn rootworm and tolerance to the herbicide glyphosate, and MON810 has resistance to the ECB. Therefore, MON88017×MON810 event maize has three advantageous features: resistance to corn rootworm, tolerance to the herbicide glyphosate and resistance to the ECB. Therefore, we presumed that the MON88017×MON810 event maize may be more productive than other conventional events and so the area planted with MON88017×MON810 may have been increased. So far, there is little information on the ratio of areas planted with each GM maize event, or which GM maize event

is the main planted maize in the USA. NASS reported that the percentage of the cultivation area planted with insect-resistant (Bt) traits in 2005, 2008 and 2009 in the USA was 26%, 17% and 17%, respectively, and the percentage of herbicide-tolerant traits in 2005, 2008 and 2009 was 17%, 23% and 22%, respectively [101, 102]. The data do not indicate which events for insect-resistant (Bt) traits or herbicide-tolerant traits were planted. However, the data suggests that herbicide-tolerant traits are becoming more popular than insect-resistant (Bt) traits over the period from 2005 to 2009. In addition, Marra *et al*. reported a survey of the average shares of total corn acres planted by the survey respondents to each GM maize event by agronomic zone in the USA in 2009. The report indicated that MON88017×MON810 (Monsanto's YieldGard VT Triple hybrids) made up the largest share of total planted acres in the survey in 2009 [103]. The stacked GM maize event with the second largest share by agronomic zone varied, with MON810×NK603 (TieldGard Con Borer Roundup Ready hybrids) in the western corn belt agronomic zone (13.8%), and TC1507×DAS59122 (Herculex Xtra) in the central corn belt agronomic zone (8.2%) and the east corn belt agronomic zone (13.1%). Among single GM maize events, NK603 made up 15% of planted corn acres in the western corn belt agronomic zone, 12.2% in the central corn belt agronomic zone, and 13.2% in the east corn belt agronomic zone [103]. According to this information, we considered that MON88017×MON810 is the largest share event and TC1507×DAS59122 or MON810×NK603 is the second largest share event among stacked GM maize events, and NK603 is the largest share event as a single GM maize event, in GM maize planted in the USA in 2009. Considering the survey [103] and Figure 4-3, we presumed that MON88017 grains or MON810 grains mainly detected as single GM events in the present study could be derived from the cultivation of MON88017×MON810 maize seeds. Consequently, it appears that the results of the present analysis of GM maize events are reasonably consistent with

the data on actual planting in the USA in 2009 (Figure 4-3). It should be noted that there are limitations to the present study in terms of estimation of the ratio of GM maize content and GM maize events in a non-IP maize sample. There are many factors that would influence the estimation, such as the time and place of the sampling for non-IP maize. The present study is only based on analyses of limited numbers of samples obtained in 2009 in Japan. However, to our knowledge, this is the first report of analysis of GM maize content and GM maize events in recent maize samples on a kernel basis, except for our study published in 2008 [78]. Although, the samples used in the present study may not be a representative group, they provide important information about the GM maize content and the main GM maize events in non-IP maize samples in 2009. In conclusion, we successfully determined the GM maize grain content on a kernel basis in non-IP maize samples imported from the USA in 2009 using the individual kernel detection system [77, 97]. In addition, we analyzed the GM events in GM maize grains of non-IP maize samples in 2009 using two multiplex qualitative PCR detection methods [98, 100] coupled to microchip electrophoresis and partially real-time PCR array analyses [72]. MON88017 and NK603 were the major single GM events and MON88017×MON810 was the major stacked GM event in the non-IP samples. This type of study should provide useful information on GM maize mixing in imported maize samples on a kernel basis and the method permits precise quantification of the GM maize content in GM maize kernels for labeling regulation. It will be necessary to obtain the latest information on the GM maize ratio and GM maize events in non-IP maize grains to investigate the level of GM mixing and the probability of stacked GM maize mixing.

GENERAL CONCLUSION

In this study, we focused on the four issues (Chapter 1-4) in detecting recombinant DNA in GM crops, and resolved these issues using genetic engineering techniques.

In Chapter 1, we developed a positive control plasmid, pBT63, which can be used for qualitative PCR testing of Bt63 rice. pBT63 was designed to allow an analyst to easily confirm a false-positive result. Validity of the test results can be checked by a straightforward procedure involving the digestion of amplified DNA products using a restriction enzyme and analysis using agarose gel electropheresis. pBT63-derived PCR products were cleaved in two, as designed. Therefore, this approach offers a simple way to distinguish PCR products derived from GM crops from those derived from contamination by pBT63. The risk of incorrectly interpreting false-positive results caused by contamination with the positive control in various qualitative PCR testing procedures would be reduced through development of positive controls similar to pBT63.

In Chapter 2, we developed a novel silica membrane-based DNA extraction method suitable for processed foods. The developed method was evaluated against four conventional methods using six kinds of processed food as analytical samples. The developed method showed wide applicability to various process foods and it gave sufficient amounts of DNA with high purity. Also, the user-friendliness of the method was extremely high because of the short handling time, the small number of pipette operations and non-use of toxic organic solvents. It is expected that our novel method will be practically used in food testing to detect GMOs, allergens and pathogenic microorganisms.

In Chapter 3, we successfully developed a qualitative detection system for the RT73 *B. rapa* canola line. Our system delivered reliable results by single kernel analysis of canola grain

samples that could potentially contain RT73 *B. rapa*. Approximately 92 canola kernels could be individually detected within one day. The proposed system could accurately monitor contamination by RT73 *B. rapa* canola, and might therefore be useful for its governmental regulation.

In Chapter 4, we investigated the GM maize grain content of non-identity preserved (non-IP) maize samples produced in 2009 in the USA using our individual kernel detection system, involving two multiplex qualitative PCR methods and partially real-time PCR array analysis, to clarify how many GM event maize grains were present in the samples and which GM events frequently appeared in 2009. The average percentage and standard deviation of GM maize grains on a kernel basis in five non-IP sample lots were 81.9%±2.8%, the average percentage of single GM event grains was 46.9%, and the average percentage of stacked GM event grains was 35.0%. MON88017 grains and NK603 grains were the most frequently observed as single GM event grains. The most frequent stacked GM event grains were MON88017×MON810 grains. This study shows that our method can provide information about GM maize events present in imported maize samples on a kernel basis.

Methods of the genetic engineering techniques established in our study have been practically applied in the field of recombinant DNA detection in GMO. We hope our accomplishment will be useful in improvement of reliability in GM detection at laboratory.

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