distributed several hundred tons of Bt10 maize between 2001 and 2004. According to the information provided by the company, this GM maize event Bt10 is a transformation product of the same recombinant DNA sequence that was used to transform its 'sister' event Bt11 maize. Safety assessment for Bt11 maize has already been completed, and many countries have approved of the distribution of this GM maize event for use in foods and feeds. Since the closely related sister event Bt10 was never intended to be commercialized, the developer had not initiated the customary procedures for approval of this new GM event. In response to this regulatory inconsistency, the European Community Commission took emergency measures against the inadvertent distribution of Bt10 maize products in Europe (*6*). Likewise, the Japanese Ministry of Health, Labour and Welfare (MHLW) took action according to the mandatory safety assessment of GMOs in Japan and prohibited import of the unauthorized Bt10 maize into Japan (*7*). To ensure foolproof implementation of these decisions, the development and evaluation of methods for specific detection of Bt10 maize event was urgently required.

Polymerase chain reaction (PCR) is currently established as a reliable methodology to detect and identify trace amounts of biotic materials. Together with immunoassay applications, PCR is the most widely used analytical technique for quantitative and qualitative detection of GM crops (*8*−*11*). We have previously reported PCR systems for the detection of GM soy, maize, papaya, and potatoes (*12*−*18*). Since the very closely related sister events Bt10 and Bt11 were not expected to lend themselves to distinction by immunoassay methods, such as ELISA or lateral flow strip analysis, it was determined that PCR would be the most suitable technique for the task at hand. The event-specific PCR system using the primer pair JSF5/JSR5 for the detection of trace amounts of Bt10 maize was provided by Syngenta in 2005. This method was evaluated in-house by the developer Eurofins GeneScan using the reference material of Bt10 maize provided by Syngenta (data not shown) and by the European Community Reference Laboratory for GM Food and Feed (http://gmo-crl.jrc.it//summaries/ Bt10%20validation %20report%20version2.pdf). This PCR system had not been examined in multilaboratory collaborative studies as is customary for official GMOs detection methods published by competent Japanese authorities, mainly owing to time constraints. Moreover, in accordance with the requirements in Japan, a second, independent PCR system for specific detection of the unapproved GMOs event was required. Therefore, we designed a new primer pair (Bt10LS3′ and Bt10LS5′) based on a junctional sequence between the recombinant DNA and maize genomic DNA and optimized the PCR conditions to develop another PCR system for event-specific detection of Bt10 maize.

The aim of this paper is to report the development of qualitative PCR systems for the specific detection of Bt10 maize and also the precise results of evaluation of the performance characteristics of the systems. Therefore, the specificity and sensitivity of these two PCR systems were examined in a single laboratory. Then, we conducted a multilaboratory collaborative study, and the data from this study were statistically analyzed to evaluate the reproducibility and sensitivity of the PCR systems for the first time. The results of this statistical analysis showed that there was no significant difference in the sensitivity of the two methods and that the limit of detection of both the methods was less than 0.05%.

MATERIALS AND METHODS

Maize (*Zea mays*) and Other Cereal Materials. Roughly milled samples of Bt10 maize seed, and seeds of two events of GM maize (Bt11 and Event176), were kindly provided by Syngenta Seeds AG (Basel, Switzerland). Seeds of seven events of GM maize including MON810, MON863, GA21, NK603 (Monsanto Co.; St. Louis, MO), T25 (Bayer CropScience AG; Monheim am Rhein, Germany), TC1507 (Dow Agrosciences LLC; Indianapolis, IN), and an event of GM soy (Roundup Ready Soy; Monsanto Co.) were kindly provided by the respective developers. Non-GM maize was imported directly from the United States. Other non-GM cereal materials including soy (*Glycine max*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), and wheat (*Triticum aestivum*) were purchased from a local market in Japan. All of these materials were ground in the Ultra-Centrifugal Mill ZM100 (Retsch GmbH, Haan Germany) using a 0.5 mm sieve ring and freeze-dried for 24 h in a FD-81 freeze dryer (Tokyo Rikakikai Co., Ltd. Tokyo, Japan). The freeze-dried samples were stored at −20 °C until further use.

Preparation of the Test Sample. In this study, two types of test samples were prepared to evaluate the sensitivity of the methods, namely, simulated DNA-mixture samples and simulated powder-mixture samples. The maize genomic DNA-mixture samples with each genomic DNA extracted from the ground materials of Bt10 maize and non-GM maize were prepared at four mixing levels containing 0, 0.01, 0.05, 0.1, and 0.5% (w/w) of Bt10 maize genomic DNA, using the serial dilution method. For the preparation of the simulated powder-mixture samples at three mixing levels containing 0, 0.05 and 0.1% (w/w) of Bt10 maize, the ground Bt10 maize was added to the ground non-GM maize in appropriate ratios and mixed well in the Ultra-Centrifugal Mill. Since it was not practical to prepare a fine powder-mixture sample at the 0.01% mixing level, the DNA extracted from the 0.05% powder-mixture sample was diluted five times with the DNA extracted from the ground non-GM maize and used as 0.01% powder-diluted solution.

Extraction and Purification of Genomic DNA. Genomic DNAs were extracted from the ground materials and Bt10 powder-mixture samples using a silica-gel membrane-type kit (DNeasy Plant Mini; QIAGEN, Hilden, Germany) according to the procedure described in our previous study with some modification (*19*). The DNA concentration was determined by measuring the UV absorption at 260 nm using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Rockland, DE). The purity of the extracted DNA was evaluated based on the ratio of absorbance at 260/280 nm and the ratio was between 1.7 and 2.0 for most of the test samples. The extracted DNA was diluted with an appropriate volume of distilled water to a final concentration of 10 ng/μL and stored at −20 °C until further use. These DNA samples were used for the subsequent PCR analysis.

Oligonucleotide Primers. According to the information provide by Syngenta, the JSF5/JSR5 primer pair could amplify a DNA sequence containing a junction between the maize genomic DNA sequence and the recombinant DNA introduced into the Bt10 maize. It was also reported that the annealing site of JSF5 was located in a maize genomic DNA sequence and that of JSR5 was located in the DNA sequence included in the recombinant DNA, and also that the JSF5/JSR5 primer pair generated a 117-bp amplicon. In this study, we noted that the Bt10LS5′/ Bt10LS3′ primer pair designed based on the sequence information provided by Syngenta, could also amplify the DNA sequence containing a junction between the maize genomic DNA sequence and the recombinant DNA introduced into Bt 10 maize, and that the Bt10LS5′/Bt10LS3′ primer pair generated a 151-bp amplified fragment. The Zein n-5′/Zein n-3′ primer pair (*24*) to amplify the DNA sequence in a maize endogenous gene was used as the analytical control to confirm the validity of the PCR technique for maize genomic DNA (*21*, *22*).

The primers were synthesized and purified on a reversed-phase column by FASMAC Co., Ltd., (Atsugi, Japan), diluted with an appropriate volume of distilled water to a final concentration of 60 μmol/L, and then stored at −20 °C until further use. The sequences of the oligonucleotides used in this study are listed in **Table 1**.

PCR Conditions. The reaction mixture for PCR was prepared in a PCR reaction tube. The reaction volume of 25 μL contained 50 ng of genomic DNA, 0.16 mM dNTP, 1.5 mM $MgCl_2$, 0.6 μM of forward and reverse primers, and 0.8 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The reactions were buffered with PCR buffer II (Applied Biosystems) and amplified in a thermal cycler

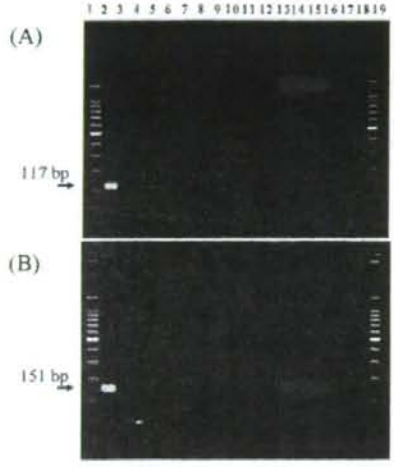

Figure 1. Specificity of the PCR systems employed to detect Bt10 maize. The Bt10 event-specific primers JSF5/JSR5 (**A**) and Bt10LS5′/3′ (**B**). Lanes 1 and 19, 100-bp ladder size standard; lane 2, Bt10; lane 3, Bt11; lane 4, GA21; lane 5, Event176; lane 6, MON810; lane 7, T25; lane 8, NK603; lane 9, MON863; lane 10, TC1507; lane 11, non-GM maize; lane 12, RRS; lane 13, non-GM soy; lane 14, barley; lane 15, wheat; lane 16, rice; lane 17, non-template DNA; lane 18, non-primers and Bt10.

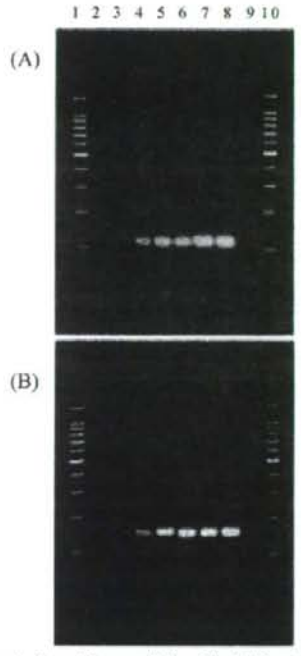

Figure 2. Examination of the sensitivity of the PCR systems to detect Bt10 maize using serial dilutions of the DNA-mixture samples. The Bt10 event-specific JSF5/ JSR5 (**A**) and Bt10LS5′/3′ (**B**) primer pairs. Lanes 1 and 10, 100-bp ladder size standard; lane 2, nontemplate DNA; lane 3, non-GM maize; lane 4, 0.01% Bt10; lane 5, 0.05% Bt10; lane 6, 0. 1% Bt10; lane 7, 0.5% Bt10; lane 8, 100% Bt10; lane 9, nonprimers and 100% Bt10.

(GeneAmp PCR System 9700; Applied Biosystems) according to the following PCR step-cycle program: preincubation at 94 °C for 10 min, denaturation at 94 °C for 25 s, annealing at 62 °C for 30 s, and extension at 72 °C for 45 s. The cycle was repeated 40 times followed by a final extension at 72 °C for 7 min. The same conditions were employed for the PCR using the Bt10LS5′/Bt10LS3′ primer pair, except that the annealing temperature was changed to 65 °C from 62 °C. The amplified products were analyzed by electrophoresis with 3.5% agarose gel.

Cloning and Sequencing of the Amplified Fragments. The amplified fragments generated using the JSF5/JSR5 or Bt10LS5′/Bt10LS3′ primer pair were subcloned into the TOPO-TA vector with TA-cloning kit (Invitrogen, Carlsbad, CA), and the recombinant plasmids were transformed into *E. coli* strain DH5α. The sequences of the clones were determined using the Dye Terminator Cycle Sequencing Kit (Beckman Coulter, Inc., San Diego, CA) and the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc.).

Interlaboratory Study. The interlaboratory study, conducted with the participation of six laboratories, was organized by the National Institute of Health and Sciences (NIHS) to evaluate the performance of the methods. The simulated powder-mixture sample containing 0, 0.05, and 0.1% (w/w) of Bt10 maize and the 0.01% powder-diluted solution as described above were used as the test samples. We prepared 10 separate tubes containing 2 g of the powder-mixture samples for each mixing level and 10 separate tubes containing 30 μL of the 0.01% powder-diluted solutions as 10-replicate samples and sent them to each participating laboratory as blind samples. A total of 40 tubes containing blind samples, solutions of the three primer pairs (6 μM each), reagents for the PCR, and the experimental protocol were provided to the six participating laboratories from the NIHS. Thus, a total 240 blind samples were analyzed by three PCR systems in the interlaboratory study. We referred to the guidelines for collaborative study to determine the general procedure of the interlaboratory study (*23*).

Statistical Analysis. To evaluate the performance of the methods, the data obtained from the interlaboratory study were analyzed statistically (*24*). The standard errors of the ratio of positive results among the laboratories were calculated as

$$\text{se}(p) = ((1/m^2L)(V_a))^{1/2}$$

where m is the number of test samples analyzed in a laboratory, a is the number of positive results obtained in a laboratory, p is the ratio of positive results to the total number of test samples analyzed in a laboratory (a/m), and V_a is variance of the number of positive result obtained among laboratories in accordance with the following formula: $V_a = (\Sigma a^2 - (\Sigma a)^2/L)/(L - 1)$. The lower limit of p was calculated at the 95 and 99% confidence levels based on the se (p).

Table 1. PCR Primers Used in This Study

PCR system	name	orientation	sequence	amplicon
JSFR5	JSF5	forward	5-CAC ACA GGA GAT TAT TAT AGG GTT ACT CA-3	117 bp
	JSR5	reverse	5-ACA CGG AAA TGT TGA ATA CTC ATA CTC T-3	
Bt10LS	Bt10LS5′	forward	5-GCC ACA ACA CCC TCA ACC TCA-3	151 bp
	Bt10LS3′	reverse	5-GAA GTC GTT GCT CTG AAG AAC AT-3	
maize endogenous gene	Zein n-5′	forward	5-CCT ATA GCT TCC CTT CTT CC-3	157 bp
	Zein n-3′	reverse	5-TGC TGT AAT AGG GCT GAT GA-3	

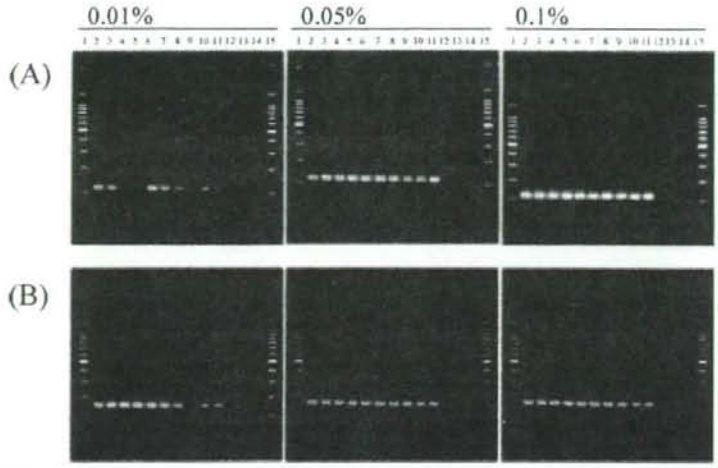

Figure 3. Examination of the sensitivity of the PCR systems to detect Bt10 maize using 10-replicate DNA-mixture samples for each of the three mixing levels. The Bt10 event-specific JSF5/JSR5 (**A**) and Bt10LS5′/3′ (**B**) primer pairs. Lanes 1 and 15, 100-bp ladder size standard; lanes 2 to 11, the DNA-mixture samples; lane 12, nontemplate DNA; lane 13, non-GM maize; lane 14, nonprimers and Bt10. The mixing levels of Bt10 DNA are shown above each of the gel photos.

The difference in the sensitivity of the two PCR systems, JSFR5 and Bt10LS, was tested by the *t*-test with the pooled variance.

RESULTS AND DISCUSSION

Specificity of Two Qualitative PCR Systems for Event-Specific Detection of Bt10 Maize. Design of suitable primers and optimization of the PCR condition are critical for the development a PCR-based method for the specific detection of GMOs. While the PCR system using the JSF5/JSR5 primer pair (the JSFR5 PCR system) was provided by Syngenta, the company released little information pertaining to design of the primers and its specificity. Therefore, we determined the annealing site of these primers by searching a DNA sequence database (NCBI/ GenBank). The results revealed that a complete homologous DNA sequence of JSR5 was contained in type of plasmid vectors (for example; Accession No. EF090407) and that the sequence was homologous with the partial DNA sequence of a gene encoding β-lactamase (ampicillin resistance gene). On the other hand, the homologous DNA sequence of JSF5 could not be found in the database. Moreover, we could not find the homologous DNA sequence of JSF5 in the public DNA sequence database even by searching for the DNA sequence identified by the isolation of the PCR product amplified using JSF5/JSR5 primer pair. However, it was found that the JSF5 primer could not anneal to the recombinant DNA sequence introduced into other GMOs. To examine the specificity of the PCR using the JSF5/JSR5 primer pair, we performed PCR with DNAs extracted from 15 kinds of cereal materials including nine events of GM maize, one event of GM soy and five kinds of non-GM cereal materials. As shown in **Figure 1A**, an amplification fragment (117 bp) was specifically detected from Bt10 maize, whereas no amplification fragment was yielded by the PCR performed with the DNAs extracted from other GM maize materials including Bt11 maize, which was transformed with the same expression vector used for the production of the Bt10 maize, the GM soy, and the other five major cereal materials. These results suggested that the PCR conditions were optimal and that the JSFR5 PCR system allowed specific detection of Bt10 maize.

To increase the reliability of the result obtained, it is mandatory for regulatory purposes in Japan to use two PCR systems targeting different DNA sequences for the testing of unapproved GMOs (*20*). Therefore, we examined another PCR system for specific detection of Bt10 maize using the Bt10LS5′/ Bt10LS3′ primer pair designed based on the sequence information provided by Syngenta. It was confirmed, based on the search of a DNA sequence database (NCBI/GenBank), that the annealing site of Bt10LS5′ and Bt10LS3′ were located within the gene encoding phosphinothricin acetyltransferase (*PAT*) included in the expression cassette (Accession No. DQ156557) and maize genomic DNA sequence (Accession No. AC152494), respectively. In regard to optimization of the PCR condition, while the heating and elongation temperatures and reaction period were the same as those used for the PCR system using the JSF/JSR5 primer pair, the annealing temperature was changed to 65 °C from 62 °C, because PCR fragments of unexpected lengths were observed occasionally. As shown in **Figure 1B**, a specific fragment (151 bp) was obtained for Bt10 maize, whereas no fragment was amplified by the PCRs for the DNAs extracted from the other GMOs or major cereals. These results suggested that the PCR system using the Bt10LS5′/ Bt10LS3′ primer pair (the Bt10LS PCR system) allows specific detection of Bt10 maize as reliably as the JSFR5 PCR system.

Sensitivity of the PCR Systems. We examined the sensitivity of the two PCR systems using the two types of sample (i.e., the DNA-mixture samples and powder-mixture samples). For the case of the DNA-mixture samples, specific PCR fragments were obtained with both the PCR systems for 100 to 0.01% samples and the intensity of the specific PCR fragments changed depending on the contents of genomic DNA derived from the Bt10 maize (**Figure 2**). On the basis of the 1C value of maize (*25*), 50 ng of maize genomic DNA, used as the template for the PCR, corresponds to approximately 18320 haploid maize genome copies (*26*). Therefore, it is estimated that 50 ng of the 0.01% DNA-mixture sample contains less than two haploid genome copies derived from Bt10 maize. In theory, only one copy of the target sequence should be required for PCR amplification, and it can be expected that the one or two copies

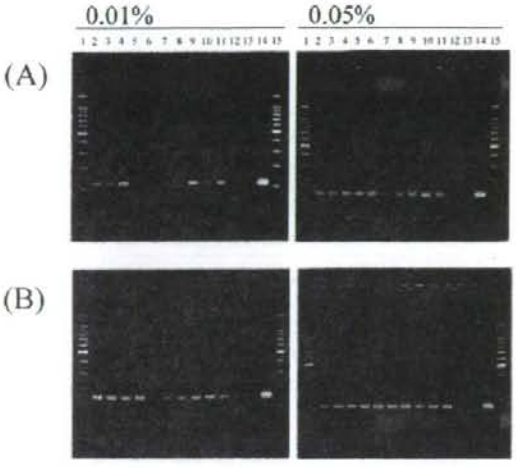

Figure 4. Examination of the sensitivity of the PCR systems to detect Bt10 maize using 10-replicates of the 0.05% powder mixture-sample and 0.01% powder diluted sample. Lanes 1 and 15, 100-bp ladder size standard; lanes 2 to 11, the samples; lane 12, nontemplate DNA; lane 13, non-GM maize; lane 14, 100% Bt10. The mixing levels of Bt10 are shown above each of the gel photos.

are distributed in the replicates of the PCR amplification with the 0.01% DNA-mixture samples with any level of uncertainty. In fact, 10 positive results were obtained with all the replicates of the PCR amplification with the 0.10 and 0.05% DNA-mixture samples, but 8 or 9 positive results were obtained with the 10 replicates of the 0.01% DNA-mixture samples using either PCR systems (**Figure 3**). In the examination using the powder-mixture samples, specific PCR fragments were obtained with all of 10 genomic DNAs, extracted from the 10 replicates of the 0.05% powder-mixture samples. Furthermore, the positivity rate obtained with the 0.01% powder-diluted solution was approximately the same as that obtained with the 0.01% DNA-mixture sample (**Figure 4**). These results suggested that the DNA extraction step slightly affected the limit of detection of the two PCR systems and that the limit of detection was in the range of less than 0.05% to over 0.01%.

Interlaboratory Study. To examine the reproducibility of the PCR systems, we conducted an interlaboratory study by sending the powder-mixture sample at the three mixing levels and 0.01% powder-diluted solution described in Materials and Methods as blind samples. All of the blind samples, including the 0, 0.10, 0.05% powder-mixture samples and the 0.01% powder-diluted solution, were prepared in 10 replicates. All the participating laboratories received the protocol, primer solutions, reagents for PCR, and 40 blind-sample tubes. Six laboratories participated in the study, and a total of 240 test samples were analyzed. As shown in **Table 2**, specific amplified fragments were detected with all the test samples with the PCR system in which the Zein n-5′/Zein n-3′ primer pair was used to detect a maize endogenous gene. These result indicated that the DNA solutions did not contain any substances that might strongly inhibit the PCR amplification. Furthermore, specific fragments were amplified in all of the 0.10 and 0.05% powder-mixture samples and no nonspecific amplification was observed in any of the 0% powder-mixture samples with either PCR system used for the specific detection of Bt10 maize. On the other hand, the positivity rate of the two PCR systems for the 0.01% powder-diluted solution showed dispersion among laboratories as expected from the results of the single laboratory examination (**Table 2**). Therefore, we statistically analyzed the data using the method reported by McClure (*24*). We calculated the standard error of the positivity rate among the laboratories and estimated the lower limit of the positivity rate for all tested samples at 95 and 99% confidence levels. As shown in **Table 3**, at the 95% confidence level, the positivity rate obtained with the 0.01% powder-diluted solution using the JSFR5 and Bt10LS PCR systems were estimated as 0.5995 and 0.6812, respectively. These results imply that the positive results were not obtained from approximately 3 to 4 of the tested samples in the 10-replicates testing of the 0.01%. From the viewpoint of the accuracy of the test results, the limit of detection of these two PCR systems may be thought of as over 0.01%. Furthermore, we compared the positive rate of two PCR systems statistically using *t*-test with pooled valiance to evaluate the sensitivities of these PCR systems. The calculated *t*-value (1.75) indicated that there was no significant difference in the sensitivities between the two PCR systems at either the 95 or 99% confidence level. These results suggested that both the PCR systems could yield the positive results with approximately the same sensitivity and that no discrepancy of the test results was likely to be caused by a difference in the sensitivity between the two PCR systems.

In this study, we examined two event-specific qualitative PCR methods to detect an unapproved GM maize event (Bt10 maize) and evaluated the performance of the methods. This is the first study to report the results of a statistical analysis of qualitative results obtained with PCR-based assays conducted to evaluate the performance of the methods. The highly specific PCR systems examined in this study are simple, sensitive, and useful for identifying trace amounts of Bt10 maize in grain samples.

Table 2. Results for All Test Samples in the Interlaboratory Study[a]

lab. code no.	ratio of positive results for the test samples in PCR systems											
	0%			0.01 %			0.05 %			0.10 %		
	JSFR5	Bt10LS	Zein	JSFR5	Bt10LS	Zein	JSFR5	Bt10LS	Zein	JSFR5	Bt10LS	Zein
I	0/10	0/10	10/ 10	6/10	7/10	10/ 10	10/10	10/10	10/ 10	10/10	10/10	10/ 10
II	0/10	0/10	10/ 10	6/10	8/10	10/ 10	10/10	10/10	10/ 10	10/10	10/10	10/ 10
III	0/10	0/10	10/ 10	7/10	9/10	10/ 10	10/10	10/10	10/ 10	10/10	10/10	10/ 10
IV	0/10	0/10	10/ 10	6/10	7/10	10/ 10	10/10	10/10	10/ 10	10/10	10/10	10/ 10
V	0/10	0/10	10/ 10	8/10	7/10	10/ 10	10/10	10/10	10/ 10	10/10	10/10	10/ 10
VI	0/10	0/10	10/ 10	7/10	7/10	10/ 10	10/10	10/10	10/ 10	10/10	10/10	10/ 10
total	0/60	0/60	60/ 60	40/60	45/60	60/ 60	60/60	60/60	60/ 60	60/60	60/60	60/ 60

[a] JSFR5 and Bt10LS; Bt10 Maize specific PCR systems in which the JSF5/JSR5 and Bt10LS5′/Bt10LS3′ primer pairs were used, respectively, Zein; Maize endogenous gene specific PCR system in which the Zein n-5′/Zein n-3′ primer pair was used.

Table 3. Statistical Analysis of Positivity Rate for the Test Sample Containing 0.01% Bt10 Maize

PCR system	m	L	V_a	se (a/m)	p	$p(0.95)^a$	$p(0.99)^a$
JSFR5	10	6	0.6667	0.0333	0.6667	0.5995	0.5545
Bt10LS	10	6	0.7000	0.0342	0.7500	0.6812	0.6351

[a] Lower limit: m, number of the test sample analyzed in one laboratory; L, number of laboratories participating in the interlaboratory study; V_a, variance of positive results among the laboratories; se, standard errors of the ratio of the positive results among the laboratories; p, the ratio of the positive results for all test samples; $p(0.95)$ and $p(0.99)$ are the estimated ratio of the positive results at 95 or 99% confidence levels.

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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 *cry1Ab/cry1Ac* 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 *cry1Ab/cry1Ac* 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 *cry1Ab* 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

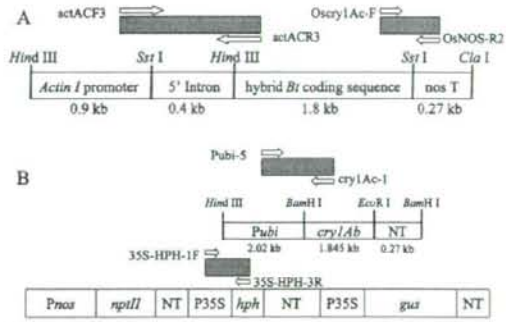

Figure 1. Diagrams of construct pFHBT1 (**A**) inserted in the GM Shanyou 63 line and construct pKUB (**B**) inserted in the Kemingdao line. The primers used in this study to generate PCR products suitable for DNA sequencing are indicated by arrows. Location of sequences taken for the alignments shown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

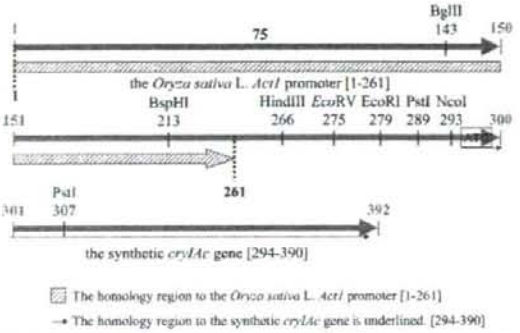

Figure 2. Nucleotide sequence alignments of PCR products generated with genomic DNAs extracted from rice vermicelli G and A samples for the junction region between the rice *Act1* promoter sequence and the *cry1Ac* gene. Restriction enzyme sites are marked.

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

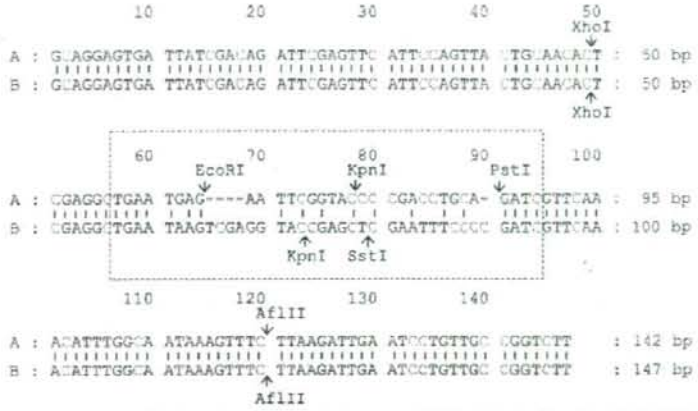

Figure 3. Nucleotide sequence alignments of PCR products generated with genomic DNAs extracted from rice vermicelli G, A, and B samples for the junction region between the *cry1Ab/cryAc* fusion gene and the *nos* sequence. *Xho*I, *Eco*RI, *Kpn*I, *Pst*I, and *Sst*I sites are denoted by arrows.

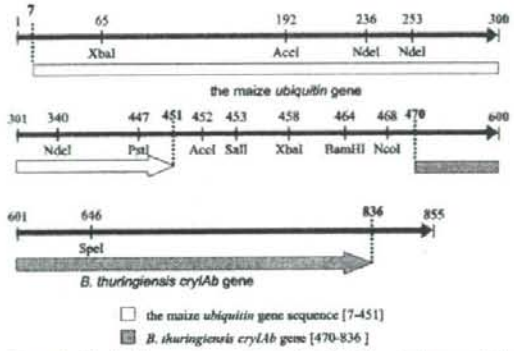

Figure 4. Nucleotide sequence alignments of PCR products generated with genomic DNAs extracted from rice vermicelli B and C samples for the junction region between the *Pubi* and *cry1Ab* genes. Restriction enzyme sites are marked.

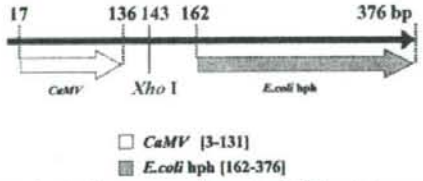

Figure 5. Nucleotide sequence alignments of PCR products generated with genomic DNAs extracted from rice vermicelli B and C samples for the junction region between the *CaMV* sequence and the *hph* gene. The *Xho*I site at position 143 is marked.

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

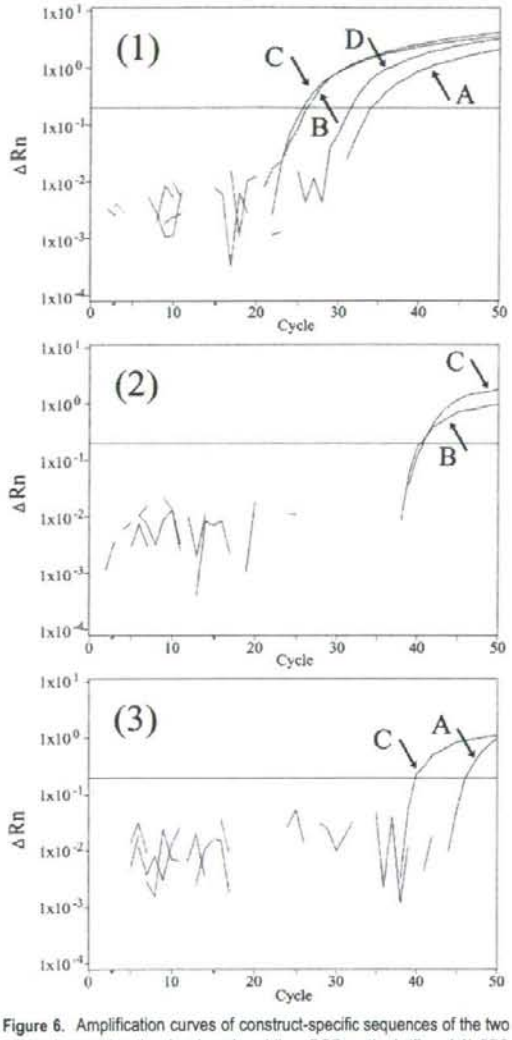

Figure 6. Amplification curves of construct-specific sequences of the two Bt rice lines using the developed real-time PCR method. (**Panel 1**) SPS gene detection in all rice vermicelli samples. (**Panel 2**) Specific detection for unknown Bt rice, which has a construct similar to that of Kemingdao in 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

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経口感作および経口惹起によるマウスの食物アレルギーモデル

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Murine Food Allergy Model with Oral Sensitization and Oral Challenge

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We found an increase of ovalbumin (OVA)-specific IgG1 antibody in the serum of mice sensitized orally with OVA emulsified with the mixture of linoleic acid and lecithin combined with sodium salicylate (SA) treatment. These mice showed severe symptoms of systemic anaphylaxis and the histological alteration in small intestine after oral challenge of OVA. In this study, we investigated suitable conditions for sensitization and for challenge in order to establish a test system for prediction of allergic potentials of food proteins.

It was demonstrated that mice were well sensitized by oral route with 10μg to 1 mg of OVA emulsified with the mixture of linoleic acid and soy been lecithin combined with 0.3 mg of SA twice or more during 3 weeks. One to 4 days after the last oral sensitization, mice were challenged orally with 4 to 100 mg of OVA emulsified with the mixture of linoleic acid and soy been lecithin. The sensitized mice showed allergic responses by the oral challenge of 4 to 100 mg of OVA emulsified with the mixture of linoleic acid and egg yolk lecithin. In addition, this test system was able to detect the allergic potentials of lactoglobulin and wheat gliadin as well as OVA.

The test system will be useful for evaluation of the allergic potentials of food proteins.

緒言

遺伝子組換え食品の安全性の評価においては，導入された組換えタンパクのアレルギー惹起性の有無を調べることが重要である[1]．組換えタンパクを含む食品は経口で摂取されることから，そのアレルゲン性の評価には，経口投与したタンパク質によって感作が成立し，特異抗体の産生や再びそのタンパク質を経口投与（惹起）した時のアレルギーの臨床症状を指標として成立した感作を検出できる試験系（以下，食物アレルギーモデル）が適していると考えられる．これまでにわれわれは，動物を用いた食物アレルギーモデルを確立することを目的として，経口投与したタンパク質に対して感作が成立する実験条件を検討した．まず，アトピー性皮膚炎，喘息，食物アレルギーなどのアレルギーへの関与の可能性が報告されている要因[2-4]の中から，複数のアレルギーの併発，腸管からの物質の吸収を高める作用を持つ薬剤の併用あるいは摂取する脂肪酸量の増加や摂取時の性状の変化といった条件を検索した．次いで，アレルゲン性タンパクである卵白アルブミン（OVA）[5,6]をマウスに経口投与する実験系に，検索した条件を単独あるいは重複して用いた．その結果，投与媒体にリノール酸とレシチンの乳化液（LL）を用いることによって脂肪酸の摂取量を高めたことに加えて乳化液の性状で摂取する条件，および腸管からの高分子量物質の吸収を高める作用を有するサリチル酸ナトリウム（SA）を併用する条件によって，マウスの血清中にOVA特異抗体を認めた．さらに，LLを用いたOVAの投与にSAを併用したところ，単独条件では認められなかったアレルギー反応の惹起による全身性アナフィラキシー症状の発現および消化管に変化を認

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めた[7-9].

これまでの検討で得られた知見を基に再現性のある食物アレルギーモデルを確立するためには，実験条件を詳細に選定することが必要となる．食物アレルギーを含む免疫系は，抗原提示細胞，T細胞やB細胞などの多種多様な細胞やサイトカインなどの情報伝達物質のネットワークで形成されている[10]．生体が抗原に曝露される時，抗原量や投与部位あるいは頻度によって活性化する細胞の種類やその働きが異なるため，感作の状態も変化する．また，免疫系の中で感作の成立に伴って活性化部位が移動するため，アレルギー反応の惹起条件によって発現する反応が異なる．したがって，感作およびアレルギー反応惹起の両方の実験条件が食物アレルギーモデルの再現性に大きく影響する．そこで本研究では，感作時の抗原量，感作投与の頻度，併用するSAの投与経路および惹起時の溶媒，惹起用量といった条件の影響を検討し，食物アレルギーモデルとしての実験条件を設定した．さらに，本食物アレルギーモデルでOVAとは異なる種類のアレルゲン性タンパクである牛乳中のラクトグロブリン（LG）[5,6]や小麦中のグリアジン（GD）[5,6]による反応を確認した．

材料および方法

生理食塩液に溶解したOVA（Grade Ⅲ, Sigma-Aldrich, St. Louis, MO）と等容量のLL（リノール酸：レシチン＝4：1）を乳化して抗原液を調製した．LLは適宜，由来の違いから混在する不純物が異なると考えられる大豆あるいは卵黄由来のレシチン（いずれも和光純薬工業，大阪）で調製した（それぞれSLLあるいはELL）．媒体対照液は，生理食塩液とSLLあるいはELLを乳化して調製した．1群6～7匹の7週齢の雌性BALB/cマウス（日本チャールス・リバー，横浜）を用い，1匹あたり0.1 mLの抗原液あるいは媒体対照液を1日1回，3週間，以下に示す頻度で経口投与した．感作時にはOVA投与群と媒体対照群のいずれにも，注射用水に溶解した2あるいは3 mg/mL SA（和光純薬工業）を1匹あたり0.1 mLの容量で腹腔内あるいは経口投与した．反応を増幅する目的で，OVA投与群には感作投与終了から1～4日後に大量のOVAを経口投与した．その約2週間後に再び大量のOVAを投与してアレルギー反応を惹起し，表れた全身性アナフィラキシー症状を観察した．症状は，無症状を0，立毛や鼻こすりを1，嘔吐様症状や下痢を2，努力呼吸やチアノーゼを3，24時間以内の死亡を4としてスコア化[11]した．本食物アレルギーモデルで感作したマウスに観察される全身性アナフィラキシー症状は，弱い症状を複数併発しながら次第に強い症状の発現へと推移することから，動物個体毎に観察された症状のスコアをすべて加算して個体スコアとした[7]．各群の個体スコアの合計を動物数で割って平均スコアを算出し，抗原投与群と媒体対照群の平均スコアが1.0以上異なる場合に抗原投与群の平均スコアの上昇とした．また，異なる媒体対照群を対照とする抗原投与群間の比較は，それぞれの媒体対照群の平均スコアを差し引いた抗原投与群の平均スコアが1.0以上異なる場合に差が認められるとした．一方，惹起前にそれぞれのマウスから採血し，血清中抗OVA IgG1抗体価を酵素免疫測定法（ELISA）で測定した．

初めに，感作条件を検討した．抗原量の影響を調べるために，SLLを媒体として10 μgあるいは1 mgのOVAを投与した（図1a）．また，投与頻度の影響を調べるために，3週間に2回（間欠）あるいは1週間に5回（連続）の頻度で1 mgのOVAを投与した（図2a）．さらに，SAの投与経路の影響を調べるために，OVAの投与にSAを腹腔内あるいは経口投与で併用した（図3a）．いずれの実験もOVA投与群にはELLを媒体として調製した250 mg/mL OVA，媒体対照群にはELLのみを1匹あたり0.4 mLの容量で経口投与してアレルギー反応を惹起した．惹起による全身性アナフィラキシー症状と血清中抗OVA IgG1抗体価を指標にして感作の成立状態を比較した．

次いで，惹起条件を検討した．ELLを媒体として1 mgのOVAで感作したマウスに大量のOVAを投与した時の反応において（図4a），惹起時の媒体の影響を調べた．ELL，生理食塩液あるいはSLLでそれぞれ250 mg/mL OVAを調製し，OVA投与群と媒体対照群に0.4 mLずつ経口投与した．大量投与時の全身性アナフィラキシー症状を指標にして媒体の影響を比較した．また，惹起時の抗原量の影響を調べるために，OVA投

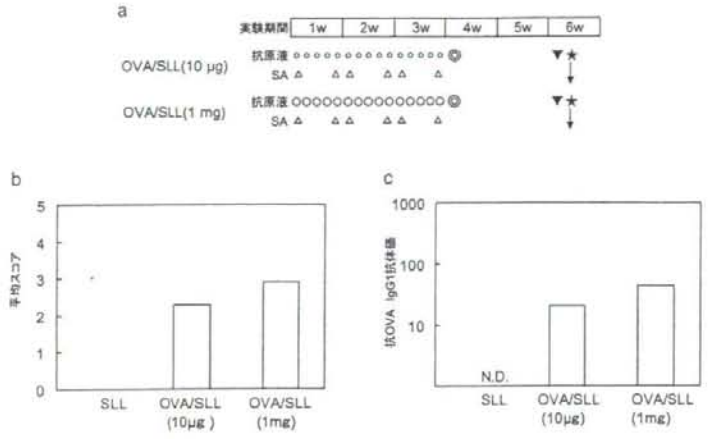

図1　抗原量の違いによる感作成立状態の比較

aは媒体にリノール酸と大豆レシチンを用いた卵白アルブミン投与群（OVA/SLL）の実験スケジュールを示す．○および◯はOVA 10 μgあるいはOVA 1 mgの経口投与，△はサリチル酸ナトリウム（SA）0.2 mgの腹腔内投与，◎はOVA 100 mgの大量投与，▼は採血，★はOVA 100 mg経口投与によるアレルギー反応の惹起，↓はアナフィラキシー症状の観察を示す．媒体対照群（SLL）には同じスケジュールで媒体のみを投与し，OVA投与群と同容量の媒体を用いて大量投与およびアレルギー反応を惹起し，採血を行った．

bはアレルギー反応の惹起時の各群におけるアナフィラキシー症状の平均スコア，cは採血時の各群における抗OVA IgG1抗体価の平均値を示す．N.D.は抗体価が検出されなかったことを示す．

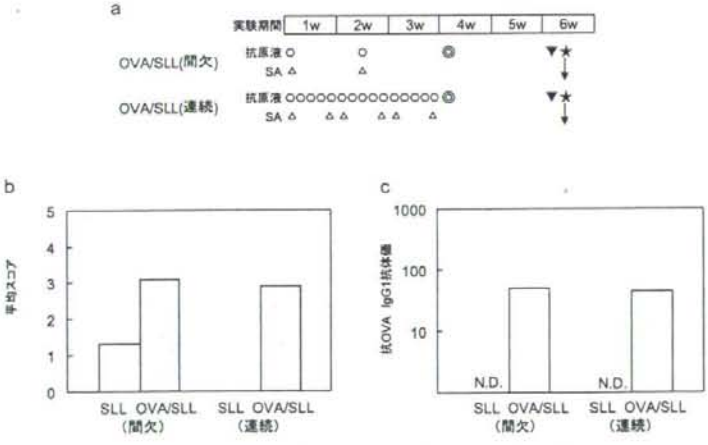

図2　投与頻度の違いによる感作成立状態の比較

aは媒体にリノール酸と大豆レシチンを用いた卵白アルブミン投与群（OVA/SLL）の実験スケジュールを示す．○はOVA 1 mgの経口投与，△はサリチル酸ナトリウム（SA）0.2 mgの腹腔内投与，◎はOVA 100 mgの大量投与，▼は採血，★はOVA 100 mgの経口投与によるアレルギー反応の惹起，↓はアナフィラキシー症状の観察を示す．媒体対照群（SLL）には同じスケジュールで媒体のみを投与し，OVA投与群と同容量の媒体を用いて大量投与およびアレルギー反応を惹起し，採血を行った．

bはアレルギー反応の惹起時の各群におけるアナフィラキシー症状の平均スコア，cは採血時の各群における抗OVA IgG1抗体価の平均値を示す．N.D.は抗体価が検出されなかったことを示す．

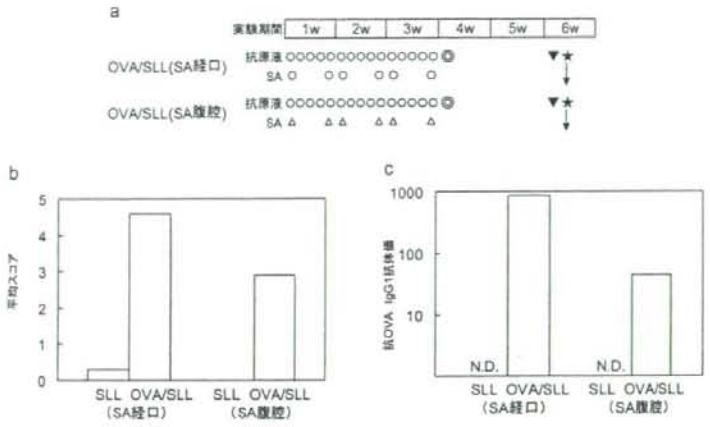

図3　サリチル酸の投与経路の違いによる感作成立状態の比較

aは媒体にリノール酸と大豆レシチンを用いた卵白アルブミン投与群（OVA/SLL）の実験スケジュールを示す．○はOVA 1 mgあるいはサリチル酸ナトリウム（SA） 0.3 mgの経口投与，△はSA 0.2 mgの腹腔内投与，◎はOVA 100 mgの大量投与，▼は採血，★はOVA 100 mgの経口投与によるアレルギー反応の惹起，↓はアナフィラキシー症状の観察を示す．媒体対照群（SLL）には同じスケジュールで媒体のみを投与し，OVA投与群と同容量の媒体を用いて大量投与およびアレルギー反応を惹起し，採血を行った．

bはアレルギー反応の惹起時の各群におけるアナフィラキシー症状の平均スコア，cは採血時の各群における抗OVA IgG1抗体価の平均値を示す．N.D.は抗体価が検出されなかったことを示す．

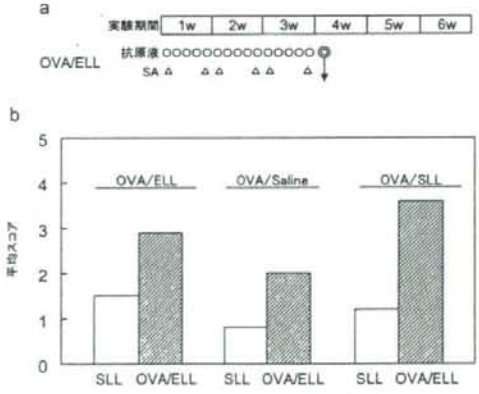

図4　惹起時の媒体の違いによるアナフィラキシー症状の比較

aは媒体にリノール酸と卵黄レシチンを用いた卵白アルブミン投与群（OVA/ELL）の実験スケジュールを示す．○はOVA 1 mgの経口投与，△はサリチル酸ナトリウム（SA） 0.3 mgの腹腔内投与，◎はbに示した条件での大量投与，↓はアナフィラキシー症状の観察を示す．媒体対照群（ELL）には同じスケジュールで媒体のみを投与した．

bはOVA大量投与時に観察した各群におけるアナフィラキシー症状の平均スコアを示した．1対のELLとOVA/ELL投与群に1匹あたりOVA 100 mgをそれぞれELLを媒体（OVA/ELL），生理食塩液を媒体（OVA/Saline）あるいはリノール酸と大豆レシチンを用いた媒体（OVA/SLL）で投与してアレルギー反応を惹起した．

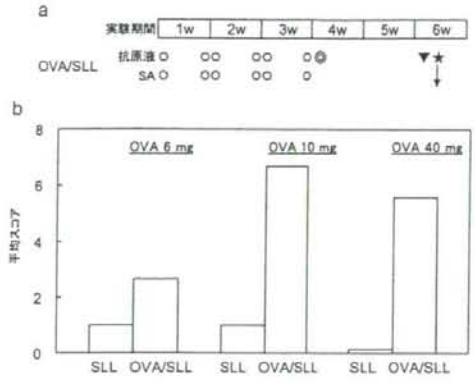

図5　惹起時の抗原量の違いによるアナフィラキシー症状の比較

aは媒体にリノール酸と大豆レシチンを用いた卵白アルブミン投与群（OVA/SLL）の実験スケジュールを示す．○はOVA 1 mgあるいはサリチル酸ナトリウム（SA） 0.3 mgの経口投与，◎はOVA 6，10あるいは40 mgの大量投与，▼は採血，★はbに示した条件でのOVAの経口投与によるアレルギー反応の惹起，↓はアナフィラキシー症状の観察を示す．媒体対照群（SLL）には同じスケジュールで媒体のみを投与し，OVA投与群と同容量の媒体を用いて大量投与およびアレルギー反応を惹起した．

bはアレルギー反応の惹起時に観察した各群におけるアナフィラキシー症状の平均スコアを示す．OVA/SLL投与群にリノール酸と卵黄レシチンを用いた媒体（ELL）を用いてOVA 6，10あるいは40 mgのOVAを経口投与してアレルギー反応を惹起した．

与群に6 mg，10 mgあるいは40 mgのOVAを経口投与してアレルギー反応を惹起し，全身性アナフィラキシー症状を観察した（図5a）．媒体対照群には媒体のみを経口投与した．

異なる種類のアレルゲン性タンパクでの反応を調べるために，LGあるいはGD（いずれもSigma-Aldrich）を抗原としてマウスに投与した．生理食塩液に溶解したOVA，LGおよび0.1 M 水酸化ナトリウム（NaOH）溶液に溶解したGDとSLLをそれぞれ乳化して抗原液を調製した．生理食塩液とSLLあるいはNaOH溶液とSLLを乳化してそれぞれ媒体対照液を調製した．各抗原0.4 mgを図6aに示したスケジュールで経口投与した後，反応を増幅する目的で4 mgの抗原を投与した．その2週間後，ELLを媒体とした4 mgの抗原を経口投与してアレルギー反応を惹起した．血清中抗LG IgG1および抗GD IgG1抗体価はELISAで測定した．

結果および考察

経口感作に用いられる一般的な投与量である1 mgおよびその1/100量である10 μgを微量用量として設定したOVA投与群では，いずれも惹起時の全身性アナフィラキシー症状において媒体対照群との平均スコアの差がそれぞれ2.3および2.9と増加し，かつ血清中に抗OVA IgG1抗体価が検出されたことから，感作が成立したと考えられた（図1bおよびc）．また，異なる頻度で抗原液を経口投与した間欠投与と連続投与のいずれのOVA投与群においても媒体対照群との平均スコアの差がそれぞれ1.8および2.9に増加し，かつ血清中に抗OVA IgG1抗体価が検出されたことから，感作が成立したと考えられた（図2bおよびc）．アレルゲン性の評価対象となるタンパク質の性状は様々であり，溶解性が低く高濃度の溶液が調製できない，あるいは中間精製物などその純度が低い場合も想定される．本食物アレルギーモデルは，微量の抗原によって感作が成立することから，目的とするタンパク質含量の低い試料にも適

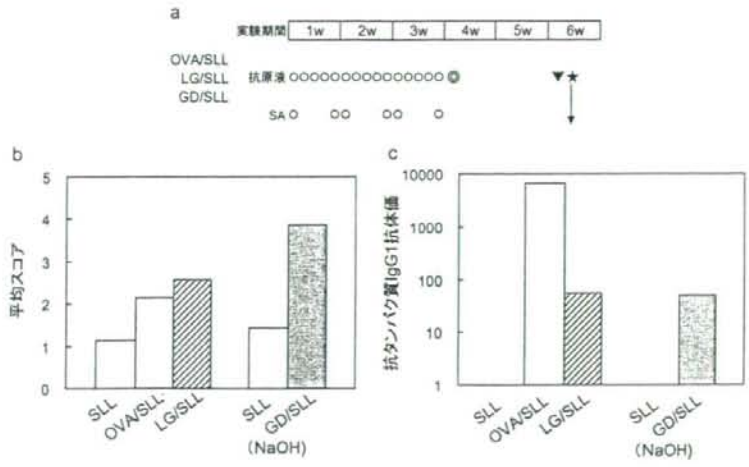

図6　抗原種の違いによる感作成立状態の比較

aは媒体にリノール酸と大豆レシチンを用いた卵白アルブミン投与群（OVA/SLL），ラクトグロブリン投与群（LG/SLL）およびグリアジン投与群（GD/SLL）の実験スケジュールを示す．○はそれぞれの抗原 0.4 mgの経口投与あるいはサリチル酸ナトリウム（SA） 0.3 mgの経口投与，◎は各抗原 4 mgの大量投与，▼は採血，★は各抗原 4 mgの経口投与によるアレルギー反応の惹起，↓はアナフィラキシー症状の観察を示す．媒体対照群（SLL）には同じスケジュールで媒体のみを投与した．GD/SLLおよびその媒体対照群は感作のための投与，大量投与およびアレルギー反応の惹起のための投与のいずれにも0.1 M水酸化ナトリウム（NaOH）を含む媒体中を用いた．

bはアレルギー反応の惹起時の各群におけるアナフィラキシー症状の平均スコア，cは採血時の各群における投与したタンパク質に対するIgG1抗体価の平均値を示す．

用可能であると考えられた．また，20 mg/mL以上の溶解度を得られるタンパク質においては，投与回数を減らしたスケジュールでも感作が可能であると考えられた．

食物アレルギーモデルにおいては，陽性対照物質としたOVAの反応と比較して目的とするタンパク質のアレルゲン性を評価することが想定される．したがって，OVAで強い症状や高い抗体価がみられる実験条件は，アレルゲン性が未知のタンパク質の検出感度を上げると考えられる．そこで，OVAの吸収量に影響すると考えられるSAを異なる経路で投与し，より強いアレルギー反応を示す投与経路を調べた．図3に示したように，OVA投与群と媒体対照群との平均スコアの差は腹腔内SA併用群2.9，経口SA併用群4.3と腹腔内より経口SA併用群が高かった．抗OVA IgG1抗体価も腹腔内より経口SA併用群が高い値を示した．消化管からの物質の吸収を高める作用が報告されているSAは[12,13]，経口投与での併用が食物アレルギーモデルとしての実験条件に適していると考えられた．

同様に，アレルギー反応の惹起時の全身性アナフィラキシー症状は，OVA投与群と媒体対照群の平均スコアの差を評価することから，両群の平均スコアの差が大きくなる惹起時の媒体の選択はアレルギー反応の検出感度を上げると考えられる．同一条件でELLを媒体として感作したマウス（図4a）に異なる3種の媒体を用いて大量の抗原を投与したところ，OVA投与群と媒体対照群の平均スコアの差はELLで1.4，生理食塩液で1.8であったが，これらに比べてSLLでは2.4と最も大きかった（図4b）．データは示さなかったがSLLを媒体として感作したマウスでは，SLLや生理食塩液よりELLを用いたアレルギー反応の惹起でOVA投与群と媒体対照群の平均スコアの差が最も大きくなった．微量の抗原でも感作が

成立する本食物アレルギーモデルは，媒体中の卵黄と大豆由来のレシチンにそれぞれ混入している微量の夾雑タンパク質に対する感作も成立している可能性がある．抗原投与時とアレルギー反応の惹起時の媒体に用いるレシチンの由来を変えた場合，媒体対照群の夾雑タンパク質による感作の影響は検出されない．媒体対照群のレシチン由来と考えられる反応を除外し，OVA投与群との差を明確にすることによって，アレルギー反応の惹起時の検出感度を上げることができると考えられた．

本食物アレルギーモデルは，感作時に比べてアレルギー反応の惹起時に大量のタンパク質を必要とする．より少ないタンパク質量でアレルゲン性の評価を可能にする目的で，順次抗原量を減らした投与での全身性アナフィラキシー症状の強度を調べた．同一条件で感作したマウス（図5a）にOVA 10あるいは40 mgを経口投与して惹起した時，OVA投与群と媒体対照群の平均スコアの差は5.7と5.5と同レベルであり（図5b），OVA 100 mgを投与した時の平均スコアの差4.3（図3b，SA経口）より高かった．したがって，反応の強度を下げることなく10 mgまでは惹起抗原量を減らせると考えられた．一方，抗原量6 mg（図5b）および4 mg（図6b）の投与においても，OVA投与群の媒体対照群との平均スコアの差は1.7および1.0と増加した．全身性アナフィラキシー反応の強度は低下するものの，感作の成立は検出できることから，少ない抗原量での惹起も可能であると考えられた．

以上の結果から，実験スケジュールとして，経口投与によるSAの併用下，3週間に2回以上の頻度で抗原タンパクを経口投与する条件，感作終了時に大量の抗原タンパクを経口投与することによって反応を増幅した2週間後に経口投与によって抗原タンパクを惹起する条件を設定した．用いる抗原タンパクは，SLLを媒体として感作時10 μg～1 mg，大量の抗原タンパクの投与時4～100 mg，ELLを媒体としてアレルギー反応の惹起時4～100 mgを設定した．全身性アナフィラキシー症状と血清中抗OVA IgG1抗体価を指標として，設定した条件の範囲でOVAによる感作が再現したことから，この実験条件は食物アレルギーモデルとなると考えられた．

本食物アレルギーモデルを用いてOVA以外のアレルゲン性タンパクの反応性を調べたところ，LGおよびGD投与群とそれぞれの媒体対照群の平均スコア差は1.4および2.4と増加し（図6b），血清中にはそれぞれの特異IgG1抗体価が検出された（図6c）．本食物アレルギーモデルはOVAと同様にLGおよびGDのアレルゲン性を検出できることが明らかとなった．また，GDのように中性付近の溶液に難溶のタンパク質においては，NaOHを含む溶液の使用も可能であった．以上の結果から，本食物アレルギーモデルは多種類のタンパク質の様々な状態での適用が可能であり，アレルゲン性の評価に有用であると考えられた．

ヒトやマウスにおいては，通常，経口投与したタンパク質に対しては経口免疫寛容が働くため[14]，感作が成立することはない．食物アレルギーモデルを作製する場合，いかに経口免疫寛容を抑えて経口投与した抗原に対して感作を成立させることができるかが問題となる．この問題を解決する方法として，強力な粘膜アジュバントであるコレラトキシン[15]を用いるマウスの食物アレルギーモデルが報告されている[11]．このモデルでピーナッツ抗原とコレラトキシンの混合液を1週間に1回の頻度で6週間経口投与したマウスは，血清中の特異IgE抗体が増加し，アレルギー反応の惹起によってチアノーゼや呼吸困難といった重篤な全身性アナフィラキシー症状を発現する．われわれが食物アレルギーモデルとしたマウスにおいても同様の反応が認められることから，LLで乳化した抗原摂取とSAの組み合わせは，コレラトキシンと同様に経口免疫寛容を抑えて感作を成立させると考えられた．ヒトが日常の生活において摂取する可能性のある物質の組み合わせによって作製した本食物アレルギーモデルは，ヒトの食物アレルギーの機序解明への利用も期待できると考えられた．

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平成19年度 厚生労働科学研究(食品の安心・安全確保推進研究)

遺伝子組換え食品の安全性評価

Safety Assessment of Genetically Modified Food

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Ⅰ はじめに

組換え食品，通常遺伝子組換え食品と呼ばれているものは，遺伝子組換え技術を応用した(Genetically modified(GM))食品のことである。遺伝子組換え食品の開発は食料の増産などを目的として，世界の多くの研究者が取り組んでいる。米国において平成6年5月，日持ちのよいトマトがFDAの承認を得て以来，さまざまな遺伝子組換え食品，食品添加物の開発が進んできた。平成20年2月までに，わが国において，安全性審査の手続きを経た遺伝子組換え食品は88種類，食品添加物は14種類に及ぶが，これらはすべて国外において生産されたものであり，国内において生産されたものはない。

わが国において，消費者の遺伝子組換え食品の安全性に対する不安はまだ根強いものがあり，食品安全委員会が平成15年度に行った食品安全モニターアンケート「食の安全性に関する意識調査」においても，遺伝子組換え食品には49％の消費者が不安であると答えている。これら消費者の方への不安に応えるためにも，遺伝子組換え食品の安全性評価の概要，国際的な安全性評価の動向についての地道な説明が重要であると考える。

本稿では，わが国における遺伝子組換え食品の安全性評価について述べ，次いで，厚生労働科学研究費の食品安全確保研究で行っている遺伝子組換え(バイオテクノロジー応用)食品の安全性確保研究の概要，組換え食品の開発の状況について述べる。

Ⅱ わが国における遺伝子組換え食品の安全性評価の考え方

1 安全性評価基準の基本的考え方

組換えDNA技術などを用いたバイオ食品などの安全性を確保するために，厚生省(当時)では，平成3年12月に「組換えDNA技術応用食品・食品添加物の安全性評価指針」を策定した。この指針の対象は，組換えDNA技術を応用して製造された食品