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Indicated Detection of Two Unapproved Transgenic Rice Lines Contaminating Vermicelli Products

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We analyzed the DNA fragments extracted from four rice vermicelli products. The *Bacillus thuringiensis* (Bt) rice line, which has a construct similar to the GM Shanyou 63 line, was detected in some vermicelli products by identification of the junction region sequence between rice *Act1* promoter and the *Cry1Ac* gene, and that between *Cry1Ac* and *nos*. In addition, we also detected a different Bt rice line by means of the junction region sequence between the maize ubiquitin promoter and *cry1Ab* gene and that between the cauliflower mosaic virus 35S promoter and the hygromycin phosphotransferase in some vermicelli products. Accordingly, we for the first time have detected the two transgenic Bt rice lines contaminating rice vermicelli samples. Furthermore, we developed a duplex real-time polymerase chain reaction (PCR) method for the simultaneous detection of both Bt rice lines.

KEYWORDS: Genetically modified rice; Bt toxin; detection method; real-time PCR; rice vermicelli; *Bacillus thuringiensis*

INTRODUCTION

In recent years, there has been great progress in food biotechnology, including transgenic crop breeding and genetic modification for food production. In some countries, the acceptance of these genetically modified (GM) foods by consumers is still controversial, and concerns about their safety persist among the public. GM foods have been authorized for food and/or feed by many countries based on their own criteria for safety assessment. In the EU, the authorization and use of GM foods and feed is stipulated by the provisions in regulations (EC) 1829/2003 and (EC) 1830/2003 (*1*, *2*). 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 authorized is prohibited from import or sale in Japan. Therefore, qualitative detection methods of regulated and unauthorized GM foods are required for unauthorized GM food regulation. We previously reported qualitative detection methods for GM maize, GM potatoes (NewLeaf Plus, NewLeaf Y), and GM papayas (Line 55-1 or its derivatives), including qualitative polymerase chain reaction (PCR) methods and a histochemical assay (*3*−*10*).

The Bt crops are GM crops in which *cry* genes derived from *Bacillus thuringiensis* (Bt) conjugated with a suitable plant expression promoter and terminator are transformed, expressing the Bt toxin protein to confer tolerance against insects. To date, Bt crops of cotton (*11*), maize (*12*), and potatoes (*13*) that have insect resistance have been commercialized in some countries, including Japan (*14*, *15*). However, no developed Bt rice has yet been authorization for food use in the European Union, Korea, and Japan (*16*−*18*).

In the present study, we analyzed DNA fragments extracted from four rice vermicelli products and detected two lines of unauthorized Bt rice harboring the Bt toxin *cry* gene, one of which has a construct similar to the previously reported GM Shanyou 63 line (*19*−*21*) and the other is an unknown Bt rice line, which has a construct similar to the Kemingdao. Furthermore, we developed a detection system to monitor these Bt rice lines using a real-time PCR method.

MATERIALS AND METHODS

Samples. One rice vermicelli sample (G) was kindly provided by Greenpeace International. Three rice vermicelli samples (A, B, and C) (imported products from China), which were suspected to be contami-

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nated with Bt rice on the basis of testing at a quarantine inspection center, were obtained through the Ministry of Health, Labor, and Welfare (MHLW) of Japan. The rice vermicelli samples (D) imported from Thailand were commercially purchased in Tokyo as the negative control.

Extraction and Purification of Genomic DNA. The samples were ground with an electric mill. DNA extraction and purification were carried out by use of the Nippon Gene GM quicker 2 kit (Nippon Gene, Toyama, Japan) according to the manufacturer's manual with the following modification: The ground samples (500 mg) were suspended in 2.1 mL of GE1 buffer, 60 μL of proteinase K (20 mg/mL), 6 μL of α-amylase, and 30 μL of RNase A (100 mg/mL) by use of a vortex mixer for 30 s and then heated at 65 °C for 30 min. A 255-μL aliquot of GE2-K buffer was added to the mixture, which was sufficiently mixed by use of a vortex mixer, followed by standing on ice for 10 min. After centrifugation at 6000*g* for 15 min at 4 °C, the collected supernatant was transferred into a fresh tube (LF tube; Prescribe Genomics Co., Tsukuba, Japan), and the mixture was centrifuged again at 13000*g* or above at 4 °C for 5 min. To 1 mL of the supernatant placed in a new LF tube were added 375 μL each of GE3 buffer and 2-propanol, and the solution was then gently mixed by being shaken 10−12 times. The mixed solution was applied onto a spin column included in the kit and centrifuged at 13 000*g* and 4 °C for 30 s to discard the eluate. This procedure was repeated until the entire eluate was loaded. The spin column was washed with 650 μL of GW buffer by centrifugation at 13 000*g* and 4 °C for 1 min. The column was transferred to a new tube, 50 μL of TE buffer was added, and the mixture was allowed to stand for 3 min at room temperature. Finally, the tube was inserted into the column and centrifuged at 13 000*g* and 4 °C for 1 min, and the eluate was then used as the DNA sample solution in the following experiments.

Polymerase Chain Reaction. The PCR reaction mixture (25 μL) in the tubes consisted of 2.5 μL of PCR buffer II (Applied Biosystems, CA), 0.16 mM dNTP, (Applied Biosystems), 1.5 mmol/L $MgCl_2$, 1.2 μmol/L 5′ and 3′ primers, and 0.8 unit of AmpliTaq Gold (Applied Biosystems). PCR was performed by preincubation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s, and terminal elongation at 72 °C for 7 min by use of the GeneAmp PCR System 9700 (Applied Biosystems).

To determine the nucleotide sequence of the transgenic construct harbored in the Bt rice, DNA fragments were amplified by PCR with a primer set of actACF3 (5′-GGG GAA TGG GGC TCT CGG ATG TAG-3′) and actACR3 (5′-GGA GAT GTC GAT GGG AGT GTA ACC-3′) for the junction region between the rice actin 1 (*Act1*) promoter sequence and the *cryIAb/cryIAc* fusion gene; a primer set of Oscry1Ac-F (5′-GCA GGA GTG ATT ATC GAC AG-3′) and OsNOS-R2 (5′-AAG ACC GGC AAC AGG ATT CA-3′) for the junction between the *cryIAb/cryIAc* fusion gene and the nopaline synthase terminator (*nos*) sequence; a primer set of Pubi-5 (5′-ATG TTG ATG CGG GTT TT-3′) and Cry1Ab-1 (5′- TCG CGG AGA GCT GGG TTA GTA-3′) for the junction region between the maize ubiquitin promoter (*Pubi*) sequence and the synthetic *cryIAb* gene sequence; and 35S-HPH-1F (5′-ACG TTC CAA CCA CGT CTT CA-3′) and 35S-HPH-3R (5′-CAA AGT GCC GAT AAA CAT AAC GA-3′) for the selection marker genes, by use of DNA extracts prepared from rice vermicelli products as templates.

After PCR amplification, the amplified products were analyzed by agarose gel electrophoresis according to previous reports (*7*−*10*, *18*).

DNA Sequencing. The DNA fragments amplified from rice vermicelli products were extracted and purified by use of a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany), and both strands of the DNA fragment were directly sequenced on an ABI Prism 3700 DNA analyzer (Applied Biosystems). In the case of two different amplified DNA fragments mixed together, after purification of the DNA fragments by use of a QIAquick PCR purification kit, the DNA fragments were treated with T4 polynucleotide kinase followed by T4 DNA polymerase. Each DNA fragment was then ligated with the pUC118/*Hin*cII BAP vector (TaKaRa Bio Inc., Shiga, Japan) and introduced into *Escherichia coli* DH5α. The plasmids containing the inserts were subjected to analysis to determine the nucleotide sequences on the ABI Prism 3700 DNA analyzer (Applied Biosystems). The nucleotide sequences were analyzed with the Lasergene v. 7.0 software (DNASTAR Inc.).

Table 1. Primer and Probe Sequences of Real-Time PCR Systems Developed in the Present Study

name	oligonucleotide sequence (5′−3′)	final concn (nM)
For Simultaneous Detection of Bt Rice Line (like GM63-Taq) and the Other Bt Rice Line (like NGMr-Taq)		
T51-SF	GCAGGAGTGATTATCGACAGTTC	750
OsNOS-R2	AAGACCGGCAACAGGATTCA	750
GM63-Taq	FAM-AATAAGTCGAGGTACCGAGCTCGAATTTCCC-TAMRA	150
NGMr-Taq	VIC-AATGAGAATTCGGTACCCCGACCTGCA-TAMRA	150
For Detection of Taxon-Specific Rice Reference Genes		
SPSF	TTGCGCCTGAACGGATAT	750
SPSR	CGGTTGATCTTTTCGGGATG	750
SPS-Taq	FAM-GACGCACGGACGACGGCTCGGA-TAMRA	300

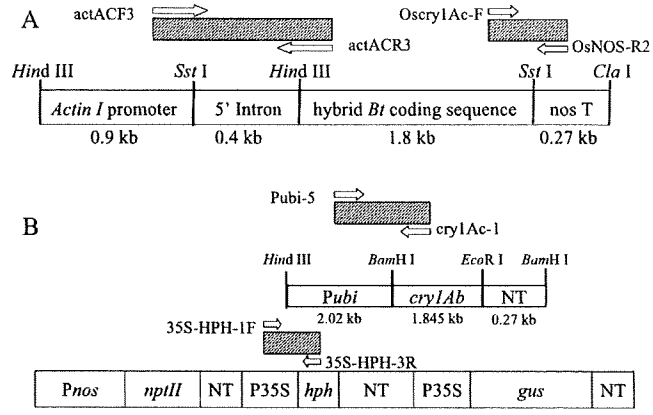

Figure 1. Diagrams of construct pFHBT1 (**A**) inserted in the GM Shanyou 63 line and construct pKUB (**B**) inserted in the Kemingdao line. The primers used in this study to generate PCR products suitable for DNA sequencing are indicated by arrows. Location of sequences taken for the alignments shown in **Figures 2**–**4** are indicated by the hatched boxes.

Real-Time PCR. Real-time PCR was performed on an ABI Prism 7900 instrument (Applied Biosystems). All reactions were run as duplicates in 96-well plates. PCR reaction mixtures were placed in a 25-μL final volume containing 50 ng of the template DNA, 12.5 μL of the universal master mix (Applied Biosystems), 0.75 μM primer pair, and two kinds of probes (150 nM) designed by the Primer Express 2.0 software (Applied Biosystems) (**Table 1**). The reaction conditions included the initiation step for 10 min at 95 °C, followed by 45 cycles of 20s at 95 °C and 1 min at 60 °C. The primer and probe sequences for the detection of the sucrose phosphate synthase (*SPS*) gene, a taxon-specific rice reference gene, were used as previously reported (*22*).

RESULTS

Identification of GM Rice Lines Contaminating Rice Vermicelli. After Greenpeace announced the contamination of rice vermicelli products by Bt rice, we began to establish a DNA extraction method from the rice vermicelli products and analyzed the nucleotide sequence of the transgene for the predicted GM Shanyou 63 line, using the rice vermicelli G sample provided by Greenpeace.

According to previously published reports, we examined the construct of the transgenic DNA sequence for the GM Shanyou 63 line as shown in **Figure 1A**. Some researchers have already reported the junction region in the construct of the transgenic DNA sequences of the GM Shanyou 63 line (*16*, *17*). We

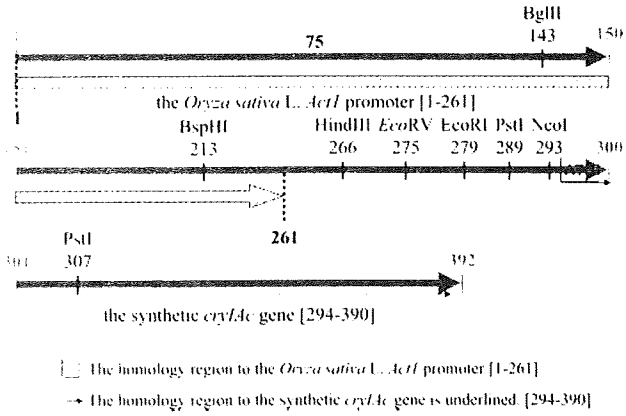

Figure 2. Nucleotide sequence alignments of PCR products generated with genomic DNAs extracted from rice vermicelli G and A samples for the junction region between the rice *Act1* promoter sequence and the *cry1Ac* gene. Restriction enzyme sites are marked.

therefore attempted to amplify some construct-specific fragments from the rice vermicelli sample DNAs using various primer pairs. Several different primer combinations targeting the presumed transgenic construct inserted in the GM Shanyou 63 were used to generate PCR products for direct DNA sequencing in the rice vermicelli samples. The nucleotide sequences of these products were analyzed by use of BLASTN. The two regions selected for alignments of the transgenic sequences with the identical GenBank sequences are shown in **Figures 2** and **3**. For the DNA fragments obtained from samples G, A, and C by use of the primer set actACF3/actACR3, as shown in **Figure 2**, the 5′ sequence part [1−261] of the amplified fragment showed 100% homology to the rice *Act1* promoter sequence reported previously (*23*, *24*). The sequence was followed by a 32-bp fragment containing multiple restriction enzyme sites. The next 96-bp region [294−390] showed 100% homology to a synthetic *cry1Ac* gene (GenBank accession number Y09787). The sequences consistent with the parts of plasmid pFHBT1 were used for the production of the GM Shanyou 63 line (*17*). In addition, we attempted to generate PCR products in the junction region between the *cry1Ab/cry1Ac* fusion gene and *nos* from the genomic DNAs extracted in all the rice vermicelli samples, using the Oscry1Ac-F/OsNOS-R2 primer pair designed by Kim et al. (*16*). The approximate expected stretched PCR products can be detected in all the rice vermicelli samples. In the PCR products from the rice vermicelli G and A samples, the direct DNA sequence was consistent with that previously reported for pFHBT1 of the GM Shanyou 63 line (*16*, *17*). However, in the PCR product sequences cloned from the rice vermicelli B sample, the junction region sequences between the *cry1Ab/cry1Ac* fusion gene and *nos* were different from that of GM Shanyou 63. These sequences are shown in **Figure 3**. Both the 5′ sequence part [1−61] and the 3′ sequence part [91−147] of the amplified fragment showed 100% homology to those previously reported for pFHBT1. The middle part [62−90] of the major amplified fragments cloned from the rice vermicelli B sample PCR products (142 bp) is slightly shorter than that (147 bp) of the GM Shanyou 63. As shown in **Figure 3**, the sites and varieties of the restriction enzyme digestion in the middle part [62−90] of the amplified fragment are different from that of the GM Shanyou 63. In rice vermicelli C sample, we obtained 91 clones from the amplified products in terms of the junction sequences between the *cry1Ab/cry1Ac* fusion gene and *nos*. Fourteen of these clones are the sequence derived from pFHBT1, and 77 of these clones are predicted to be the sequence derived from another unknown Bt rice line construct. These results suggest that the rice vermicelli C sample contains both Bt rice lines, one similar to the GM Shanyou 63 line and the other an unknown Bt rice line.

Since the unknown Bt rice line could be predicted to be the Kemingdao line, which has the *Pubi*-driven *cry1Ab* gene (**Figure 1B**) (*25*), we attempted to generate PCR products in the junction region between *Pubi* and *cry1Ab* from the genomic DNAs extracted from the rice vermicelli B samples, using the designed primer pair combinations. As shown in **Figure 4**, the 5′ sequence portion [7−451] of the amplified fragment shows 98% homology to the maize polyubiquitin gene sequence (GenBank accession number S94464). The sequence is followed by a 21-bp fragment containing multiple restriction enzyme sites. The next 365 bp region [471−836] shows 79% homology to a *B. thuringiensis cry1Ab* gene for insecticidal crystal protein gene (GenBank accession number X54939). Furthermore, as shown in **Figure 5**, the 5′ sequence part [17−136] of the amplified fragment shows 99% homology to the cauliflower mosaic virus 35S promoter (*CaMV*) sequence (GenBank accession number S51061). In addition, the 3′ sequence part [162−376] of the amplified fragment shows 100% homology to the plasmid pJR225 *E. coli* hygromycin phosphotransferase (*hph*) gene (GenBank accession number K01193). These results suggest that the junction region sequence between the *Pubi* and *cry1Ab* gene and the junction region between the *CaMV* and *hph* gene could be detected by use of the designed primer pair combinations in the rice vermicelli B and C samples, not in the rice vermicelli G and A samples.

Duplex Construct-Specific Detection of Two Unauthorized Bt Rice Lines in Rice Vermicelli. To simultaneously detect the two different Bt rice lines contaminating the rice vermicelli samples, we attempted to develop a duplex real-time PCR method. On the basis of the two-line sequence data identified from the rice vermicelli A and B samples, two probes were designed for the specific sequence of the Bt rice lines. We first confirmed that the amplification curves of SPS-Taq labeled with the FAM dye were detected in all the rice vermicelli product DNAs for evaluation of the quality of the extracted genomic DNAs. In addition, to discriminate both lines, we designed a probe for the detection of the similar construct rice line of GM Shanyou 63 labeled with the FAM and TAMRA dyes (GM63-Taq) and a probe for the detection of the unknown Bt rice line labeled with VIC and TAMRA (NGM-Taq). Consequently, one primer pair and two probes for both line detections were considered to be mixed in one reaction tube to simultaneously detect both Bt lines in the genomic DNA extracted from the rice vermicelli samples. As shown in **Figure 6**, the amplification curves of GM63-Taq labeled with the FAM dye and NGM-Taq labeled with the VIC dye were observed in rice vermicelli products A and B, respectively. The amplification curves of both GM63-Taq and NGM-Taq were observed in rice vermicelli product C, presumably contaminated with both Bt rice lines. These results suggest that rice vermicelli G and A samples primarily contain the similar construct line of GM Shanyou 63;rice vermicelli B sample primarily contains the unknown Bt rice, which has a construct similar to that of the Kemingdao line; and rice vermicelli C sample contains both Bt rice lines.

DISCUSSION

We have for the first time clarified that the unauthorized Bt rice line, which has a construct similar to that of GM Shanyou 63, is contaminating rice vermicelli samples, based on detection of the junction region sequence between rice *Act1* promoter and

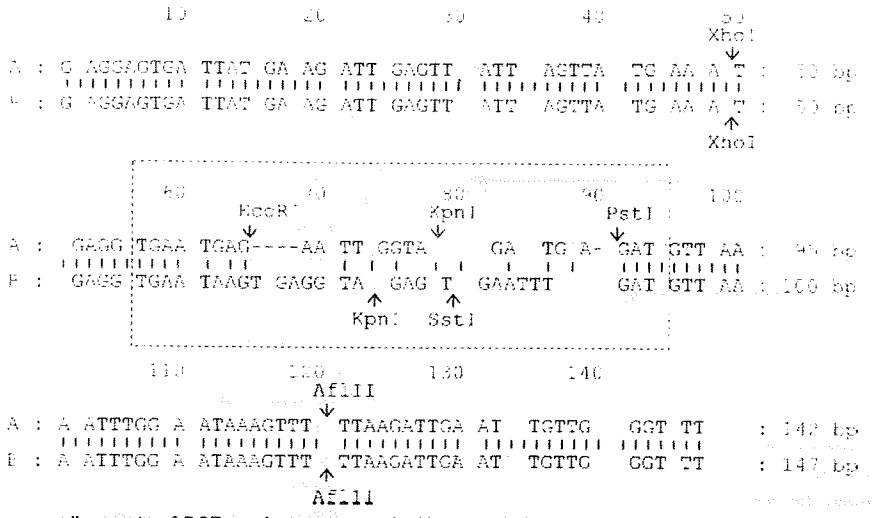

Figure 3. Nucleotide sequence alignments of PCR products generated with genomic DNAs extracted from rice vermicelli G, A, and B samples for the junction region between the *cry1Ab/cryAc* fusion gene and the *nos* sequence. *Xho*I, *Eco*RI, *Kpn*I, *Pst*I, and *Sst*I sites are denoted by arrows.

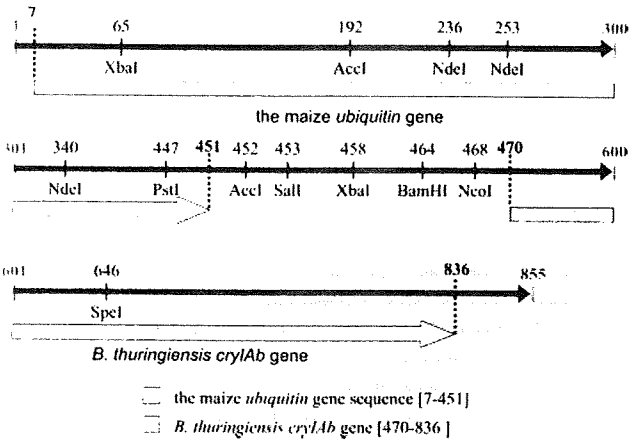

Figure 4. Nucleotide sequence alignments of PCR products generated with genomic DNAs extracted from rice vermicelli B and C samples for the junction region between the *Pubi* and *cry1Ab* genes. Restriction enzyme sites are marked.

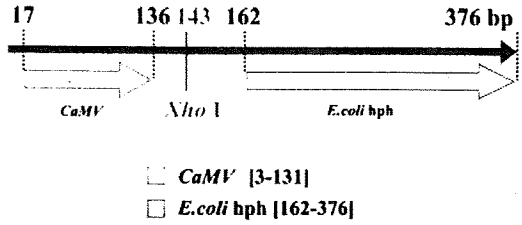

Figure 5. Nucleotide sequence alignments of PCR products generated with genomic DNAs extracted from rice vermicelli B and C samples for the junction region between the *CaMV* sequence and the *hph* gene. The *Xho*I site at position 143 is marked.

Cry1Ac gene and that between *Cry1Ac* and *nos*. We also detected a junction region sequence between *cry* and *nos* different from that of the GM Shanyou 63 on the PCR fragments in the rice vermicelli B and C samples, as shown in **Figure 3**. The presence of the different junction region indicated that the detected Bt rice line had been developed by use of a different vector, not the pFHBT that is used in GM Shanyou 63. In other words, rice vermicelli B and C samples contained another unknown, but similar, Bt rice line, not GM Shanyou 63. We also suggested that the unknown Bt rice line contaminating the rice vermicelli B and C samples may have the construct of the *Pubi*-driven *cry1Ab* gene due to the detection of the junction region between the *Pubi* and *cry1Ab* gene in them. Furthermore, the junction region between *CaMV* and the selectable marker *hph* could be detected in B and C samplea but not in G and A samples. These results suggest that the *CaMV–hph* sequences would be derived from the unknown Bt rice line contaminating rice vermicelli B and C samples, because the construct in the pFHBT for the GM Shanyou 63 line should not contain *CaMV–hph*, and removal of the sequence by segregation has been reported for the parental elite restorer rice Minghui 63 (**Figure 1B**). The presence of the DNA sequence provides the evidence that the unknown Bt rice line, which has a construct similar to that of the Kemingdao line, might be contaminating the rice vermicelli B and C samples because the *Pubi*-driven *cry1Ab* gene and the 35S promoter-driven *hph* marker gene have been used in plasmid vector pKUB for production of the Kemingdao line (**Figure 1B**) (*25*).

In China, some GM rice varieties have already been developed and tested in the field and environmental trials (*15*, *26*). The Bt rice expresseing the Bt toxin has been developed in China and approved for environmental release trials, and at least two Bt rice lines, GM Shanyou 63 and Kemingdao, entered preproduction trails in 2001 (*19*, *27*). Contamination by the GM Shanyou 63 line has been detected in Chinese rice in the European Union and Korea. However, the Kemingdao line has not yet been detected in rice and rice products. Our findings suggest that the unknown Bt rice line found in this study in rice vermicelli sample might have a construct similar to the Kemingdao line (*25*).

Furthermore, we developed a duplex real-time PCR method for the simultaneous and rapid qualitative detection of both unauthorized Bt rice lines. German researchers have already developed a real-time PCR method for detection of the GM Shanyou 63 line (*17*). They designed a reverse primer on a multiple cloning site in the junction between the *cry1Ab/cry1Ac* gene and *nos*. Therefore, the unknown Bt rice line we found in this study presumably may not be detectable by their method because a reverse primer cannot be annealed, although the GM Shanyou 63 line can be sensitively detected.

However, we cannot identify and estimate the detection limit of the real-time PCR method for both Bt rice lines because we do not have any authentic reference material for the Bt rice lines, and because transgenic sequences in processed food products usually can be degraded. Further studies are required to examine the feasibility of detecting lower levels of the two Bt lines by

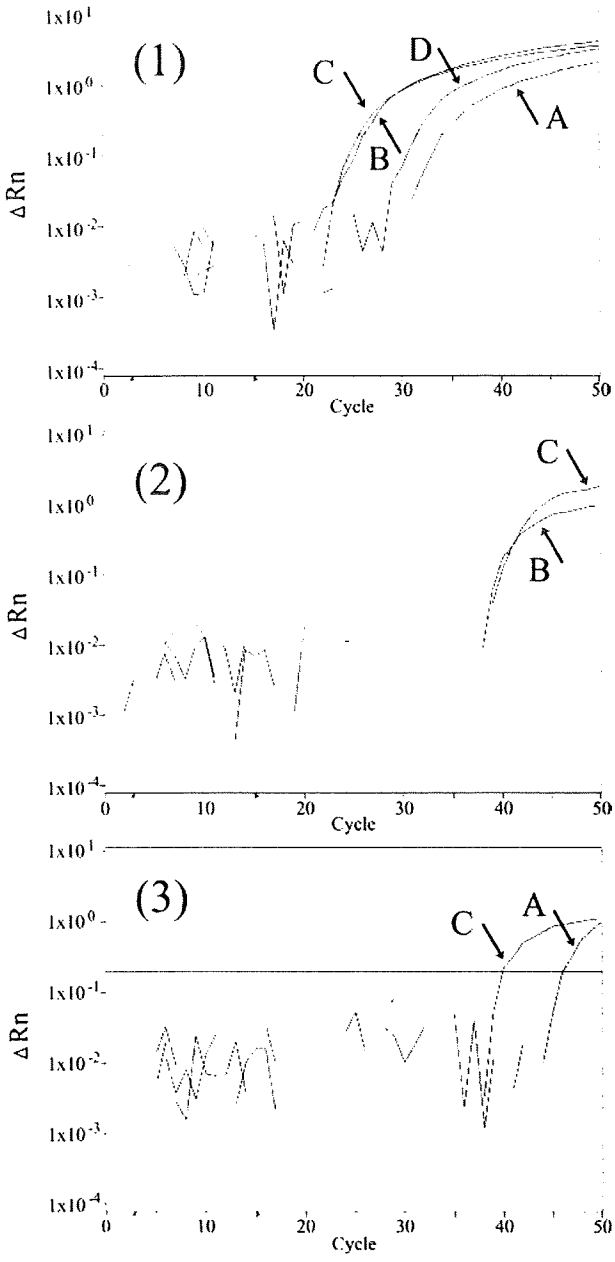

Figure 6. Amplification curves of construct-specific sequences of the two Bt rice lines using the developed real-time PCR method. **(Panel 1)** SPS gene detection in all rice vermicelli samples. **(Panel 2)** Specific detection for unknown Bt rice, which has a construct similar to that of Kemingdao in the rice vermicelli samples B and C. **(Panel 3)** Specific detection for GM unknown Bt rice, which has a construct similar to Shanyou 63 specific detection in the rice vermicelli sample. **(A)** Rice vermicelli sample A containing the Bt rice line, which has a construct similar to that of the GM Shanyou 63 line; **(B)** rice vermicelli sample B containing an unknown Bt rice line, which is similar to the Kemingdao; **(C)** rice vermicelli sample C, containing the two Bt rice lines; **(D)** rice vermicelli sample D, containing non-GM rice.

use of reference materials and to extend the applications of the developed method to more complex processed food products.

In conclusion, we detected, for the first time, two transgenic Bt rice lines contaminating rice vermicelli samples. One was the Bt rice line, which has a construct similar to that of the GM Shanyou 63 line, and the other was an unknown Bt rice line, which is similar to the Kemingdao line. In addition, we developed a duplex real-time PCR method for the simultaneous detection of both Bt rice lines. We consider this developed method to be a reasonable assay for monitoring Bt rice in processed food products.

ABBREVIATIONS USED

GM, genetically modified; Bt, *Bacillus thuringiensis*; *nos*, nopaline synthase terminator; *Pubi*, maize ubiquitin promoter; *CaMV*, cauliflower mosaic virus 35S promoter; *SPS*, sucrose phosphate synthase; *hph*, hygromycin phosphotransferase.

ACKNOWLEDGMENT

We are grateful to Greenpeace International for providing the rice vermicelli G sample.

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A Screening Method for the Detection of the 35S Promoter and the Nopaline Synthase Terminator in Genetically Modified Organisms in a Real-Time Multiplex Polymerase Chain Reaction Using High-Resolution Melting-Curve Analysis

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To screen for unauthorized genetically modified organisms (GMO) in the various crops, we developed a multiplex real-time polymerase chain reaction high-resolution melting-curve analysis method for the simultaneous qualitative detection of 35S promoter sequence of cauliflower mosaic virus (35SP) and the nopaline synthase terminator (NOST) in several crops. We selected suitable primer sets for the simultaneous detection of 35SP and NOST and designed the primer set for the detection of spiked ColE1 plasmid to evaluate the validity of the polymerase chain reaction (PCR) analyses. In addition, we optimized the multiplex PCR conditions using the designed primer sets and EvaGreen® as an intercalating dye. The contamination of unauthorized GMO with single copy similar to NK603 maize can be detected as low as 0.1% in a maize sample. Furthermore, we showed that the present method would be applicable in identifying GMO in various crops and foods like authorized GM soybean, authorized GM potato, the biscuit which is contaminated with GM soybeans and the rice which is contaminated with unauthorized GM rice. We consider this method to be a simple and reliable assay for screening for unauthorized GMO in crops and the processing food products.

Key words maize; nopaline synthase terminator; genetically modified organism; real-time multiplex polymerase chain reaction; high-resolution melting-curve analysis; 35S promoter

In recent years, many types of genetically modified organisms (GMO), including microorganisms, animals and plants, have been put into practical use, and the number of commercially available genetically modified (GM) crops is increasing rapidly.[1] In Japan, over 70 lines of GM crops have been approved for open field cultivation or as food, feed and ornamental plants and classified as living modified organisms (LMO) under the Cartagena Protocol domestic law that came into effect in Japan on January 31, 2008.

Due to the commercialization, other GMO that have not been approved for human consumption, such as those intended for industrial processes, bioremediation, or the production of pharmaceuticals, may enter the market. Therefore, the ability to trace these organisms or to verify their absence in food will need to be assured.

GM foods have been authorized for food and/or feed by many countries based on their own criteria for safety assessment. In the EU, the authorization and use of GM foods and feed are stipulated by the provisions in regulations (EC) No. 1829/2003 and (EC) No.1830/2003.[2,3] Japan 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 authorized is prohibited from import or sale in Japan. Therefore, qualitative detection methods of regulated and unauthorized GM foods are required to regulate unauthorized GM food. Previously, we reported the development of qualitative detection methods for GM maize, GM potatoes (NewLeaf Plus, NewLeaf Y), GM papayas (Line 55-1 or its derivatives) and GM rice (LL rice and Chinese Bt rice lines), including qualitative polymerase chain reaction (PCR) methods and a histochemical assay.[4—13] Especially, in China, some GM rice varieties have been developed and tested in the field and environmental trials.[14—16] Bt rice expressing the Bt toxin has been developed in China and approved for environmental release trials. We have detected at least two Bt rice lines, 'GM Shanyou 63' and 'Kemingdao,' which entered pre-production trails in 2001[16,17] and developed the detection methods for these rice.[7]

To date, no strategic method for the detection of the unauthorized GMO has been fully discussed and internationally accepted. However, to screen for unauthorized GMO in the various crops, several theoretical approaches have been proposed. One of these approaches, the "indirect subtractive approach," is based on the presumption that a positive screening test and no authorized GMO in a sample constitutes indirect evidence for the presence of unauthorized GMO.[18] In another approach, Cankar *et al.* reported the detection of unauthorized GMO based on differential quantitative PCR, which is an extension of qualitative differential PCR for detecting the 35S promoter sequence of cauliflower mosaic virus (35SP).[19] The application of this approach has been limited to a case study of GM maize events.

While, high-resolution melting-curve analysis (HRM) is a simple and cost-effective post-PCR technique that can be used.[20] The technique requires the use of standard PCR reagents and double-stranded DNA (dsDNA)-binding dyes that are used at saturating concentrations without inhibiting PCR amplification.

In the present study, we developed a screening detection method for both 35SP and the nopaline synthase terminator

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(NOST) in GM crops and the processed foods using HRM with EvaGreen® as an intercalator in a real-time multiplex PCR to predictably detect unauthorized GMO.

MATERIALS AND METHODS

Samples The rice sample (imported product from China), which was suspected to be contaminated with Bt rice based on testing at a quarantine inspection center, and the biscuit, which was contaminated with GM soybean, were obtained through the Ministry of Health, Labor and Welfare (MHLW) of Japan. The biscuit consisted mainly of wheat ingredients. Rice produced in Japan was used as a negative control and was purchased commercially in Tokyo. The certified reference materials of the representative GM maize line, NK603 (0% (w/w, non-GM), 0.1%, 0.5%, 1%, 2% and 5%) in powder and the representative GM soybean line, 40-3-2 (Roundup Ready Soybean) were purchased from the Institute for Reference Materials and Measurement (IRMM; Retieseweg, Belgium). GM potato (New Leaf) was kindly provided by the Monsanto Co. (St. Louis, MO, U.S.A.). The ColE1 plasmid was purchased from NIPPON GENE (Toyama, Japan).

Extraction and Purification of Genomic DNA The samples were ground using an electric mill. All the plant genomic DNA, except for the rice, was extracted using a silica-gel membrane-type kit (DNeasy Plant Mini; QIAGEN, Hilden, Germany). The homogenized samples (each 1 g) were incubated at 65 °C for 10 m in 10 ml of the buffer AP1 (QIAGEN) and 20 μl of RNase A (100 mg/ml; QIAGEN), and mixed 2—3 times during incubation by inverting the tube. After adding buffer AP2 (QIAGEN), the resultant mixture was incubated for 10 min on ice. The mixture was centrifuged at 4 °C for 20 min at 4000×***g***. The clear supernatant was transferred to a QIA shredder spin column (QIAGEN) and centrifuged for 4 min at 10000×***g***. The mixture was pipetted after adding 1.5 volumes of buffer AP3/ethanol, and was applied to a mini spin column. The column was centrifuged for 1 min at 10000×***g***, the flow-through was discarded, and the column was washed 3 times with buffer AW (QIAGEN). The DNA was then eluted twice from the column with 70 μl of the pre-warmed DW. The rice DNA extraction and purification were carried out using the NIPPON GENE GM quicker 2 kit (NIPPON GENE) according to the manufacturer's manual with the following modification. The ground samples (500 mg) were suspended in 2.1 ml of buffer GE1, 60 μl of proteinase K (20 mg/ml), 6 μl of α-amylase solution (attached in kit) and 30 μl of RNase A (100 mg/ml) using a vortex mixer for 30 s and then heated at 65 °C for 30 min. A 255 μl aliquot of the buffer GE2-K was added to the mixture and sufficiently mixed using a vortex mixer followed by standing on ice for 10 min. After centrifugation at 6000×***g*** for 15 min at 4 °C, the collected supernatant was transferred into a new tube, and centrifuged again at 13000×***g*** or above at 4 °C for 5 min. To 1 ml of the supernatant placed in a new tube, 375 μl of both the buffer GE3 and isopropanol were added, and then the solution was gently mixed by being shaken 10—12 times. The mixture was applied onto the spin column included in the kit and centrifuged at 13000×***g*** and 4 °C for 30 s to discard the eluate. This procedure was repeated until the entire eluate was loaded. The spin column was washed with 650 μl of the buffer GW by centrifuging at 13000×***g*** for 1 min. The column was transferred to a new tube and 50 μl of the TE buffer was added and allowed to stand for 3 min at room temperature. Finally, the tube with the spin column was centrifuged at 13000×***g*** and 4 °C for 1 min, and the eluate was then used as the DNA sample solution in the following experiments. The extracted DNA was diluted with an appropriate volume of DW to a final concentration of 10 ng/μl, and stored at −20 °C until use.

PCR Conditions The PCR reaction mixture (25 μl) in the tubes contained 50 ng genomic DNA, 2.5 μl of PCR buffer II (Applied Biosystems, CA, U.S.A.), 0.16 mM of deoxyribonucleotide triphosphate (dNTP) (Applied Biosystems), 1.5 mmol/l $MgCl_2$, 1.2 μmol/l of 5′ and 3′ primers (P35S 1—5′ and P35S 2—3′ for 35SP, NOS ter 3—5′ and NOS ter 3—3′ for NOST, ColE1 F1 and ColE1 R1 for ColE1), 2 μl of 5×10^{-2} pg/μl ColE1 plasmid (approximately 13.9×10^3 copies/reaction mixture) and 0.8 units of AmpliTaq Gold (Applied Biosystems). PCR was performed by pre-incubation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s, and terminal elongation at 72 °C for 7 min using the GeneAmp PCR System 9700 (Applied Biosystems). After PCR amplification, the amplified products were analyzed by agarose gel electrophoresis according to the previous reports.[4,7,9—12]

Multiplex Real Time PCR Using HRM The multiplex real time PCR reaction mixture (20 μl) in the tubes contained 20 ng genomic DNA, 1X Multiplex PCR Mix (QIAGEN, Hilden, Germany), 0.5X Q-solution, (QIAGEN), 1X EvaGreen® (WAKO, Tokyo, Japan), and 0.2 μmol/l of 5′ and 3′ primers (P35S 1—5′ and P35S 2—3′ for 35SP, NOS ter 3—5′ and NOS ter 3—3′ for NOST, ColE1 F1 and ColE1 R1 for ColE1). The reaction was performed by pre-incubation at 95 °C for 15 min, followed by 50 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 90 s (45 s for uniplex real time PCR), extension at 72 °C for 90 s, and terminal elongation at 72 °C for 10 min. After the latter run, the sample was held at 95 °C for 30 s and then held at 72 °C for 5 s, and melt curve data was generated by raising the temperature from 72 to 97 °C at 0.3 °C increments, while pausing for 5 s per step using the Rotor gene™ 6000 (QIAGEN). Positive identification of an isolate containing a gene target was determined by the corresponding melt temperature. The Rotor gene™ 6000 software (Ver 1.7) calculates melt peaks by plotting the negative first derivative of the primary melt curve (fluorescence *vs.* temperature).

RESULTS

Design of the PCR Systems and the Selection of EvaGreen® as an Intercalator Dye We selected primers that were appropriate for the simultaneous detection of 35SP and NOST in screening for unauthorized GMO. In addition, we further designed the primers to detect ColE1 spiked to assess the validity of the PCR run.[21] The selected primer pairs are described in Table 1. Selection of primers was performed on the basis of the results obtained from conventional PCR and electrophoresis using the NK603 maize sample. Electrophoresis analysis showed no formation of primer dimers

(Fig. 1). Next, we examined SYBR® Green I and EvaGreen® as intercalating dyes. Both fluorescence dyes provided similar amplification and melting curve data, although the shape of the melting curve using EvaGreen® was detected more clearly (data not shown). Considering the reliability and sensitivity of the analysis, we chose to use EvaGreen® in further experiments.

Table 1. Primer Sequences and Product Tm for the Multiplex Real Time PCR

Name	Oligonucleotide sequence	Product Tm (°C)
P35S 1—5'	ATT GAT GTG ATA TCT CCA CTG ACG T	80.18
P35S 2—3'	CCT CTC CAA ATG AAA TGA ACT TCC T	80.18
NOS ter 3—5'	GTC TTG CGA TGA TTA TCA TAT AAT TTC TG	77.09
NOS ter 3—3'	CGC TAT ATT TTG TTT TCT ATC GCG T	77.09
ColE1 F1	CGG TTA CTT GAA CGC TGT GA	90.59
ColE1 R1	CAC CTT ACG GGC TGT CTG AT	90.59

In uniplex PCR, the specific amplification of either 35SP, NOST or ColE1 was performed on the NK603 maize and ColE1 plasmid. All sequences were correctly identified as possessing the correct sequence for each primer set as observed by distinct melt peaks at specific temperatures. Melt peak height and shape were consistent for each sequence target. Melt temperatures relative to one another were in agreement with the expected amplicon size, with larger amplicons exhibiting higher Tms. Non-GM rice sample was considered negative due to the melt profiles.

Multiplex PCR Systems We examined the suitability of the proposed real-time PCR systems to be used in a Tm melting curve determination. After careful analysis of the respec-

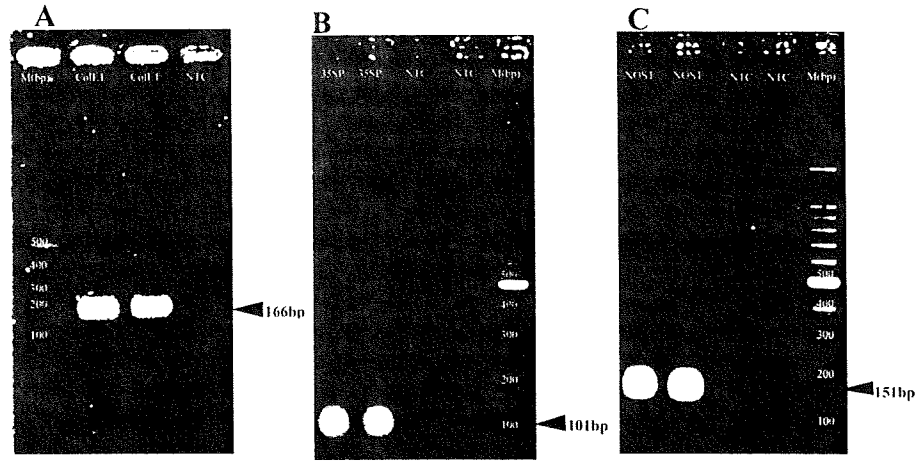

Fig. 1. Targeted Gene Amplification by PCR

PCR reactions were run using total genomic DNA from GM maize line (NK603) as the template and primers that were designed specific to ColE1 (A), 35SP (B) and NOST (C), and analyzed by 3% (w/v) agarose gel electrophoresis in TAE buffer. Arrows indicate the amplified PCR products stained by ethidium bromide. NTC, non-template control; M, 100 bp DNA marker.

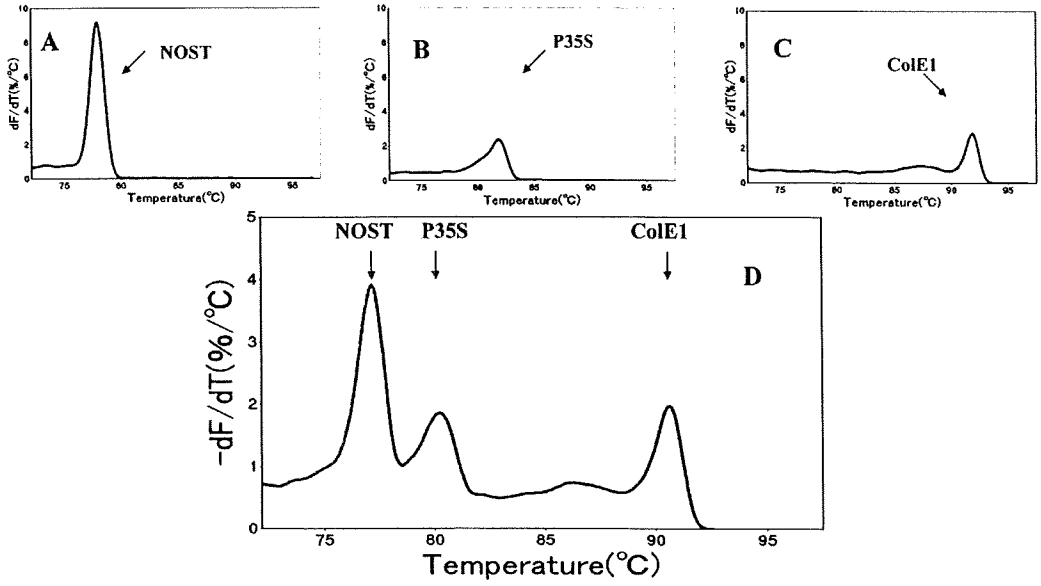

Fig. 2. Melting Curve of Each Uniplex PCR Assay for NOST (A), P35S (B), ColE1(C) and 3-Plex PCR Assay (D)

NK603 maize genomic DNA was amplified as the template by thermal conditions (initial denaturation at 95 °C for 15 m, followed by 50 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 90 s and extension at 72 °C for 90 s, and terminal elongation at 72 °C for 10 min). Melting curves were analyzed by Rotor gene™ 6000 using EvaGreen® as an intercalating dye, and the corresponding Tm was assigned to each product by arrow.

tive experimental Tm, we optimized the PCR conditions in the multiplex condition using the NK603 maize sample. In particular, we examined the time of the annealing step since that step is critical for the optimization of the multiplex condition. We found that 90 s was the optimal time for annealing in the multiplex format. Multiplex reactions using the 3 primer sets in the same reaction tube showed positive melting curves in the sample solution prepared from NK603 maize sample. For the examination of the level of spiked ColE1, we found that the optimized level of the spiked ColE1 is approximately, 2 μl of 5×10^{-2} pg/μl ColE1 plasmid (approximately 13.9×10^{3} copies) in the PCR reaction mixture (25 μl).

The Tm of the melt curve profile can identify which target sequences were amplified by PCR (Fig. 2). Peaks were clearly separated from one another and the average Tms of the different peaks were significantly different in the multiplex reaction. On the other hand, no significant shifts of the average Tms of 35SP, NOST and ColE1 were observed between the uniplex and multiplex reactions. The sample solution prepared from non-GM maize did not produce the correct amplicon when viewed on agarose gels and did not show the correct melt profiles.

To assess the sensitivity of the proposed method using the NK603 maize samples, we tested different amounts of the NK603 maize samples: 0% (non-GM), 0.1%, 0.5%, 1%, 2% and 5%. As shown in Fig. 3, all of the positive melting curves were obtained stably except for the 0% sample. These results suggest that the contamination of unauthorized GMO with single copy of the transgenic gene similar to NK603 maize as low as 0.1% can be detected in a maize sample.

Application to Various Other Samples To assess the application of the established method to other crops, the authorized GM soybean (Roundup Ready Soybean), the authorized GM potato (NewLeaf), the biscuit which is contaminated with GM soybean and the rice which is contaminated with unauthorized GM rice were analyzed using the established method. As shown in Fig. 4, the positive melting curves for 35S and NOST were detected in GM soybean,

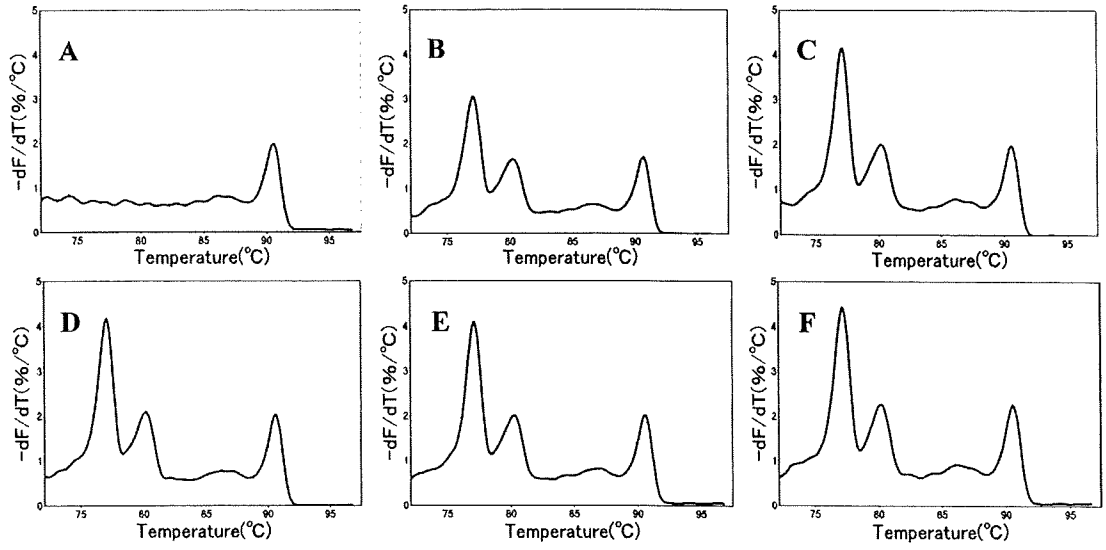

Fig. 3. Sensitivity of the 3-Plex Realtime PCR

The melting curves were tested for detection of different amounts of NK603 maize samples: A) 0% (w/w, non-GM), B) 0.1% (w/w), C) 0.5% (w/w), D) 1% (w/w), E) 2% (w/w) and F) 5% (w/w).

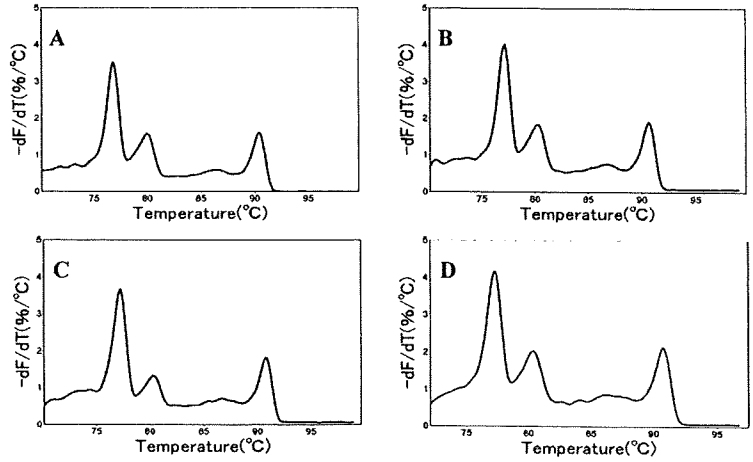

Fig. 4. Application to Other GM Products for Detection

Melting curves represent 3-plex PCR using total genomic DNA samples from: A) GM soybean (Roundup Ready Soybean), B) GM potato (NewLeaf), C) rice contaminated with unauthorized GM rice and D) biscuit contaminated with GM soybean of unknown amount.

GM potato, the biscuit which is contaminated with GM soybean and the rice which is contaminated with unauthorized GM rice. The melt profiles did not produce peaks, except for ColE1 as a reference, in non-GM soybean, non-GM rice and non-GM potato as negative samples (data not shown). These results suggest that the method established here may be applicable in identifying GMO in various crops and foods.

DISCUSSION

A simultaneous qualitative detection method for 35SP and NOST using HRM was developed for the screening detection of unauthorized GMO in several crops. We selected suitable primer sets for the simultaneous detection of 35SP and NOST and designed the primer set for the detection of spiked ColE1 plasmid to evaluate the validity of the PCR analyses. In addition, we optimized in the multiplex PCR conditions using the designed primer sets and EvaGreen® as an intercalating dye. We considered that using EvaGreen® would be more accurate because EvaGreen® is a generally less inhibitory toward PCR and less likely to cause nonspecific amplification compared with SYBRGreen I®.

Simple and accurate screening methods for detection are necessary to search the unauthorized GM crops in various foods. Many studies have employed PCR to target multiple genes for the detection of GM crops. However, conventional PCR requires post-PCR processing to visualize the amplification products, and usually including agarose gel electrophoresis and nucleic acid staining. The handling of PCR products is not only time-consuming, but also has the potential of cross-contamination of the amplified products. Real-time PCR has solved many of these problems, allowing faster cycling times and closed-tube detection during the amplification process. Previous studies have demonstrated the detection of GM crops using specific probes.[7,13] However, the use of fluorescence labeled probes adds significant cost to an assay comprising multiple targets. The closed-tube screening method using HRM has advantages over current techniques because it requires no post-PCR handling, which minimize the risk of PCR contamination, and no separation step, both of which improve analysis time.

The intercalating dyes, such as SYBR® Green I and EvaGreen®, have been used successfully in real-time PCR HRM.[22–24] They bind all amplified dsDNA and do not confer sequence specificity to a desired target as oligonucleotide probes. However, the amplicons can be distinguished by melt curve analysis, whereby the melting temperature of PCR products is determined by the reduction in relative fluorescence as all dsDNA is denatured to the single-stranded form. The shape and peak location of the melt curve are functions of the GC/AT ratio, length, and sequence of the fragment.[23] Therefore, optimized reaction conditions and sufficient differences in amplicon length and GC/AT ratio are necessary to discriminate clearly between amplification products in a melt analysis. A real-time PCR HRM using EvaGreen® was developed to simultaneously detect 35SP, NOST and ColE1. Each product differed sufficiently in size and sequence to be distinguished by melt curve analysis and agarose gel electrophoresis.

In addition, we applied the method established in this study to several GM crops. To assess unauthorized GM crop contamination, GM maize, GM soybean, GM rice and the biscuits contaminated with GM soybeans were used as selected targets. We showed that the present method is applicable for GM maize, GM soybean, GM rice and even the detection of GM soybean in the biscuit, which consisted mainly of wheat. The present method has the potential to screen for novel types of unauthorized GM crops, as well as authorized GM crops, since 35SP and NOST are major sequences in GM crops and widely inserted into various GM crops.

Recently, we detected unauthorized GM crops that have become major concerns such as CBH 351 maize, Bt10 maize, LLRice601, and Chinese Bt rice. The present method may not meet the absolute detection limit of unauthorized GM crops, because the contaminated crops may contain unknown recombinant-DNA (r-DNA) sequence or r-DNA sequences with modified nucleotide sequences. However, after confirming the contamination of unauthorized GM crops using the present method, it may be necessary to analyze the detailed construct sequence close to 35SP and NOST using inversed PCR and anchored PCR techniques.

In conclusion, we developed a multiplex real-time PCR HRM method for the simultaneous qualitative detection of 35SP and NOST in several crops. We consider this method to be a simple and reliable assay for screening for unauthorized GMO in crops and the processing food products.

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論　　文
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PCR 法を用いた米加工品の安全性未審査遺伝子組換え米の検知法

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A conventional PCR method to detect recombinant DNA from genetically modified rice lines not approved for use in processed foods

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Abstract

Detection methods using a conventional polymerase chain reaction (PCR) were developed to detect two lines of genetically modified (GM) rice that have not been approved as safe for use in foods in Japan. Two GM rice lines were *Bacillus thuringiensis* (Bt) rice lines (Bt63 and NNBt) in which *cry* genes derived from Bt conjugated with a suitable plant expression promoter and terminator are transformed, expressing the Bt toxin protein confers a tolerance against insects. We designed primer pairs to detect the two lines of GM rice based on the common *cry* gene sequence. We also designed the primer pairs to specifically detect Bt63 and NNBt rice, respectively, on the sequences we had already clarified. The proposed method was applied to the detection of the two Bt rice lines in rice vermicelli and glutinous rice samples. We showed that the present conventional method was a reasonable assay for monitoring two Bt rice lines in processed rice products.

Keywords : 遺伝子組換え米、Bt トキシン、もち米、検知法、*Bacillus thuringiensis*
genetically modified rice, Bt toxin, detection method, PCR, glutinous rice, *Bacillus thuringiensis*

I　緒言

平成 12 年の厚生省告示 232 号、233 号により、安全性審査がされていない遺伝子組換え (GM) 食品並びにそれらを原料とする加工食品が国内で流通しないよう、食品衛生法の規格基準が改正され、安全性審査が法的に義務化された[1,2]。これにより、平成 13 年 4 月から、安全性審査を受けていない GM 食品又はこれを原材料に用いた食品は、輸入、販売等が法的に禁止された。EU や韓国においても GM 食品及び GM 飼料に関して安全性審査の規制が行われている[3,4]。我々は、これまでに PCR を用いた安全性未審査のトウモロコシ[5]、パパイヤ[6]、ジャガイモ[7,8] の検知法について報告してきた。

中国で開発された遺伝子組換え米 (GM Shanyou 63 (Bt63 米)) [9-12] は、殺虫活性を示す *Bacillus thuringiensis* (Bt) 由

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来 Bt toxin タンパク質の遺伝子（CryIAc: *CryIAc* と *CryIAb* の融合 DNA 塩基配列）を導入した遺伝子組換え米である。我が国では Bt63 米に関して食品の安全性未審査であるため、輸入時に米及び米を含む加工品に対しモニタリング検査を行い、国内流通を未然に防ぐ必要がある[13-15]。我々は既に PCR を用いた解析と文献情報や DNA データベース情報に基づいた解析により、中国からの輸入ビーフンから米の Actin I promoter により Bt toxin タンパク質が発現誘導するカセットが挿入されている Bt63 米の混入を同定し、さらにトウモロコシの maize ubiquitin promoter により Bt63 米の Bt toxin タンパク質とほぼ同様の構造である Bt toxin タンパク質（CryIAb）が発現誘導される Kemingdao 系統[16]の Bt 米（NNBt 米）についても検出した[17]。本研究では、我々の解析結果をもとに従来の PCR 法を用いたもち米粉及びビーフン加工品中の Bt63 米と NNBt 米の定性検知法を開発したので報告する。

Ⅱ　実験方法

1. 試料及び試薬

試験試料としてはグリーンピースから提供されたビーフン試料（Bt63 米陽性検体）1 検体及び厚生労働省モニタリング検査において擬陽性と判断されたもち米試料 1 検体、市販の陰性検体（ビーフンともち米）の各 1 検体を用いた。DNA の抽出精製には、㈱ニッポンジーン製シリカゲル膜タイプキットの NIPPON GENE GM quicker 2 を用いた[17]。DNA ポリメラーゼとしてはライフテクノロジーズジャパン㈱製の AmpliTaq™ Gold を、dNTP、10 × PCR bufferII、ならびに塩化マグネシウムは AmpliTaq™ Gold に付属のものを用いた。アガロースはタカラバイオ㈱製 LO3「TAKARA」を用いた。DNA マーカーはタカラバイオ㈱製 100 bp ラダーを用いた。水は日本ミリポア㈱製 Milli-Q Synthesis A10 で精製した超純水を 120℃、20 分の条件でオートクレーブ滅菌したものを用いた。他の試薬はすべて市販特級品を用いた。プライマーの合成は、㈱グライナージャパンに委託し、逆相カートリッジ精製品を用いた。

2. 機器

粉砕機：ミキサーミル MM200（㈱レッチェ製）、恒温槽：ドライサーモユニット DTU-1B（タイテック㈱製）、冷却遠心機：Avanti HP-25（ベックマン・コールター㈱製）、卓上遠心機：KR-1000（フナコシ㈱製）、タッチミキサー：MT-51（ヤマト科学㈱製）、分光光度計：GeneQuant II（GE ヘルスケア・ジャパン㈱製）、サーマルサイクラー：GeneAmp PCR System 9700（ライフテクノロジーズジャパン㈱製）、電気泳動装置：Mupid-α（㈱アドバンス製）、ゲルイメージ解析装置：Diana システム（Raytest 社製）

3. DNA 溶液の調製

試料を粉砕器で均質に細粉砕し、500 mg を DNA の抽出に供した。DNA 抽出は、NIPPON GENE GM quicker 2（㈱ニッポンジーン製）を用い、添付のプロトコールの一部を改変して行った。すなわちポリプロピレン製遠沈管（15 mL 容）に量り採り、GE1 緩衝液 2.1 mL、Proteinase K（20 mg/mL）60 μL、α-アミラーゼ（高濃度品）6 μL および RNase A（100 mg/mL）30 μL を加え、試料塊が無いようにボルテックスミキサーで 30 秒間混合した後、65℃の条件で 30 分間加温した。GE2-K 緩衝液 255 μL を加え、ボルテックスミキサーで十分に混和後、氷上に 10 分間静置した。6,000 x g 以上、4℃の条件で 15 分間遠心した。上清を新しいチューブ（2 mL 容）に移し、13,000 x g 以上、4℃の条件で 5 分間遠心した。次いでその上清を新しいチューブ（15 mL 容）に移し、上清 1 mL に対して GB3 緩衝液 375 μL 及びイソプロパノール 375 μL を添加した後、10 ～ 12 回転倒混和した。混合液を 700 μL ずつ spin column に負荷した後、13,000 x g 以上、4℃の条件で 30 秒間遠心し、溶出液を捨てた。すべての混合液を負荷するまでこの操作を繰り返した。次いで GW 緩衝液 650 μL を負荷し、13,000 x g 以上、4℃の条件で 1 分間遠心し、溶出液を捨てた。spin column を新しいチューブ（1.5 mL 容）に移し、TE 緩衝液 50 μL を加え 3 分間室温で静置した後、13,000 x g 以上、4℃の条件で 1 分間遠心し、得られた溶出液を DNA 試料原液とした。希釈後、230、260、280 および 320 nm における O.D. を分光光度計を用いて測定した。また、O.D. 260 nm の値 1 を 50 ng/μL DNA と規定し DNA 濃度を算出した。更に、抽出された DNA の質的評価を行なうために O.D. 260/280 nm を算出し、DNA の精製度の確認を行った。本吸光度比が 1.7 ～ 2.0 のとき良好な精製が行われたものと判断した。

4. PCR 条件

既報と同様の条件を用いた[7,8]。PCR 反応液は、PCR 緩衝液（タカラバイオ㈱製）、0.20 mmol/L dNTP、3.0 mmol/L 塩化マグネシウム、0.5 μmol/L プライマー及び 0.625 units Taq DNA ポリメラーゼを含む液に、DNA 溶液 2.5 μL を氷中で加え、全量を 25 μL にした。反応条件は以下の通りである。95℃で 10 分間反応後、95℃ 30 秒間、60℃ 30 秒間、72℃ 30 秒間を 1 サイクルとして 40 サイクルの PCR 増幅を行う。次いで 72℃で 7 分間保った後、4℃で保存した。

5. 検出方法

既報と同様に行った[7,8]。泳動には、100 mL 当たり 10 μg のエチジウムブロマイドを含む 2 % アガロースゲルを用いた。PCR 反応液の 7.5 μL を TAE（tris-acetate EDTA）緩衝液中で 100 V 定電圧で電気泳動を行った。次いで、ゲルイメージ解析装置を使用し、UV（312 nm）照射下で画像を取り込み、増幅される DNA の検知を行った。また、予想される長さの DNA が増幅された場合はその増幅産物を QIAquick PCR purification kit（㈱キアゲン製）により精製し、該当する PCR 産物を ABI PRISM 3700 DNA analyzer（ライフテクノロジーズジャパン㈱製）を用いてシークエンス解析を行った。

Ⅲ 結果及び考察

1. DNA 抽出の検討

ビーフンあるいはもち米からの DNA 抽出について検討した。既報においてシリカゲル膜タイプキット（NIPPON GENE GM quicker 2）を用いて、良好に抽出可能であることを見いだしているが[17]、さらに DNA 収量を増やすために、シリカゲル膜カラムへ供する前にエタノール沈殿操作を加え、シリカ膜カラムへのゲノミック DNA の保持効率の改善を試みた。その結果、ビーフン試料においては DNA 収量が有意に増加した。しかし、もち米試料においては収量に関する増加はみられなかった。

2. プライマーの設計

米に特異的な遺伝子として、米ゲノムに内在する sucrose phosphate synthase（SPS）遺伝子から 90 bp の増幅バンド長が得られるプライマー対（SPS-F/SPS-R）を用いた[18]。次に Bt63 米を検知する目的でプライマー対を設計した。*Cry1Ac*（GenBank™ Accession No.：Y09787）遺伝子情報を基に Bt toxin 遺伝子（*Cry1Ac* と *Cry1Ab* の融合 DNA 塩基配列）領域を認識するプライマー対（AC-3F/AC-3R）の設計を行った。また Bt63 米を特異的に検知するために、既報[17]で明らかになった Bt63 米の構造配列で actin I promoter から Bt toxin 遺伝子の境界領域において、加工品に適用するように短い断片長の増幅産物が得られるプライマー対（actACS-F/actACS-R）を設計した。また既に Kim ら[13]は GM Shanyou 63 系統の Bt 米に挿入されている Bt toxin 遺伝子から nopaline synthase terminator（NOS-T）の境界領域を解析し、プライマー対（Oscry1Ac-F/OsNOS-R2）を設計しており、本研究では Kim らが開発したプライマー対を用いた。GM63 米に導入された DNA 構造と本研究で報告するプライマーの導入 DNA 上の位置を Fig. 1 に示す。

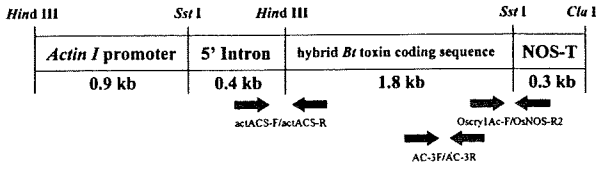

Fig. 1. Structure of the GM cassette in Bt63 rice
Primer sites designed to hybridize are drawn with arrows in the direction for PCR amplification. Restriction enzyme sites are indicated by their names. NOS-T; nopaline synthase terminator

Bt toxin 遺伝子領域を認識するプライマー対である AC-3F/AC-3R を用いて、ビーフン 2 検体（陽性 1 検体と陰性 1 検体）及びもち米 2 検体（擬陽性 1 検体と陰性 1 検体）からの抽出 DNA により PCR 増幅をしたところ、ビーフン陽性検体ともち米擬陽性検体から増幅断片長 90 bp のバンドが検出された（data not shown）。actACS-F/actACS-R のプライマー対を用いた試験ではビーフン陽性検体のみから増幅断片長 120 bp のバンドが検出された。次に Bt toxin 遺伝子から NOS-T の境界領域を認識する Oscry1Ac-F/OsNOS-R2 のプライマー対では、ビーフン陽性検体ともち米擬陽性検体から、おおよそ増幅断片長 150 bp 付近のバンドが検出された。両方の増幅産物をダイレクトシークエンス解析した結果、ビーフン陽性検体からの増幅産物の断片長は 147 bp で、そのシークエンスは Bt63 米の配列と一致したが、もち米擬陽性検体から検出された増幅産物の断片長は 142 bp で、シークエンスは Bt63 米の配列と一致しなかった。この結果から、もち米検体から得られた PCR 増幅産物は Bt63 米に使われている発現ベクターとは異なる発現ベクターが挿入された Bt 系統が混入されている可能性が示唆された。我々は既報で他のビーフン陽性検体から、Bt63 米と NNBt 米の混入を検出している[17]。もち米擬陽性検体から検出された増幅産物断片長 142 bp のシークエンスは、既報[17]で示したビーフン検体から検出された NNBt 米のシークエンスと一致した。このことから、もち米検体から検出された Bt 米は既報[17]においてビーフン検体で検出された NNBt 米であることが示唆された。

もち米擬陽性検体には NNBt 米の混入が示唆されたので、もち米中の NNBt 米の特異的プライマー対を確立することを検討した。既報[17]でビーフン陽性検体から明らかになった Maize ubiquitin promoter 配列と CryIAb タンパク質のコード領域との境界領域の配列において、加工品に適用するように短い断片長の増幅産物が得られるプライマー対（Pubi-F1/Cry1Ab-R1）を設計した。また同様に既報[17]でビーフン陽性検体から明らかになった cauliflower mosaic virus 35S promoter（CaMV）の配列と、選別マーカーである hygromycin phosphotransferase（hph）遺伝子配列との境界領域の配列において、加工品に適用するように短い断片長の増幅産物が得られるプライマー対（N35S-F1/HPH-R1）を設計した。NNBt 米に導入された DNA 構造と本研究で報告するプライマーの導入 DNA 上の位置を Fig. 2 に示す。また設計したプライマー配列と増幅バンド長を Table 1 に示す。

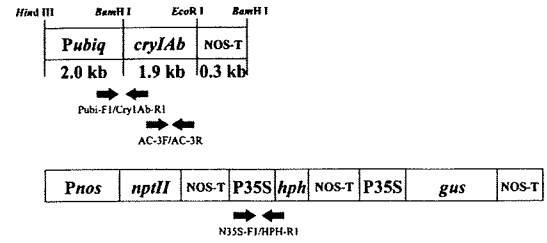

Fig. 2. Structure of the GM cassette in NNBt rice
Primer sites are drawn with arrows in the direction for PCR amplification. Restriction enzyme sites are indicated by their names. P*ubiq*; Maize ubiquitin promoter, P*nos*; nopaline synthase promoter, NOS-T; nopaline synthase terminator, P35S; 35S promoter sequence of cauliflower mosaic virus, *gus*; β-glucuronidase gene, nptII; neomycin phosphotransferase II gene

Table 1. Specific primers used for the detection of Bt rice lines

Primer Name	Sequence (5' → 3')	Specificity	Amplicon size
SPS-F	5'-TTG CGC CTG AAC GGA TAT-3'	SPS / sense	81 bp
SPS-R	5'-CGG TTG ATC TTT TCG GGA TG-3'	SPS / anti-sense	
AC-3F	5'-GTT CGC CTA TGG AAC CTC TT-3'	Cry1Ac / sense	90 bp
AC-3R	5'-TTC TGT GGT GGG ATT TCG TC-3'	Cry1Ac / anti-sense	
actACS3-F	5'-TTC ATC GGT AGT TTT TCT TTT CAT-3'	5'Intron / sense	120 bp
actACS3-R	5'-GGC CTG CAG TTG TCC AT-3'	Cry1Ac / anti-sense	
Oscry1Ac-F	5'-GCA GGA GTG ATT ATC GAC AG-3'	Cry1Ac / sense	147 bp
OsNOS-R2	5'-AAGACCGGCAACAGGATTCA-3'	T-nos / anti-sense	
Pubi-F1	5'-TTTTTAGCCCTGCCTTCA-3'	Pubi / sense	203 bp
Cry1Ab-R1	5'-ACCGGTTTCAATGCGTTCT-3'	Cry1Ab / anti-sense	
N35S-F1	5'-CCACTGACGTAAGGGATGA-3'	P35S / sense	154 bp
HPH-R1	5'-GGCTTTTTCATATCTCATTG-3'	HPH / anti-sense	

3. 設計したプライマー対の擬陽性検体への適用

米内在性遺伝子プライマー対（SPS-F/SPS-R）、Bt 米検知用プライマー対（AC-3F/AC-3R）、Bt63 米検知用プライマー対（actACS-F/actACS-R、Oscry1Ac-F/OsNOS-R2）及び NNBt 米特異的検知用プライマー対（Pubi-F1/Cry1Ab-R1、N35S-F1/HPH-R1）を用いて、ビーフン検体及びもち米検体からの抽出 DNA より PCR 増幅をしたところ、ビーフン陽性検体の抽出 DNA では、Bt 米検知用プライマー対、Bt63 米検知用プライマー対（actACS-F/actACS-R、Oscry1Ac-F/OsNOS-R2）によりバンドが検出された（Fig. 3B, C, D）。もち米擬陽性検体の抽出 DNA では Bt 米検知用プライマー対及び NNBt 米特異的検知用プライマー対によりバンドが検出された（Fig. 3B, E, F）。Oscry1Ac-F/OsNOS-R2 のプライマー対を用いた検討において、もち米擬陽性検体の抽出 DNA でも増幅産物が検出されたが、ビーフン陽性検体の抽出 DNA からの増幅産物よりも断片長が短かった（Fig. 3D）。また、そのシークエンス解析では、既報のビーフン擬陽性検体に混入していた NNBt 米のシークエンスと一致し、断片長は 142 bp であった。もち米擬陽性検体の抽出 DNA により NNBt 米特異的検知用プライマー対を用いて得た増幅産物をシークエンス解析したところ、既報のビーフン擬陽性検体から得られた NNBt 米のシークエンスとすべて一致した。このことから、もち米検体に混入している Bt 米は NNBt 米であることが示された。他のもち米擬陽性検体 2 検体からも同様に NNBt 米が検出された（data not shown）。このことから、ビーフン検体は主に Bt63 米が混入する可能性があり、もち米検体には主に NNBt 米が混入する可能性があることが示唆された。

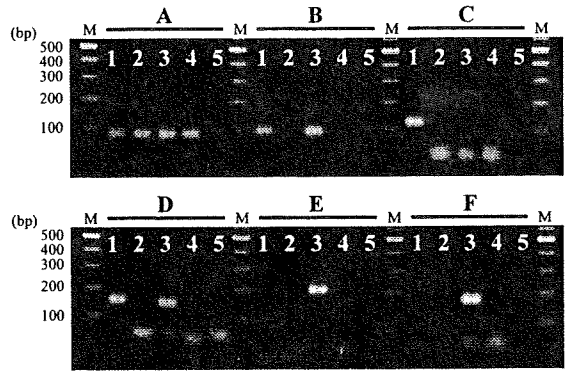

Fig. 3. PCR application of the primers designed to detect GM rice lines

Primer sets were designed for the specific detection of the rice endogenous gene (**A**; SPS-F/SPS-R), the Bt rice line (**B**; AC-3F/AC-3R), the Bt63 rice line (**C**; actACS-F/actACS-R, **D**; Oscry1Ac-F/OsNOS-R2) and the NNBt rice line (**E**; Pubi-F1/Cry1Ab-R1, **F**; N35S-F1/HPH-R1), respectively. Total genomic DNA isolated from rice vermicelli (lane 1; positive sample, lane 2; negative sample) and glutinous rice (lane 3; positive sample, lane 4; negative sample) was used as a template.

Lane 5, no-template control of each PCR reaction mixture. M, 100 bp ladder DNA marker

Ⅳ　結語

安全性未審査中国産遺伝子組換え米の Bt63 米と NNBt 米ついて、ビーフン及びもち米を検体対象として、PCR を用いた検知法を検討した。Bt63 米と NNBt 米について検知可能なプライマー対や各系統に特異的なプライマー対を設計し、ビーフン陽性検体及びもち米擬陽性検体から、各々混入している当該系統の Bt 米が検出された。

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遺伝子組換え食品の検知法

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遺伝子組換え食品の検知法

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厚生労働省では，平成13年4月から食品衛生法に基づく表示について義務化するに伴い，「組換えDNA技術応用食品の検査方法について」[注1]で遺伝子組換え(GM)食品の検査方法を定めた（通知検査法）。本方法は，世界で初めて国が定める標準法として，我が国に輸入の可能性がある安全性未審査GM食品の検知法を定め，さらに表示制度に対応した安全性審査済みのGM食品の定量法を規定している。農林水産省では，農林物資の規格化及び品質表示の適正化に関する法律（JAS法）の定める品質表示制度のもとで，安全性が確認されたGM農作物とその加工食品に対する表示を行い，厚生労働省と歩調をあわせ平成13年4月より表示制度を実施し，これに対応し，JAS分析試験ハンドブック「遺伝子組換え食品検査・分析マニュアル」[注2]を作成している[1]。本稿では，上記の通知検査法の検知法について紹介する。

1　GM食品の検知法の基礎

GM食品の開発企業は染色体挿入部位とそのコピー数の違いから，多くの組換えイベント（event）を分離取得する。組換え体は，そのイベントごとに増殖・分化されるが，この一つのイベント由来の後代植物を系統と言う[1]。我が国では系統ごと（スタック品種ごと）に特性評価及び安全性評価が実施される。従って系統ごとに検知法を確立することが必要になっている。食品中に存在する遺伝子組換え体を検知する基本的手法としては，大きく分けて導入遺伝子から作られるタンパク質を検出対象とした方法（protein-based method）と，遺伝子組換え体に使用されたDNAの領域配列を検知するポリメラーゼ連鎖反応（polymerase chain reaction；PCR）を利用した方法（DNA-based method）がある。

2　安全性未審査のGM食品の検査法

現在，医薬食品局食品安全部長通知法としては，トウモロコシとしてCBH351系統（害虫抵抗性）[2]，DAS59132系統（除草剤耐性），Bt10系統（害虫抵抗性）[3]が検査対象となっている。またハワイ産抗ウイルスパパイヤ（55-1系統）は定性PCR法[4]と組織染色法[5]を採用している。また監視安全課長通知法として，米国産長粒種米LLライス（LL601系統）と中国産害虫抵抗性Btコメ（Bt63系統及び未知系統）[6]が検査対象になっている。表1に各検査法を示す。

表1　安全性未審査のGM食品の検査法

作　物	系統名	方　法	タンパク質あるいはDNA抽出法
とうもろこし	CBH350; Starlink	ラテラルフロー法	水でタンパク質を抽出
とうもろこし加工品	CBH351; Starlink	定性PCR法	イオン交換樹脂タイプのDNA抽出精製キット（QIAGEN Genomic-tip）
とうもろこし	Bt10	定性PCR法	シリカゲル膜タイプキット法（QIAGEN DNeasy Plant Mini Kit）
とうもろこし	DAS59132	リアルタイム定性PCR法	シリカゲル膜タイプキット法（QIAGEN DNeasy Plant Mini Kit）
パパイヤ	55-1	定性PCR法	CTAB法または，シリカゲル膜タイプキット法（QIAGEN DNeasy Plant Mini Kit），缶詰パパイヤはイオン交換樹脂タイプのDNA抽出精製キット（QIAGEN Genomic-tip）
		組織免疫染色法（GUS試験法）	
米	米国産LLRICE	リアルタイム定性PCR法	シリカゲル膜タイプキット法（NIPPON GENE GM quicker 2）
米	中国産Bt米	ラテラルフロー法	水でタンパク質を抽出
米加工品	中国産Bt米	定性PCR法，リアルタイム定性PCR法	シリカゲル膜タイプキット法（NIPPON GENE GM quicker 2変法）

注1　http://www.mhlw.go.jp/topics/idenshi/index.html
注2　http://www.famic.go.jp/technical_information/jashandbook/index.html