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Development and Evaluation of Event-Specific Qualitative PCR Methods for Genetically Modified Bt10 Maize

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In 2005 it was reported that the genetically modified (GM) maize strain or 'event' called Bt10 had been distributed inadvertently in the United States over the previous 4 years. In order to ensure that grain for food and feed production did not contain trace amounts of Bt10 maize and complied with the applicable regulation, highly sensitive and specific detection of Bt10 maize was required. Accordingly, we developed a novel qualitative PCR system for specific detection of Bt10 maize. Moreover, we amply evaluated the performance characteristics of two PCR systems, our own and the one provided by the developer of Bt10, Syngenta Co. Ltd. It was confirmed that both of the qualitative PCR systems can specifically detect Bt10 maize, and the results of a single-laboratory examination suggested that the limit of detection was approximately less than 0.05% for both methods. To evaluate the reproducibility of the methods, we organized an interlaboratory study with the participation of 6 laboratories and analysis of 240 blind test samples. In this paper, we report, for the first time, the statistical analysis of the qualitative PCR data obtained from the interlaboratory study. The results of this analysis also revealed that there was no significant difference in the sensitivity between the two aforementioned methods and that the limit of detection of both the methods was less than 0.05%. Thus, we conclude that both of the methods are equally suitable for correct identification and sensitive detection of the unapproved GM maize Bt10 event in test samples.

INTRODUCTION

Recent progress in recombinant DNA and plant transformation techniques has accelerated the development of various genetically modified (GM) organisms (GMOs) and of products derived from them. GM crops, in particular, herbicide-tolerant or insect-resistant soy, maize, rapeseed, and cotton, have already been approved by many countries for agricultural use for about a decade (*1*). However, the safety, in terms of use in foods/feeds, and the environmental effects of GM crops has been a matter of public concern because of anxiety regarding new technologies and lack of understanding of technical information. Therefore, an increasing number of countries have implemented regulatory frameworks to assess the safety of these GMOs prior to declaring them safe for the environment, for human consumption, and for use in animal feed. In addition, some countries have adopted the policy of informing their consumers of the GM origin of a food product's ingredients by labeling, supported by testing, and traceability of GM and conventional crops throughout the supply chain (*2*, *3*). Sensitive and specific analytical methods to test commercialized GM crops strains or 'events' are available at commercial and governmental laboratories (*4*).

On the other hand, no specific analytical methods are, in general, required for testing the presence of particular GM events that have not undergone safety assessment for approval, since these events are not intended to be commercialized for agricultural use and do not enter the food chain. However, in the event of accidental contamination of an unapproved GM event due to unforeseen circumstances, regulatory organizations should be well prepared with a testing system to monitor the distribution of the unapproved GM event. It will be challenging or perhaps impossible for commercial and governmental laboratories to develop such testing system without the assistance of the developer. Development and evaluation of a new analytical method is likely to be required, based upon experiments with authentic material containing the GM event in question. An incident where specific testing system for an unapproved GM event became necessary was the accidental distribution of Bt10 maize reported in 2005 (*5*). Syngenta Co. Ltd., one of the world's largest agricultural biotechnology companies, reported that they inadvertently produced and

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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

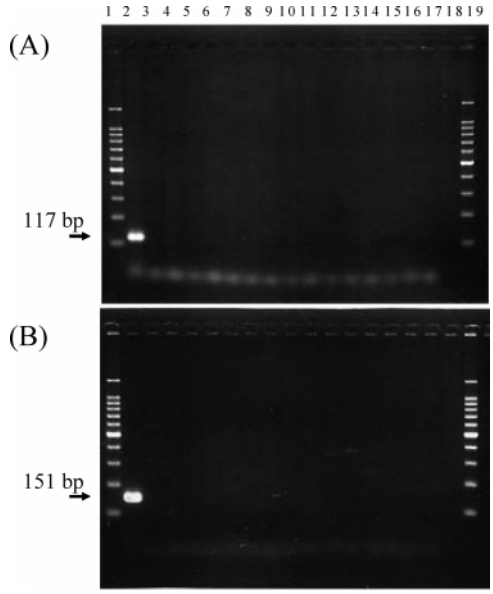

Figure 1. Specificity of the PCR systems employed to detect Bt10 maize. The Bt10 event-specific primers JSF5/JSR5 (**A**) and Bt10LS5′/3′ (**B**). Lanes 1 and 19, 100-bp ladder size standard; lane 2, Bt10; lane 3, Bt11; lane 4, GA21; lane 5, Event176; lane 6, MON810; lane 7, T25; lane 8, NK603; lane 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.

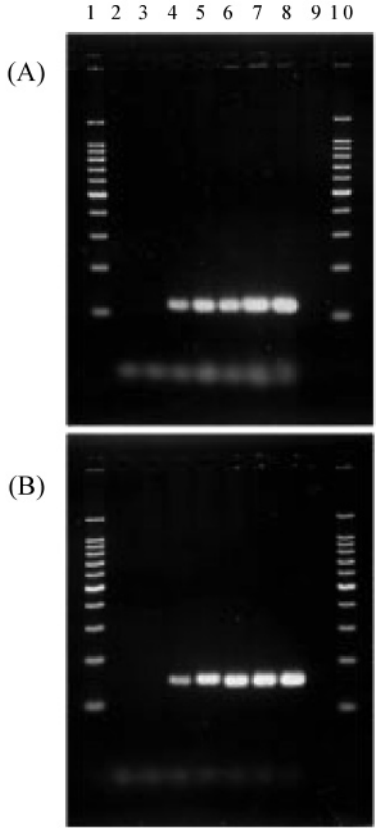

Figure 2. Examination of the sensitivity of the PCR systems to detect Bt10 maize using serial dilutions of the DNA-mixture samples. The Bt10 event-specific JSF5/ JSR5 (**A**) and Bt10LS5′/3′ (**B**) primer pairs. Lanes 1 and 10, 100-bp ladder size standard; lane 2, nontemplate DNA; lane 3, non-GM maize;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

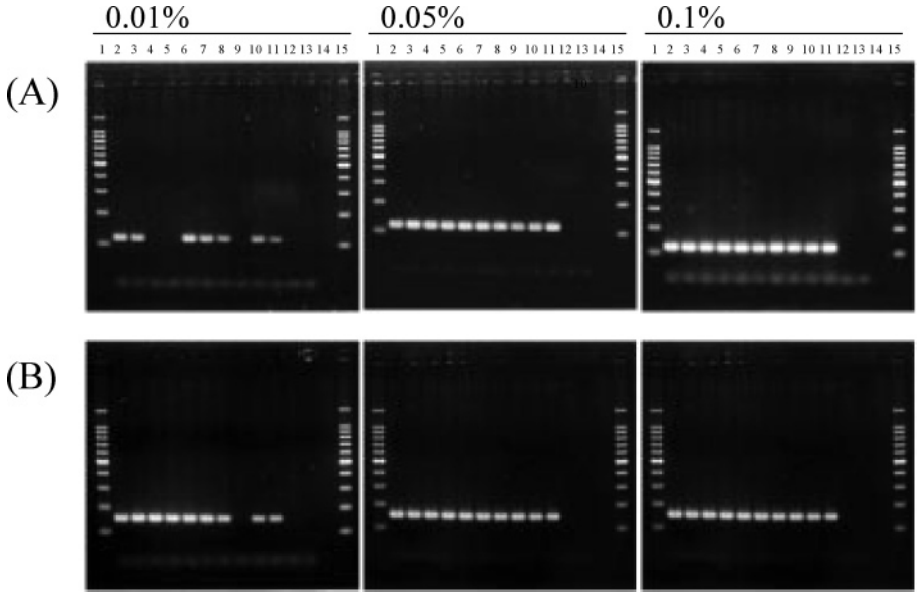

Figure 3. Examination of the sensitivity of the PCR systems to detect Bt10 maize using 10-replicate DNA-mixture samples for each of the three mixing levels. The Bt10 event-specific JSF5/JSR5 (**A**) and Bt10LS5′/3′ (**B**) primer pairs. Lanes 1 and 15, 100-bp ladder size standard; lanes 2 to 11, the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

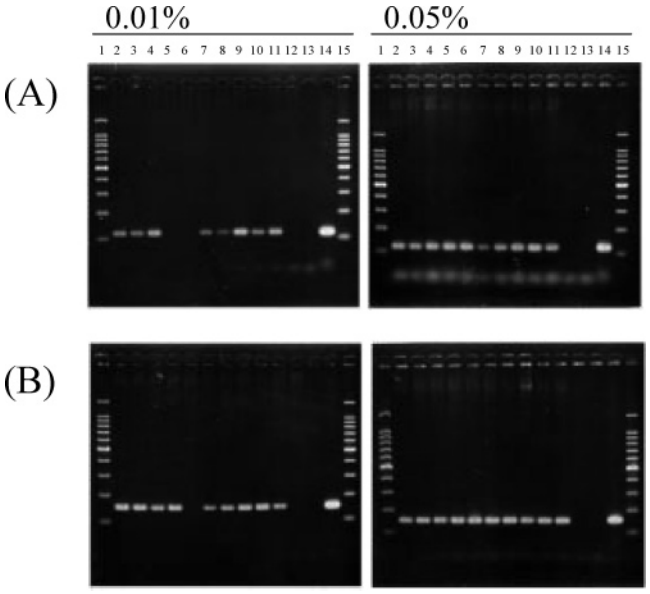

Figure 4. Examination of the sensitivity of the PCR systems to detect Bt10 maize using 10-replicates of the 0.05% powder mixture-sample and 0.01% powder diluted sample. Lanes 1 and 15, 100-bp ladder size standard; lanes 2 to 11, the samples; lane 12, nontemplate DNA; lane 13, non-GM maize; lane 14, 100%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

(1) James, C. *Global Status of Commercialized Transgenic Crops: 2005*; ISAAA: Ithaca, NY, 2005; No. 34.

(2) Hino, A. Safety assessment and public concerns for genetically modified food products: The Japanese experience. *Toxic Pathol.* **2000**, *30*, 126−128.

(3) Miraglia, M.; Berdal, K. G.; Brera, C.; Corbisier, P.; Holst-Jensen, A.; Kok, E. J.; Marvin, H. J. P.; Schimmel, H.; Rentsch, J.; van Rie, J. P. P. F.; Zagon, J. Detection and traceability of genetically modified organisms in the food production chain. *Food Chem. Toxicol.* **2004**, *42*, 1157−1180.

(4) Lipp, M.; Shillito, R.; Giroux, R.; Spiegelhalter, F.; Charlton, S.; Pinero, D.; Song, P. Polymerase chain reaction technology as analytical tool in agricultural biotechnology. *J. AOAC Int.* **2005**, *88*, 136−155.

(5) *Nature* **2005**, *434*, 423.

(6) Decision (EC) No.317/2005. *Off. J. Eur. Union* **2005**, *L101*, 14−16.

(7) Bulletin No. 232, The Ministry of Health, Labour and Welfare of Japan: Tokyo, Japan, 2001.

(8) Gachet, E.; Martin, G. G.; Vigneau, F.; Meyer, G. Detection of genetically modified organisms (GMOs) by PCR: A brief review of methodologies available. *Trends Food Sci. Technol.* **1999**, *9*, 380−388.

(9) Ahmed, F. E. Detection of genetically modified organisms in foods. *Trends Biotechnol.* **2002**, *20*, 215−213.

(10) Holst-Jensen, A.; Rønning, B. S.; Løvseth, A.; Berdal, G. K. PCR technology for screening and quantification of genetically modified organisms (GMOs). *Anal. Bioanal. Chem.* **2003**, *375*, 985−993.

(11) García-Cañas, V.; Cifuentes, A.; González, R. Detection of genetically modified organisms in food by DNA amplification techniques. *Crit. Rev. Food Sci. Nutr.* **2004**, *44*, 425−436.

(12) Goda, Y.; Asano, T.; Shibuya, M.; Hino, A.; Toyoda, M. Detection of recombinant DNA from genetically modified papaya. *Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Jpn.)* **2001**, *42*, 231−236.

(13) Matsuoka, T.; Kuribara, H.; Takubo, K.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. Detection of recombinant DNA segments introduced to genetically modified Maize (*Zea mays*). *J. Agric. Food Chem.* **2002**, *50*, 2100−2109.

(14) Kuribara, H.; Shindo, Y.; Takubo, K.; Matsuoka, T.; Takubo, K.; Futo, S.; Aoki, N.; Hirano, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Novel reference molecules for quantitation of genetically modified maize and soybean. *J. AOAC Int.* **2002**, *85*, 1077−1089.

(15) Shindo, Y.; Kuribara, H.; Matsuoka, T.; Futo, S.; Sawada, C.; Shono, J.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Validation of real-time PCR analyses for line-specific quantitation of genetically modified maize and using new reference molecules. *J. AOAC Int.* **2002**, *85*, 1119−1126.

(16) Watanabe, T.; Kuribara, H.; Mishima, T.; Kikuchi, H.; Kubo, M.; Kodama, T.; Futo, S.; Kasama, K.; Toyota, A.; Nouno, M.; Saita, A.; Takahashi, K.; Hino, A.; Akiyama, H.; Maitani, T. New qualitative detection methods of genetically modified potatoes. *Biol. Pharm. Bull.* **2004**, *43*, 301−305.

(17) Akiyama, H.; Watanabe, T.; Wakabayashi, K.; Nakade, S.; Yasui, S.; Sakata, K.; Chiba, R.; Spiegelhalter, F.; Hino, A.; Maitani T. Quantitative Detection System for Maize Sample Combined-Trait Genetically Modified Maize. *Anal. Chem.* **2005**, *77* (22), 7421−7428.

(18) Onihshi, M.; Matsuoka, T.; Kodama, T.; Kashiwaba, K.; Futo, S.; Akiyama, H.; Maitani, T.; Furui, S.; Oguchi, T.; Hino, A. Development of a multiplex polymerase chain reaction method for simultaneous detection of eight events of genetically modified maize. *J. Agric. Food Chem.* **2005**, *53*, 9713−9721.

(19) Watanabe, T.; Kasama, K.; Kikuchi, H.; Suzuki, T.; Tokishita, S.; Sakata, K.; Matsuki, A.; Hino, A.; Akiyama, H.; Maitani, T. Laboratory-performance study of quantitative PCR methods to analyze an approved genetically modified maize (mon810 line). *Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Jpn.)* **2006**, *47*, 15−27.

(20) Notification No. 0517001. Department of Food Safety, The Ministry of Health, Labour and Welfare of Japan: Tokyo, Japan, 2005.

(21) Kirihara, J. A.; Petri, J. B.; Messing, J. Isolation and sequence of a gene encoding a methionine-rich 10-kDa zein protein from maize. *Gene* **1988**, *71*, 359−370.

(22) Watanabe, T.; Kasama, K.; Wakui, C.; Shibuya, M.; Matsuki, A.; Akiyama, H.; Maitani, T. Laboratory-performance study of the notified methods to detect genetically modified maize (CBH351) and potato (NewLeaf Plus and NewLeaf Y). *Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Japan)* **2003**, *44*, 281−288.

(23) *Official Methods of Analysis of AOAC International*, 17th ed.; AOAC International: Gaithersburg, MD, 2000; Appendix D, 2−11.

(24) McClure, F. D. Design and analysis of qualitative collaborative studies: minimum collaborative program. *J. Assoc. Off. Anal. Chem.* **1990**, *73* (6), 953−960.

(25) Bennett, M.; Smith, J. B. Nuclear DNA in angiosperms. *Philos. Trans. R. Soc. London Ser. B: 334*, 309−345.

(26) Hernández, M.; Rodriguez-Lázaro, D.; Zhang, D.; Esteve, T.; Pla, M.; Prat, S. Interlaboratory transfer of a PCR multiplex method for simultaneous detection of four genetically modified maize lines: Bt11, MON810, T25, and GA21. *J. Agric. Food Chem.* **2005**, *53*, 3333−3337.

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