body-associated protein in soybean, has been identified as one of the major allergenic proteins and named Gly m Bd 30K. Our ELISA method is highly specific for this soybean protein, with the LOD of 0.47 ng/mL (equivalent to 0.19 μg/g food) and limit of quantification (LOQ) of 0.94 ng/mL (equivalent to 0.38 μg/g food). Recovery ranged from 87.7% to 98.7%, while the intra- and interassay coefficients of variation were less than 4.2% and 7.5%, respectively. These results show that this ELISA method is specific, precise, and reliable for quantitative analysis of the soybean protein in processed foods. Five types of incurred samples (model processed foods: rice gruel, sausage, sweet adzuki-bean soup, sweet potato cake, and tomato sauce) containing 10 μg soybean soluble protein/g food were prepared for use in interlaboratory evaluations of the soybean ELISA kit (Sakai *et al.*, 2009). The kit displayed a sufficient RSD_r value (interlaboratory precision: 9.3–13.4% RSD_r) and a high recovery (97–114%) for all incurred samples. The RSD_r value for the incurred samples was mostly $<4.8\%$. The results of this interlaboratory evaluation suggest that the soybean kit can be used as a precise and reliable tool for determination of soybean proteins in processed foods.

A sensitive qualitative detection method for soybeans in foods using PCR was also developed (Yamakawa *et al.*, 2007b). For specific detection of soybeans with high specificity, the primer pair was designed using the gene encoding the *Glycine max* repetitive sequence. Trace amounts of soybeans in commercial food products could be qualitatively detected by this method.

2. Walnut

Tree nuts are regarded as one of the most potent of all known food allergens and are often attributed as the cause of severe food anaphylaxis and death. Walnut (*Juglans regia*) is the most common allergenic tree nut and this allergy can be observed in all age groups (Bock *et al.*, 2001). In addition, the walnut allergy is extremely potent, inducing life threatening allergic reactions similar to peanut allergy (Clark and Ewan, 2003; Pumphrey, 2000; Pumphrey and Roberts, 2000). According to Japanese regulations, the labeling of food products containing walnut is recommended. To ensure proper labeling, a novel sandwich ELISA kit for the determination of walnut protein in processed foods has been developed (Doi *et al.*, 2008). The sandwich ELISA method is highly specific for walnut soluble proteins. The recovery ranged from 83.4% to 123%, while the intra- and interassay coefficients of variation were less than 8.8% and 7.2%, respectively. We prepared seven types of incurred samples (model processed foods: biscuit, bread, sponge cake, orange juice, jelly, chicken meatball, and rice gruel) containing 10 μg walnut soluble protein/g food for use in interlaboratory evaluations of the walnut ELISA method (Sakai *et al.*, 2010a). The walnut kit displayed a sufficient RSD_r

(interlaboratory precision: 5.8–9.9% RSD_r) and a high level of recovery (81–119%) for all the incurred samples. All RSD_r values for the incurred samples examined were less than 6.0%. The results of this interlaboratory evaluation suggest that the walnut ELISA method can be used as a precise and reliable tool for determination of walnut proteins in processed foods.

A sensitive qualitative detection method for walnut using PCR was also developed (Yano *et al.*, 2007). For detection of walnuts with high specificity, the primer pair was designed based on walnut *matK* genes. Trace amounts of walnuts in commercial food products can be qualitatively detected using this method.

3. Kiwifruit

Kiwifruit (*Actinidia deliciosa* and *A. chinensis*) is a major fruit allergen that produces severe symptoms and is responsible for a large number of clinical cases worldwide (Lucas *et al.*, 2003; Lucas *et al.*, 2004; Möller *et al.*, 1998a). Under Japanese regulations, it is recommended for labeling as much as possible. To develop PCR-based methods for detection of trace amounts of kiwifruit in foods, we designed two primer pairs targeting the ITS-1 region of the *Actinidia* spp. using PCR simulation software (Taguchi *et al.*, 2007). On the basis of the known distribution of a major kiwifruit allergen (actinidin) within the *Actinidia* spp., in addition to reports on clinical and immunological cross-reactivities, one of the primer pairs was designed to detect all *Actinidia* spp. and the other to detect commercially grown *Actinidia* spp. (i.e., *A. arguta* and is interspecific hybrids) except for *A. polygama*. The specificity of these methods using designed primer pairs was verified by PCR on eight *Actinidia* spp. and 26 other plants, including fruits. The methods were considered to be specific enough to yield products of the target-size only from *Actinidia* spp. and sensitive enough to detect 5–50 fg of *Actinidia* spp. DNA spiked in 50 ng salmon testis DNA used as a carrier (1–10 ppm of kiwifruit DNA) and 1700 ppm (wt/wt) of fresh kiwifruit puree spiked in a commercial plain yogurt (corresponded to ca. 10 ppm of kiwifruit protein). These methods are expected to be useful in the detection of unidentified kiwifruit and its related species in processed foods.

4. Banana

Banana contains food allergens that are common to those in latex or pollens (Ito *et al.*, 2006; Sanchez-Monge *et al.*, 1999). Many clinical studies have reported cross-reactivity of banana and latex, referred to as the latex-fruit syndrome (Blanco *et al.*, 1999; Ikezawa and Osuna, 2002; Möller *et al.*, 1998b). These studies monitored the number of patients with food allergy in Japan and found that patients with banana allergy comprised the second largest population (below only kiwifruit allergy) among those with fruit allergies. We developed specific PCR methods for detection of

banana residue in processed foods. For high banana specificity, the primer pair was designed based on the large subunit of ribulose-1, 5-bisphosphate carboxylase (*rbcL*) genes of chloroplasts and used to obtain amplified products specific to banana by both conventional and real-time PCR (Sakai *et al.*, 2010b). To confirm the specificity of these methods, genomic DNA samples from 31 other species were examined; no amplification products were detected. Subsequently, eight kinds of processed foods containing banana were investigated using these methods to confirm the presence of banana DNA. Conventional PCR had a detection limit of 1 ppm (wt/wt) banana DNA spiked in 50 ng of salmon testis DNA, while SYBR Green I real-time semi-quantitative PCR had a detection limit as low as 10 ppm banana DNA. Thus, both methods show high sensitivity and may be applicable as specific tools for the detection of trace amounts of banana in commercial food products.

5. Pork, beef, chicken, mutton, and horseflesh

As the modern diet often comprises processed foods, especially minced meats, manufacturers are obligated to properly label raw materials. Hence, a rapid method of detecting meat ingredients in processed foods is needed to verify proper labeling. A rapid real-time quantitative PCR method to detect trace amounts of pork, beef, chicken, mutton, and horse meat in foods was developed (Tanabe *et al.*, 2007). The primers and TaqMan minor groove binder (MGB) probes were designed using the gene encoding cytochrome *b* for specific detection of each species. The LOQ of this method was 100 fg/μL of each mitochondrial DNA in 10 ng/μL of wheat mitochondrial DNA matrix. The calculated R^2 values of the standard curves for the five species ranged between 0.994 and 0.999. This method is particularly useful in the detection of unidentified minced meat in processed foods for verification of food labeling.

IV. PATIENT EVALUATION OF ALLERGY FOOD LABELING

To clarify the usefulness and reliability of the food-labeling system, food allergy patients (or their parents) at Sagamihara National Hospital were asked to evaluate it by questionnaire. We received responses from 169 patients. As shown in Table 4.20, patients' profiles were an average age of 49.3 ± 35.6 months, age of the first onset of symptoms of 10.1 ± 14.1 months, and average of 2.9 ± 2.5 eliminated foods. Eliminated foods included hen's eggs (135), cow's milk (79), and wheat (47), as well as peanuts and fish eggs. Of these patients, 44.2% had a past history of anaphylaxis, and 80.2% had experienced symptoms following exposure to even extremely small amounts of the causative foods.

TABLE 4.20 Characteristics of surveyed subjects

169 parents of food allergy patients at Sagamihara National Hospital	
Age of patients	49.3 ± 35.6 months M/F = 1.9
Age of first onset of symptom	10.1 ± 14.1 months
Number of eliminated foods	2.9 ± 2.5
Eliminated foods	
Hen's eggs	135
Cow's milk	79
Wheat	47
Peanuts	51
Fish eggs	28
Past history of anaphylaxis	44.2%
Incidence of symptom by extremely small amount	80.2%

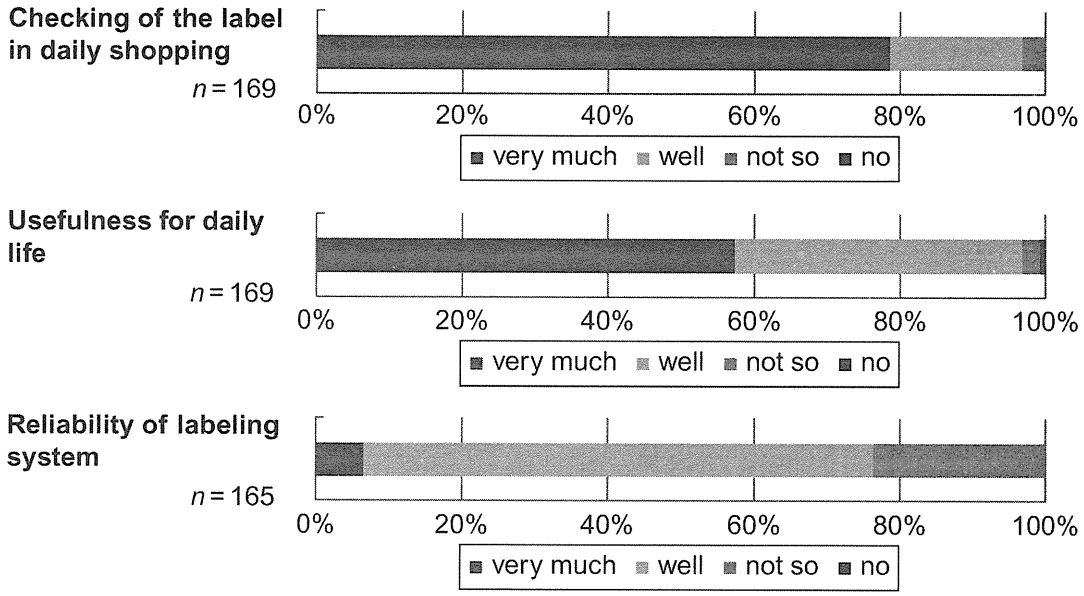

FIGURE 4.7 Evaluation of allergy food labeling.

As shown in Fig. 4.7, 97% of patients routinely checked the allergy food label during daily shopping, and 97% evaluated the allergy food labeling as "very useful" or "useful." In addition, 76.4% of the respondents relied on the allergy food-labeling system, and 79.3% had a correct understanding of the food-labeling system based on self-evaluation. On the other hand, 48.8% of respondents answered that the labeling system was "very easy" or "easy" to understand (Fig. 4.8). Patients who had experienced accidental intake by misreading a label or by mislabeling comprised 30.9% and 13.9%, respectively (Fig. 4.9).

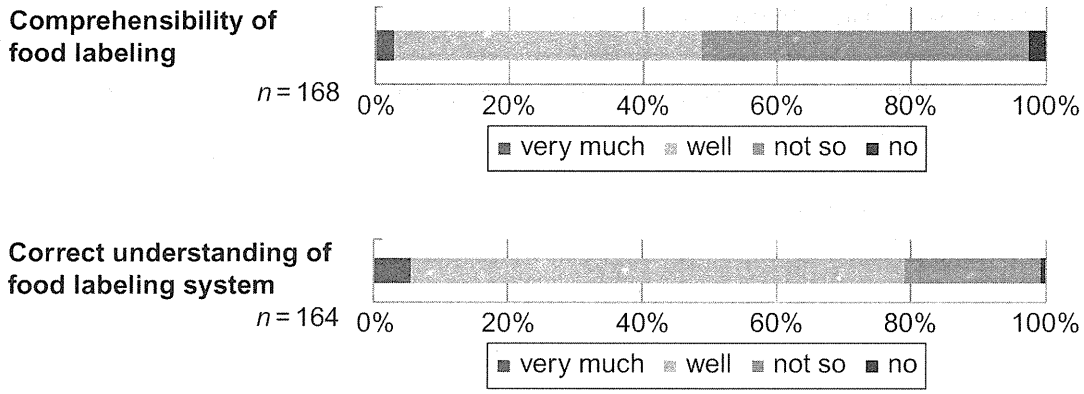

FIGURE 4.8 Comprehension and understanding of allergy food labeling.

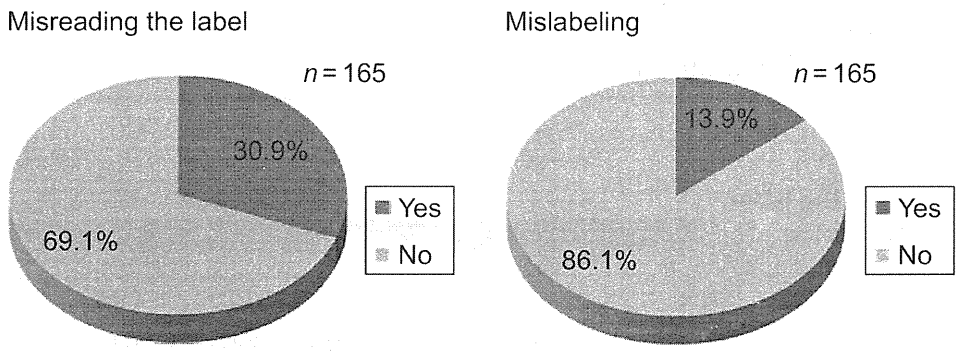

FIGURE 4.9 Incidences of accidental intake by misreading and mislabeling of food labels.

Overall, the Japanese food allergy-labeling system was highly evaluated by food allergy patients and parents. Almost all patients felt that the food-labeling system was very useful, although there were cases of accidental intake either by misreading the label or by mislabeling by food companies.

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FOOD COMPOSITION AND ADDITIVES

Interlaboratory Study of DNA Extraction from Multiple Ground Samples, Multiplex Real-Time PCR, and Multiplex Qualitative PCR for Individual Kernel Detection System of Genetically Modified Maize

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In many countries, the labeling of grains, feed, and foodstuff is mandatory if the genetically modified (GM) organism content exceeds a certain level of approved GM varieties. We previously developed an individual kernel detection system consisting of grinding individual kernels, DNA extraction from the individually ground kernels, GM detection using multiplex real-time PCR, and GM event detection using multiplex qualitative PCR to analyze the precise commingling level and varieties of GM maize in real sample grains. We performed the interlaboratory study of the DNA extraction with multiple ground samples, multiplex real-time PCR detection, and multiplex qualitative PCR detection to evaluate its applicability, practicality, and ruggedness for the individual kernel detection system of GM maize. DNA extraction with multiple ground samples, multiplex real-time PCR, and multiplex qualitative PCR were evaluated by five laboratories in Japan, and all results from these laboratories were consistent with the expected results in terms of the commingling level and event analysis. Thus, the DNA extraction with multiple ground samples, multiplex real-time PCR, and multiplex qualitative PCR for the individual kernel detection system is applicable and practicable in a laboratory to regulate the commingling level of GM maize grain for GM samples, including stacked GM maize.

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Genetically modified (GM) crops have been developed and are widespread as food and feed in many countries (1). These GM crops have been assessed and authorized for food use by administrative authorities over the past two decades. Under the current regulatory conditions, the labeling of grains, feed, and foodstuff is mandatory if the GM crop content exceeds a certain level of the approved GM varieties. For instance, the European Union, Japan, and Korea have set threshold values of 0.9, 5, and 3%, respectively, of GM organism material in a non-GM background as the basis for labeling (2–8). In the United States, GM crops are not regarded as a food safety issue, and labeling is voluntary. Depending on national philosophy, governmental regulation differs on the use and application of the technology for GM crops. In Japan, non-GM crops are segregated as non-GM material and imported from the United States using an identity preserved handling system that requires document certification from U.S. farms to Japanese processing

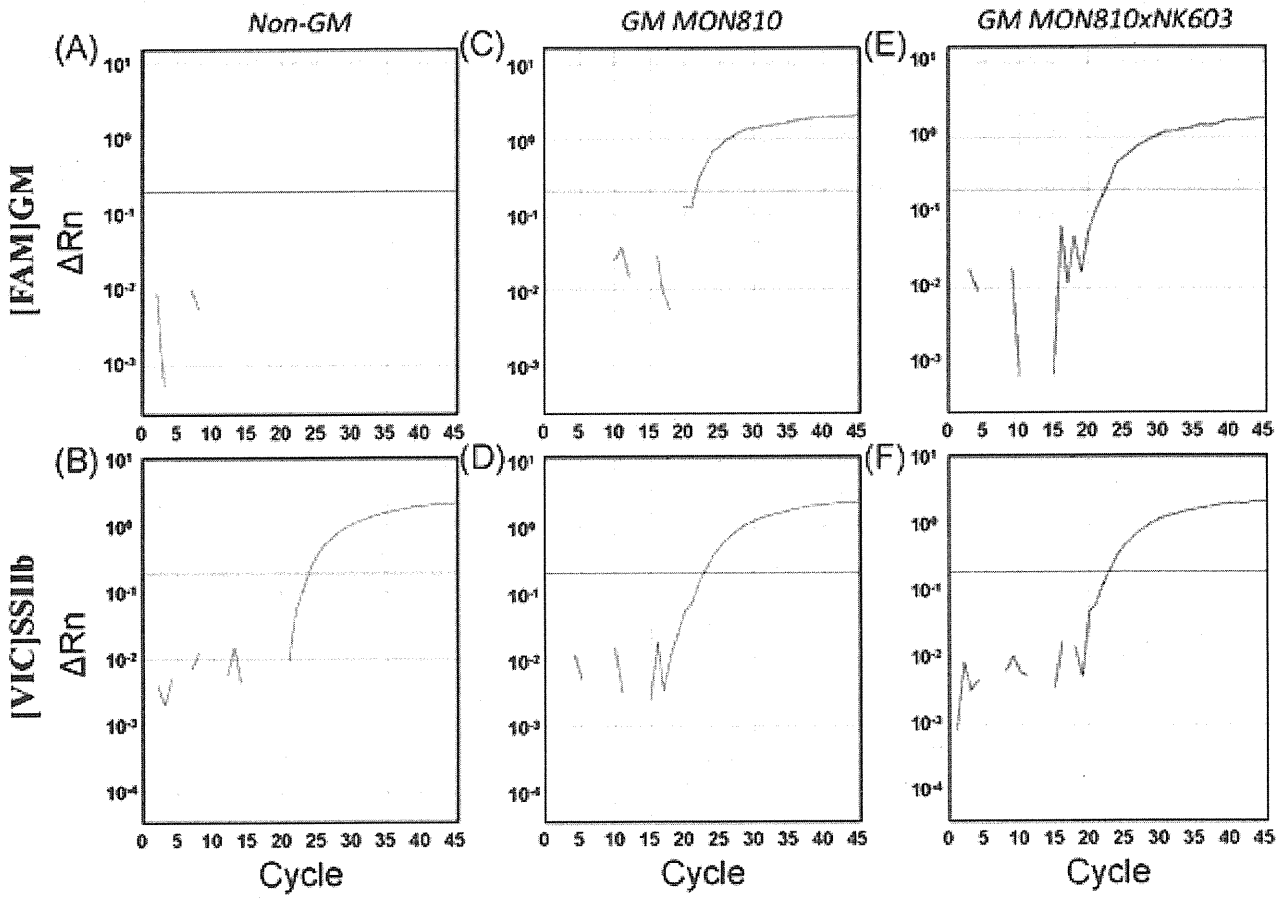

Figure 1. Typical multiplex real-time PCR amplification plots. The amplification plot was established using genomic DNA derived from (A, B) non-GM maize, (C, D) MON810 maize, or (E, F) MON810xNK603 stacked maize as the template. The probes used to specifically detect GM maize-specific gene (A, C, E) and endogenous SSIIb gene (B, D, F) in multiplex real-time PCR were labeled with VIC- and FAM-fluorescent dyes, respectively. The horizontal axis indicates the number of PCR cycles, and the vertical axis indicates Δnormalized reporter signal (ΔRn) values, which are the relative values automatically calculated by the analysis software based on the signal intensities of FAM dye, dependent on the target amplification and ROX passive reference.

traders. Recently, the production of stacked GM maize events, in which two or more different characteristic traits are inserted, has increased in the United States due to enhanced production efficiency (1). Although the levels of adventitious commingling of GM maize into non-GM maize in the labeling system refer to GM maize on a weight per weight (w/w) percentage basis, the conventional applicable detection methods, such as quantitative real-time PCR, do not directly measure the w/w percentage of GM maize. The GM maize percentages calculated using current quantitative real-time PCR methods are calculated by converting relative copy numbers between a specific rDNA sequence and a taxon-specific DNA sequence into a w/w percentage using appropriate reference materials. The GM maize content in a maize sample containing stacked GM maize events, as determined by current quantitative real-time PCR methods, is likely to be overestimated compared to the actual w/w percentage of GM maize in the sample because the relative copy numbers are calculated on a haploid basis.

We have developed an individual kernel detection system that consists of grinding individual maize kernels, DNA extraction from multiple ground maize kernels, multiplex real-time PCR using the extracted DNAs from individual ground maize kernels for GM detection, and multiplex qualitative PCR using the extracted DNAs for GM event detection to analyze the exact commingling level and varieties of GM maize (9–11). The detection system has already been implemented in Japan as an official GM organism detection method (12).

However, as a routine test in the laboratory, the single-kernel detection system appears to be time-consuming. As multitube samples are tested and the DNA of each kernel is extracted from each well in the 96-well plate in the single-kernel detection system, it is likely that the analyst will make some errors.

Therefore, in the single-kernel detection system it was necessary to demonstrate that an analyst is able to decontaminate and quantify each kernel individually, then determine the GM maize level (the percentage of kernel/kernel) on a multiple laboratory basis.

In the present study, the steps in the individual kernel

Table 1. In-house validation of the individual maize kernel test

	SSIIb[b]						GM[b]					
	Non-GM		MON810		MON810xNK603		Non-GM		MON810		MON810xNK603	
Day[a]	(+)[c]	(–)[c]	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)
1	12	0	6	0	6	0	0	12	6	0	6	0
2	12	0	6	0	6	0	0	12	6	0	6	0
3	12	0	6	0	6	0	0	12	6	0	6	0
4	12	0	6	0	6	0	0	12	6	0	6	0
5	12	0	6	0	6	0	0	12	6	0	6	0
Total	60	0	30	0	30	0	0	60	30	0	30	0

[a] Real-time PCR was run independently on 5 different days.

[b] The probes used to specifically detect endogenous SSIIb and GM maize-specific genes were labeled with 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein (VIC) and 6-carboxyfluorescein (FAM)-fluorescent dyes, respectively.

[c] (+) = Number of positive samples, (–) = number of negative samples.

detection system requiring the most attention, i.e., DNA extraction from multiple ground samples, multiplex real-time PCR, and multiplex qualitative PCR, were evaluated to clarify their applicability, practicality, and ruggedness for use in the determination of GM maize kernel samples, including stacked GM maize events, in an interlaboratory study.

Experimental

Maize (Zea mays) Materials

Non-GM maize grain, MON810 seeds, and stacked maize seeds (MON810xNK603) were kindly provided by Monsanto Co. (St. Louis, MO) as positive controls for GM maize.

Oligonucleotide Primers and Probes for Multiplex Real-Time PCR

Sets of primer pairs and probes for the construct-specific and universal GM quantification were the same as those described in our previous papers (9, 10). The SSIIb-3 system (SSIIb 3-5′ and SSIIb 3-3′ with SSIIb-TaqV) was used as the primers and probe for the detection of the taxon-specific gene encoding the maize starch synthase IIb gene sequence (SSIIb) by multiplex real-time PCR. The p35S-1 (P35S 1-5′ and P35S 1-3′ with P35S-Taq) and GA21-3 systems (GA21 3-5′ and GA21 3-3′ with GA21-Taq) were used for multiplex real-time PCR. All sets of primer pairs and probes (p35S-Taq and GA21-Taq) for detection of the cauliflower mosaic virus 35s promoter sequence (p35S) and GA21-specifc sequence, respectively, were purchased from Fasmac Co., Ltd (Kanagawa, Japan). SSIIb-TaqV, which is labeled with VIC® and 6-carboxytetramethyl-rhodamine (TAMRA) at the 5′ and 3′ ends, was synthesized by Life Technologies (St. Louis, MO) and used as a probe for detection of SSIIb. The target sequence of the p35S-1 system to detect the 35S promoter region derived from CaMV is widely found in recombinant DNA of almost all GM events, with the exception of GA21. The GA21-3 system was designed to detect the construct-specific sequence GM maize event GA21 (9, 10).

Grinding of Maize Materials

For the in-house validation study of DNA extraction from multiple ground samples and multiplex real-time PCR detection, individual maize kernels were placed in a sample tube that contained the pulverizing medium (MC0316MZ; Yasui Kikai Co., Osaka, Japan), and the tube was closed with an attached cap (ST-0350MZ; Yasui Kikai Co.). The 24 sample tubes were arrayed in the tube holder (Type SH-123; Yasui Kikai Co.). Two tube holders can be accommodated in a multibeads shocker (Model MB601NIHS; Yasui Kikai Co.) at a time. Next, the maize kernels were ground by heavily shaking the tubes using a multibeads shocker at 2500 rpm for 1 min and repeated for 1 min after the tube holder was reversed (9).

In the interlaboratory validation of DNA extraction from multiple ground samples and multiplex real-time PCR, the non-GM maize grains, MON810 seeds, and stacked maize seeds (MON810xNK603) were ground in an ultra-centrifugal mill (ZM100; Retsch GmbH, Haan, Germany) using a 0.5 mm sieve ring. Samples were stored at –20°C until further use.

Table 2. Ruggedness of DNA extraction from multiple ground samples and multiplex real-time PCR using different real-time PCR instruments

	SSIIb[a]						GM[a]					
	Non-GM (86)[b]		MON810xNK603 (2)		MON810 (2)		Total (90)		Non-GM (86)		MON810xNK603 (2)		MON810 (2)		Total (90)	
Instrument	(+)[c]	(–)[c]	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)
AB7900	86	0	2	0	2	0	90	0	0	86	2	0	2	0	4	86
AB7500	86	0	2	0	2	0	90	0	0	86	2	0	2	0	4	86
Expected	86	0	2	0	2	0	90	0	0	86	2	0	2	0	4	86

[a] Probes used to specifically detect endogenous SSIIb and GM maize-specific genes were labeled with VIC- and FAM-fluorescent dyes, respectively.
[b] Number in parentheses indicates the number of samples analyzed.
[c] (+) = Number of positive samples, (–) = number of negative samples.

DNA Extraction from Each Maize Kernel or Ground Maize Samples Using DNeasy® 96 Plant Kit

Genomic DNA extraction from finely ground maize powder was performed using the DNeasy 96 plant kit (QIAGEN, Hilden, Germany) according to previous reports (9, 10). Buffer AP1 (preheated to 65°C) and RNase A (QIAGEN; final concentration, 100 µg/mL) were combined to make the working solution. One milliliter of working solution was added to each sample tube containing the ground maize powder. The tubes were capped and incubated for 30 min at 65°C (inverted 10 times at intervals of 10 min). A 170 µL aliquot of Buffer AP2 solution was then added to each solution. After sealing to avoid leakage, the tubes were vigorously shaken for 15 s, incubated for 10 min at –20°C, and centrifuged for 20 min at 3000 rpm using a metalfuge (MBG100; Yasui Kikai Co.). A 400 µL aliquot of each supernatant was carefully transferred to a new microtube and centrifuged again for 5 min at 12 000 rpm. Each supernatant was carefully transferred to a new microtube, and 1.5 volumes (typically 600 µL) Buffer AP3/E were added to each sample. After carefully transferring 1 mL of each sample to the DNeasy 96 plates, the plates were sealed with tape and then aspirated until each DNeasy membrane was dry. After removing the tape, 800 µL Buffer AW was carefully added to each sample. The plate was again sealed with tape and aspirated until each DNeasy membrane was dry. The washing was repeated three times. An 800 µL aliquot of 100% ethanol was then added to each sample. The plate was aspirated for 15 min to dry each DNeasy membrane. After removing the tape to elute the DNA, the plate was placed in the correct orientation on a rack of elution microtubes, and then 75 µL distilled water (preheated to 65°C) was added to each sample. The plate was resealed and incubated for 5 min at room temperature, followed by aspiration until each DNeasy membrane was dry.

Multiplex Real-Time PCR Conditions

To simultaneously detect genomic DNA from ground maize samples and confirm the validity of the extracted genomic DNA, multiplex real-time PCR analyses were performed according to previous reports (9, 10). The amplification curves of the target sequence were monitored with a fluorescent dye that labeled the designed oligonucleotide probes using the ABI PRISM® 7900HT sequence detection system (Life Technologies Corp., Carlsbad, CA). The reaction volume (25 µL) contained 2.5 µL sample genomic DNA solution, 12.5 µL Universal Master Mix® (Life Technologies Corp.), 0.5 µM primer pair, and 0.2 µM probe (for p35S: 0.1 µM probe). The PCR step-cycle program was as follows: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 30 s at 95°C and 90 s at 59°C.

Table 3. Interlaboratory validation of DNA extraction from multiple ground samples and multiplex real-time PCR

		SSIIb[a]						GM[a]					
		Non-GM (86)[b]		MON810xNK603 (2)		MON810 (2)		Total (90)		Non-GM (86)		MON810xNK603 (2)		MON810 (2)		Total (90)	
Laboratory	Instrument	(+)[c]	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)
A	AB7500	86	0	2	0	2	0	90	0	0	86	2	0	2	0	4	86
B	AB7900	86	0	2	0	2	0	90	0	0	86	2	0	2	0	4	86
C	AB7900	86	0	2	0	2	0	90	0	0	86	2	0	2	0	4	86
D	AB7500	86	0	2	0	2	0	90	0	0	86	2	0	2	0	4	86
E	AB7900	86	0	2	0	2	0	90	0	0	86	2	0	2	0	4	86
Expected		86	0	2	0	2	0	90	0	0	86	2	0	2	0	4	86
Agreement, %[d]		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

[a] Probes used to specifically detect endogenous SSIIb and GM maize-specific genes were labeled with VIC and FAM-fluorescent dyes, respectively.

[b] Numbers in parentheses indicate the number of samples analyzed.

[c] (+) = Number of positive samples, (–) = number of negative samples.

[d] Value was calculated from comparison with the expected results and results obtained from five different laboratories.

Generally, the baseline was set to cycles 3 through 15. The Δnormalized reporter signal (ΔRn) threshold cycle (Ct) for plotting values was set to 0.1–0.5 during exponential amplification. The nonexponential curves, such as the rise of the baseline or the phenomenon known as 6-carboxy-X-rhodamine (ROX) dye dropping, could be observed, although it occurred with low frequency. In our previous studies, we concluded that the clearness of the exponential amplification curves after 15 cycles (last cycle of the baseline) of real-time PCR enabled the adoption of this as the threshold for the discrimination of GM from non-GM maize (9, 10). If the exponential amplification curves for GMO detection could be clearly observed after 15 cycles, the samples were judged to be positive for GM maize; otherwise, they were judged to be negative. In this study, the GM maize detection plasmid set, ColE1/TE (Nippon Gene Co., Tokyo, Japan), was used as the positive control. This plasmid set contained six concentrations of the reference plasmid pMul5, into which was inserted the amplification products of p35S, GA21, and SSIIb diluted with Tris-EDTA buffer (pH 8.0) containing 5 ng/μL ColE1 plasmid (9, 10). The ColE1 plasmid contained none of the amplification GM products and was used as the negative control. The positive controls were prepared using the two concentrations of the plasmid set with 250 000 and 1500 copies/plate. In the negative control, the ColE1 plasmid was also used as the nontemplate control for the analysis. In the reaction plate, real-time PCR was performed in duplicate using two reaction vessels for the nontemplate control as the negative control and positive control (two concentrations of the plasmid set). The other 90 reaction vessels were used for the genomic DNA samples extracted from the ground maize samples.

Multiple Qualitative PCR Conditions

To identify which GM traits are contained in genomic DNA extracted from ground maize samples, multiple qualitative PCR detection was performed according to our previously reported method with some modifications (11). The reaction mixture for PCR was prepared in a 96-well plate. The reaction volume (25 μL) contained 25 ng genomic DNA, 0.2 mmol/L deoxynucleotide triphosphate (dNTP), 1.5 mmol/L $MgCl_2$, 0.2 μmol/L each of 5′ and 3′ primers, 1.25 units AmpliTaq Gold DNA polymerase (Life Technologies Corp.), and 15 primers at the following concentrations: 0.2 μmol/L for M810 1-5′, NK603 1-3′ M863 1-5′, M863 1-3′, Bt11 1-5′, and CryIA 1-3′; 0.1 μmol/L for T25 2-5′, T25 2-3′, GA21 1-5′, GA21 1-3′, TC1507 1-5′, and TC1507 1-3′; 0.05 μmol/L for Event 176 1-5′; and 0.045 μmol/L for SSIIb 1-5′ and SSIIb 1-3′. For the combination of primers for the detection of each event, M810 1-5′ and NK603 1-3′ for NK603 detection, Event 176 1-5′ and CryIA 1-3′ for Event 176 detection, T25 2-5′ and T25 2-3′ for T25 detection, GA21 1-5′ and GA21 1-3′ for GA21 detection,

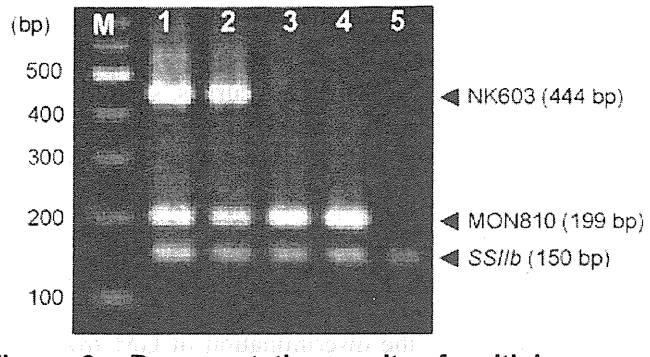

Figure 2. Representative results of multiplex qualitative PCR. PCR amplification products using genomic DNA templates purified from No. 10 (Lane 1), No. 45 (Lane 2), No. 59 (Lane 3), No. 79 (Lane 4), and non-GM (Lane 6) samples were analyzed by agarose gel [3% (w/v)] electrophoresis. Arrowheads indicate the PCR amplification products of the taxon-specific gene MON810, NK603, and SSIIb at 199, 444, and 155 bp, respectively. Lane M: DNA size marker.

M863 1-5′ and M863 1-3′ for MON863 detection, M810 1-5′ and CryIA 1-3′ for MON810 detection, SSIIb 1-5′ and SSIIb 1-3′ for SSIIb detection, TC1507 1-5′ and TC1507 1-3′ for TC1507 detection, and Bt11 1-5′ and CryIA 1-3′ for Bt11 detection were used.

The reactions were buffered with PCR buffer II (Life Technologies Corp.) and amplified in a thermal cycler (Silver 96-well GeneAmp PCR System 9700; Life Technologies Corp.) in max mode, according to the following PCR step-cycle program: preincubation at 95°C for 10 min; 10 cycles of denaturation at 95°C for 0.5 min; annealing at 65°C for 1 min; extension at 72°C for 1 min; 27 cycles of denaturation at 95°C for 0.5 min; annealing at 65°C for 1 min; extension at 72°C for 1 min; and final extension at 72°C for 7 min. The cycle was repeated 40 times followed by a final extension at 72°C for 7 min.

Interlaboratory Study

The interlaboratory study was organized by the National Institute of Health Sciences (NIHS) to evaluate the validity of DNA extraction from multiple ground samples, multiplex real-time PCR detection, and multiplex qualitative PCR detection in the individual kernel detection system in terms of its applicability, practicality, and ruggedness. The study was conducted with the participation of five laboratories. The validation test consisted of DNA extraction from individual ground maize samples, and multiplex real-time and multiplex qualitative PCR detection for the purpose of partial evaluation of the individual kernel detection system. For complete validation of the individual kernel detection system, it was necessary to distribute the maize kernel samples of GM seeds to the five laboratories. In distributing the maize kernel samples to the five laboratories, it was essential to know whether each maize kernel in the samples is GM or non-GM, as well as the events of each GM kernel prior to sample distribution. However, we could not confirm whether the maize kernel samples were GM or non-GM without grinding the seeds and subjecting the samples to detection, even though we could obtain some pure GM seeds as the positive sample. Of the several steps in the individual detection system, we consider that the steps requiring the most attention are the DNA extraction from individual ground kernels and the subsequent multiplex real-time PCR and multiplex PCR detection in terms of applicability, practicality, and ruggedness, since the steps involving the grinding of individual maize kernels appear to be uncontaminated due to the individual closed tubes.

Therefore, we weighed out the average weight of a normal maize kernel (0.37 g) from each ground sample as reference GM and non-GM samples and distributed it to individual tubes. These blind tube samples were thus sent to the laboratories. In addition to the 90 blind tube samples, solutions of the three primer pairs (each 6 μM), all reagents for PCR, a vacuum pump (DA-60D; ULVAC, Kanagawa, Japan), and the experimental protocol were provided to the five participating laboratories from the NIHS. Thus, a total of 450 blind tube samples were analyzed by the real-time PCR systems in the interlaboratory study. The guidelines for a collaborative study were referenced to determine the general procedure of this interlaboratory study (13).

Results and Discussion

In-House Validation Study of DNA Extraction from Multiple Ground Samples and Multiplex Real-Time PCR

To assess the applicability and reproducibility of DNA extraction from multiple ground samples and multiplex real-time PCR, these were assessed in an in-house study. Genomic DNA was individually extracted from 12 GM kernels (six MON810 kernels and six MON810xNK603 stacked kernels) and 12 non-GM kernels/day. Genomic DNA was amplified using multiplex real-time PCR. The tests were repeated for a total of 120 kernels (60 GM maize kernels and 60 non-GM maize kernels) on 5 different days. Figure 1 shows typical amplified products of genomic DNA from non-GM maize, MON810 maize, and MON810xNK603 stacked maize. Table 1 shows the results of in-house validation of multiplex real-time PCR for the same day and 5 different days. Neither false-positive nor false-negative results were observed (0%), verifying that both GM kernels and non-GM kernels can be clearly detected using the detection assay with good accuracy and precision at a confidence level of 95%, since the 95%

Table 4. Interlaboratory validation of multiplex qualitative PCR

Laboratory/sample	No. 10	No. 45	No. 59	No. 79
A	MON810xNK603	MON810xNK603	MON810	MON810
B	MON810xNK603	MON810xNK603	MON810	MON810
C	MON810xNK603	MON810xNK603	MON810	MON810
D	MON810xNK603	MON810xNK603	MON810	MON810
E	MON810xNK603	MON810xNK603	MON810	MON810
Expected result	MON810xNK603	MON810xNK603	MON810	MON810
Accuracy, %[a]	100	100	100	100

[a] Value was calculated from comparison with the expected result and results obtained in 5 different laboratories.

confidence interval of $P = 0.95$, calculated based on the Clopper-Pearson method, is 0.861–0.990 ($n = 60$; 14).

Interlaboratory Validation of DNA Extraction from Multiple Ground Samples, Multiplex Real-Time PCR, and Multiplex Qualitative PCR

To intentionally assess the applicability, practicability, and ruggedness of the individual maize kernel detection system in multilaboratory use, we conducted an interlaboratory validation of DNA extraction from multiple ground samples, multiplex real-time PCR, and multiplex qualitative PCR for the individual maize kernel detection system. The study consisted of three steps: DNA extraction from multiple ground samples; multiplex real-time PCR for detection of GM maize samples, including stacked GM maize; and multiplex qualitative PCR for GM event detection.

We first examined the ruggedness of the multiplex real-time PCR using an AB7900 and AB7500 at NIHS. As shown in Table 2, we confirmed that the DNA extracted from test samples (non-GM samples, 86 tubes and GM samples, two tubes of MON810, and two tubes of MON810xNK603) were detected using both real-time PCR instruments. Five laboratories were then invited to take part in the interlaboratory study. Each laboratory was equipped with either an AB7900 or AB7500 real-time PCR instrument; three laboratories had an AB7900 and two laboratories had an AB7500. Table 3 summarizes the detection of the *SSIIb* gene, and GM detection of MON810 maize and MON810xNK603 maize for all samples in the interlaboratory study.

For the detection of the *SSIIb* gene, the positive rates of non-GM samples and GM samples were both 100% (Table 3). For GM detection of MON810 maize and MON810xNK603 maize, the positive rates of both MON810 event and MON810xNK603 were 100%, although only 10 tubes of each (two tubes × five laboratories) were tested. The positive rate of non-GM samples was 0% (Table 3).

Next, it was attempted to validate the multiplex qualitative PCR method for GM event detection. Before validation, we confirmed four GM samples using multiplex qualitative PCR. As shown in Figure 2, electrophoresis of samples from tube Nos. 10 and 45 showed the three amplified products of MON810, NK603, and SSIIb, which were judged to be MON810xNK603, while tube Nos. 59 and 79 showed the two amplified products of MON810 and SSIIb, which were judged to be MON810. All five laboratories judged the four tubes, Nos. 10, 45, 59, and 79, to be GM tube samples (Table 4), corresponding to MON810xNK603, MON810xNK603, MON810, and MON810, respectively, as confirmed by electrophoresis. These results suggest that multiplex qualitative PCR for events detection would be valid and suitable for reproducibility and ruggedness.

Conclusions

This study suggested that the DNA extraction from multiple ground samples, multiplex real-time PCR, and multiplex qualitative PCR for the individual kernel detection system is suitable for enforcement purposes with respect to its interlaboratory reproducibility and transferability, applicability, and ruggedness. The interlaboratory study, including the DNA extraction step, was conducted in close adherence to internationally accepted guidelines for collaborative trials (15). In practical terms, the present study suggests these methods can be readily adopted by any laboratory. These methods would provide a practical tool for the detection of GM maize containing stacked maize. However, for complete validation of the individual kernel detection system, it is necessary to conduct the validation on a multiple laboratory basis with samples of seeds mixed to various GM levels and with various numbers of staked events such as MON810xNK603xMON863 or MON863xNK603. The present study suggests that the quantification as kernel-based measurement appears to be accurate in the case of testing pure GM seeds or non-GM bulk lot. When this proposed system can accurately monitor GM commingling level in maize samples, it will be an

appropriate tool for the implementation of respective Japanese regulatory requirements concerning authorized GM maize products.

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Original Paper

Development and Evaluation of Event-Specific Quantitative PCR Method for Genetically Modified Soybean A2704-12

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A novel real-time PCR-based analytical method was developed for the event-specific quantification of a genetically modified (GM) soybean event; A2704-12. During the plant transformation, DNA fragments derived from pUC19 plasmid were integrated in A2704-12, and the region was found to be A2704-12 specific. The pUC19-derived DNA sequences were used as primers for the specific detection of A2704-12. We first tried to construct a standard plasmid for A2704-12 quantification using pUC19. However, non-specific signals appeared with both qualitative and quantitative PCR analyses using the specific primers with pUC19 as a template, and we then constructed a plasmid using pBR322. The conversion factor (C_f), which is required to calculate the amount of the genetically modified organism (GMO), was experimentally determined with two real-time PCR instruments, the Applied Biosystems 7900HT and the Applied Biosystems 7500. The determined C_f values were both 0.98. The quantitative method was evaluated by means of blind tests in multi-laboratory trials using the two real-time PCR instruments. The limit of quantitation for the method was estimated to be 0.1%. The trueness and precision were evaluated as the bias and reproducibility of relative standard deviation (RSD_R), and the determined bias and RSD_R values for the method were each less than 20%. These results suggest that the developed method would be suitable for practical analyses for the detection and quantification of A2704-12.

Key words: A2704-12; event-specific; genetically modified (GM); real-time PCR; soybean

Introduction

The use of genetically modified (GM) crops has been increasing since their commercialization in 1996. After more than a decade, the global area of GM crops has increased approximately 80-fold, from 1.7 million hectares in six countries in 1996, to 134 million hectares in 25 countries in 2009[1]. The utilization of GM crops has generated substantial economical benefits, but, nevertheless, has been subjected to rigid control. The presence of GM products in crops or foods is obliged to be labeled in the European Union (EU), Korea, Japan, Australia, and many other countries. In Japan, the genetically modified organism (GMO) labeling system has been defined by the "JAS law"*1 and the "Food Sanitation law"*2, and the thresholds for the unintentional commingling level for approved GM soy and maize were both set at 5%[2],*3,*4.

The Japanese food self-sufficiency ratio on a calorie supply basis has been hovering around 40% in recent years, but the ratios among grains, except for rice, are extremely low*5. Among them, soybeans are one of the most important crops in Japan. The domestic consump-

*1 Notification No. 1173 (Oct. 1, 2007); Ministry of Agriculture, Forestry and Fisheries of Japan (2007)

*2 Notification No. 79 (Mar. 15, 2001); Ministry of Heath, Labour and Welfare of Japan (2001)

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

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

*5 Annual Report on Food, Agriculture and Rural Areas in Japan FY2006 Policies on Food, Agriculture and Rural Areas in Japan FY2007, Summary of Ministry of Agriculture, Forestry and Fisheries of Japan; http://www.maff.go.jp/e/pdf/fy2006_rep.pdf

tion of soybeans as oil and food is over 4 million tons/year, but the self-sufficiency ratio for this crop is only approximately 5%*6, *i.e.*, more than 90% of soybeans are imported into Japan. Most of them are imported from the United States, where GM soybeans account for more than 90% of the soybean cultivation areas[1]. There are several approved GM soybean events in Japan*7. The first commercial GM soybean was a glyphosate-tolerant soybean (GTS), event 40-3-2 [Roundup Ready® soybean (RRS)]. RRS has received regulatory approval in many countries, including Japan. Next, glufosinate-tolerant soybeans such as A2704-12 and A5547-127 were approved, and then the second generation of GTS, MON89788, was recently approved.

A2704-12 and A5547-127 contain the same transgene cassette consisting of cauliflower mosaic virus 35 S promoter (P35S), the synthetic *pat* gene which codes phosphinothricin *N*-acetyltransferase derived from *Streptomyces viridochromogenes*[3), 4)], and cauliflower mosaic virus 35S terminator (T35S)[5), 6)]. Many methods for analyzing RRS and MON89788 have been published[7)-11)], but there is no report on quantification of A2704-12, although A2704-12 is now being commercially cultivated.

In this study, we developed a new quantitative method for A2704-12, using event-specific PCR, and then evaluated the method with two multi-laboratory trials. The development of the quantitative method was mainly carried out in the National Food Research Institute, and the interlaboratory collaborative study was independently conducted with the National Institute of Health Sciences (NIHS).

Materials and Methods

Plant materials

Regarding soybean and maize seeds, A2704-12 and a non-GM isoline of A2704-12 used as a non-GM soy sample were kindly provided by the developer, MON 89788, RRS, MON810, MON863, MON88017, and NK 603 by Monsanto Company (St. Louis, MO, USA), Bt11, Event176, GA21, and MIR604 by Syngenta Seeds AG (Basel, Switzerland), TC1507 and DAS59122 by Pioneer Hi-Bred International (Johnston, IA, USA), and T25 was directly imported from the USA. QC9651 maize from Quality Technology International, Inc. (Elgin, IL, USA) was used as a non-GM maize. Seeds of rice (*Oryza sativa*) variety Kinuhikari, wheat (*Triticum aestivum*) variety Haruyutaka, and barley (*Hordeum vulgare*) variety Harrington were harvested in Japan. A5547-127 was purchased from the American Oil Chemists' Society (AOCS) (Urbana, IL, USA) as DNA extract (AOCS 0707-C; above 999.9 ng/μL of GM DNA).

Oligonucleotide primers and probes

For the specific detection of A2704-12, a pair of primers (KVM175; 5′-GCAAAAAAGCGGTTAGCTCCT-3′ and SMO001; 5′-ATTCAGGCTGCGCAACTGTT-3′) and a fluorescent dye-labeled probe (TM031; 5′-CGG-TCCTCCGATCGCCCTTCC-3′) were used for real-time PCR. The sequences of these primers and probe were taken from the report of the European Commission's Joint Research Centre (JRC, IRMM, Retieseweg, Geel, Belgium)*8. *Le1*[12)] was used as a soybean-specific endogenous reference DNA for quantitative analysis. For specific detection of *Le1*, the primers (Le1n02-5′; 5′-GCCCTCTACTCCACCCCCA-3′ and Le1n02-3′; 5′-GCCCATCTGCAAGCCTTTTT-3′) and fluorescent dye-labeled probe (Le1-Taq; 5′-AGCTTCGCCGCTTCCTT-CAACTTCAC-3′) were used[8)]. The oligonucleotide primers and TaqMan® probes were synthesized by FASMAC Co., Ltd. (Kanagawa, Japan) and Life Technologies (Carlsbad, CA, USA), respectively. The oligonucleotide probes were labeled with 6-carboxyfluorescein (FAM) at the 5′ ends and 6-carboxytetramethylrhodamine (TAMRA) at the 3′ ends.

Preparation of calibrant plasmid

Specific sequence fragments from A2704-12 and the endogenous soybean *Le1* gene were synthesized as a single oligonucleotide in tandem and inserted into a pBR322 vector. The constructed plasmid was purified by equilibrium centrifugation in a CsCl gradient to collect the covalently closed circular DNA, which was linearized by cutting at a restriction site located outside the integrated fragment. The concentration of the linearized DNA was calculated from the ultraviolet (UV) absorbance measured with a spectrophotometer, DU 800 (Beckman Coulter, Fullerton, CA, USA) as described previously[8)], and converted to the molar concentration. The solution was then diluted to theoretical concentrations of 20, 125, 1,500, 20,000, and 250,000 copies per 2.5 μL, with 5 ng/μL of ColE1 DNA (NIPPON GENE, Tokyo, Japan) solution. Finally, the copy numbers of the diluted plasmids were adjusted based on the *Le1* segment of pMulSL2, which has been adopted in the Japanese standard analytical method*9, using quantitative real-time PCR analyses.

*6 Ministry of Agriculture, Forestry and Fisheries of Japan; http://www.maff.go.jp/j/seisan/ryutu/daizu/d_data/pdf/011_juyou.pdf

*7 List of products that have undergone safety assessment and been announced in the Official Gazette (May 12, 2010) of the Department of Food Safety, Ministry of Health, Labour, and Welfare; http://www.mhlw.go.jp/english/topics/food/pdf/sec01-2.pdf

*8 Event-specific method for the quantification of soybean line A2704-12 using real-time PCR; http://gmo-crl.jrc.ec.europa.eu / summaries / A 2704-12 _ soybean _ validated _ Method.pdf

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

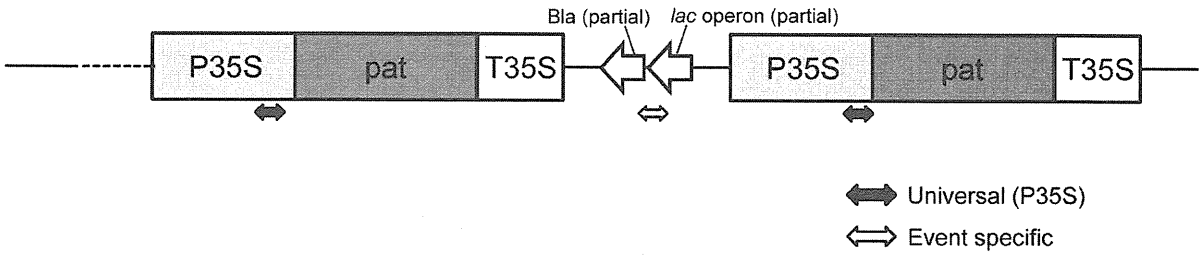

Fig. 1. Schematic diagrams of the target positions in A2704-12
The event-specific target sequence of A2704-12 is the junction region of two partial sequences derived from the *bla* gene and *lac* operon, corresponding to positions 248–267 and 2036–2056 in pUC19, between the two *pat* gene cassettes. The nucleotide sequence of pUC19 is available under accession number L09137.

Preparation of test samples and DNA extraction

To evaluate the quantitative method, we used six mixing levels of test materials containing 0%, 0.10%, 0.50%, 1.0%, 5.0%, and 10.0% A2704-12. To prepare the mixed samples, we ground A2704-12 seeds and non-GM seeds using MM200 and ZM100 grinders (Retsch, Haan, Germany), respectively, as described previously[13], and then mixed the samples on a weight-to-weight basis. DNA was extracted from the ground materials using GM quicker (NIPPON GENE) according to the manufacturer's manual. The concentration and quality of extracted DNA solutions were evaluated by measuring UV absorbance with a spectrophotometer, ND-100 (NanoDrop Technologies, Wilmington, DE, USA). Soybean genomic DNA solutions were adjusted to a concentration of 20 ng/μL.

Qualitative PCR

Qualitative PCR using a thermal cycler, GeneAmp PCR system 9700 (Life Technologies), and agarose gel electrophoresis were performed as described previously by Kuribara *et al.* (2002).

Quantitative PCR

TaqMan® real-time PCR assays were carried out using the Applied Biosystems 7900HT (AB 7900) or the Applied Biosystems 7500 (AB 7500) (Life Technologies), in 25 μL final volume, containing 50 ng of sample DNA, 12.5 μL Universal Master Mix (Life Technologies), 0.5 μM primer pairs, and 0.2 μM probe. The step-cycle program was as follows: 2 min at 50°C, 10 min at 95°C, 45 cycles, 30 s at 95°C, and 1 min at 59°C. In the reaction plate, each sample was measured in triplicate.

Multi-laboratory trial

Multi-laboratory trials were performed with the AB 7900 and the AB 7500 independently and consisted of 2 separate stages: measurement of the C_f value and a blind test. All measurements were conducted by 5 laboratories for the AB 7900 and 3 laboratories for the AB 7500. Experimental protocols were provided by the NIHS. Quantitative real-time PCR was performed with primers, probes, Universal Master Mix, and blind DNA solutions supplied by NIHS.

The first stage was the experimental determination of the C_f value as the ratio of the copy number of recombinant DNA (r-DNA) to the taxon-specific sequence in the GM plant genome. To calculate the C_f value for A2704-12, we extracted the genomic DNA from genuine GM seeds and determined the copy numbers of r-DNA and taxon-specific sequences. The measurement was conducted twice at each laboratory. The C_f value for each real-time PCR instrument was separately determined as the mean of the obtained values.

A blind test was carried out as the second stage. Blind samples designed as blind duplicates of the soybean genomic DNAs extracted from 6 different concentrations of A2704-12, 0%, 0.10%, 0.50%, 1.0%, 5.0% and 10.0%, were sent to the participants. All participants were requested to submit the data from the real-time PCR analyses. All submitted data were analyzed by means of Cochran's test[14] and Grubbs' test[15, 16].

Results and Discussion

Specificity of the PCR system for A2704-12

A2704-12 contains two copies of the *pat* gene cassette inserted in a head-to-tail configuration*[10]. The biotic transformation of soybeans was conducted by microparticle bombardment with a pUC19-based plasmid containing the *pat* gene cassette. The pUC19 plasmid contains an antibiotic resistance gene; *beta-lactamase* (*bla*), and a *lac* operon, and several fragments derived from pUC19 DNA exist in the A2704-12 genome. Partial sequences derived from the *bla* gene and *lac* operon are integrated side-by-side between the two *pat* gene cassettes*[10], and this site is unique to A2704-12. For specific detection of A2704-12, the junction site was used (Fig. 1). The specificity of the primer set was confirmed by qualitative PCR. The expected 64-bp product was detected using genomic DNA solutions from A2704-12, but not from non-GM soybeans; GM soybeans RRS, MON89788, and another glufosinate-tolerant soybean: A5547-127; non-GM maize; 11 lines of GM maize; rice, wheat, and barley; and the no template control (Fig. 2).

*[10] Agbios database. http://www.cera-gmc.org/?action=gm_crop_database&

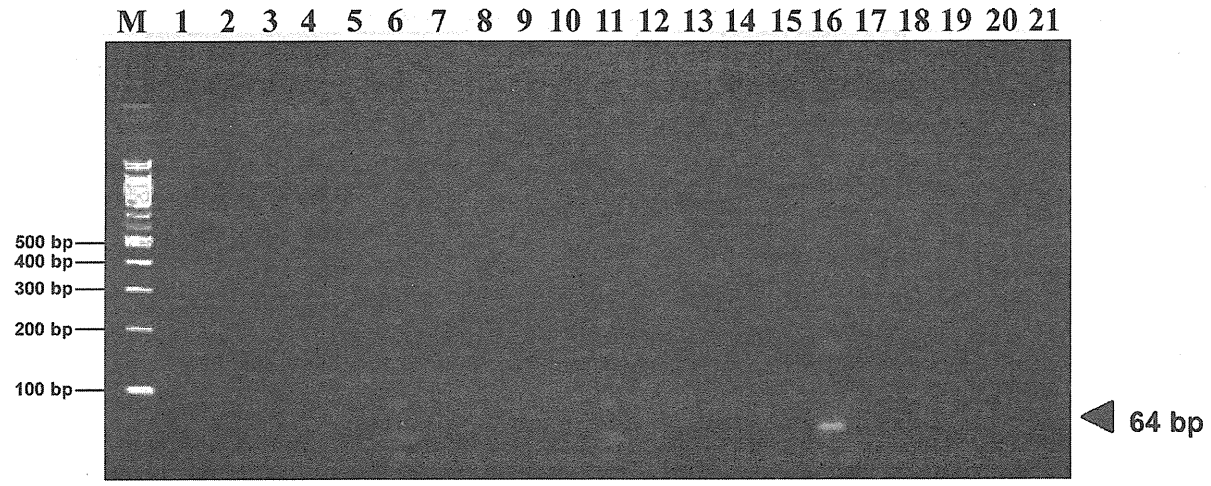

Fig. 2. Specificity test of the designed primer pair for genomic DNAs from several GM events or crops
Agarose gel (3.0%) electrophoretogram of the amplified PCR products corresponding to the 64 bp of A2704-12 DNA. Arrowhead indicates the expected amplified product. Lanes 1–11, eleven GM maize events, namely NK603, Event176, T25, GA21, MON810, TC1507, Bt11, MIR604, MON88017, DAS59122, and MON863, respectively; 12 and 13, non-GM maize and non-GM soy, respectively; 14–17, four GM soybean events, namely, RRS, MON89788, A2704-12, and A5547-127, respectively; 18–21, rice, wheat, barley, and no template, respectively. Lane M shows 100 bp ladder size markers.

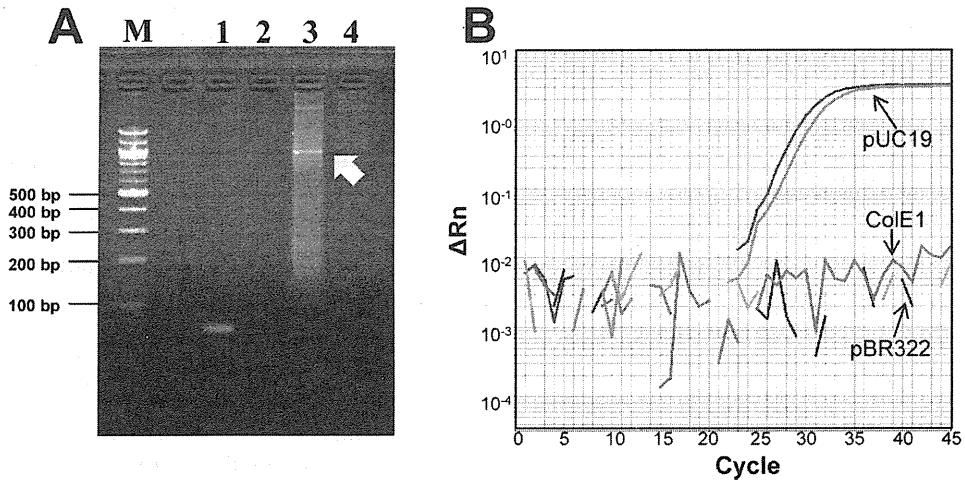

Fig. 3. Specificity test of the designed primer pair and probe for plasmid DNAs
A: Qualitative PCR analysis. Lanes 1–4, A2704-12 genomic DNA, ColE1 DNA, pUC19, and pBR322, respectively. The arrow indicates a non-specific amplification product. Lane M shows 100 bp ladder size markers.
B: Quantitative PCR analysis. pUC19-, pBR322-, or ColE1-derived signals in the amplification profile using the AB 7900 are shown. Concentrations of DNA solutions of A2704-12 genomic DNA, ColE1 DNA, pUC19, and pBR322 were 20, 5, 10, and 10 ng/μL, respectively.

Construction of calibrant plasmid for A2704-12

In Japan, one of the features of standard quantitative methods is the utilization of standard plasmid DNA as reference molecules and calibrators. We have developed several standard plasmids[8), 17)] using pUC19 or pBR322. Although pUC19 is a useful plasmid containing a high-copy-number replicon[18)], when the pUC19 plasmid was used as a template, a non-specific amplified product of around 1.0 kb was observed (Fig. 3A). Furthermore, a non-specific signal was detected by quantitative PCR analysis (Fig. 3B). As mentioned above, for the specific detection of A2704-12, the pUC19-derived sequences, KVM175 and SMO001, which are a part of the sequences of *bla* gene and *lac* operon, respectively, were used as primers. The unexpected band may have been caused by the sequences of these primers, indicating the possibility that inaccurate quantification could occur if pUC19-based plasmids were used as a calibrator for quantification. Thus, we used pBR322 plasmid for the construction of the standard plasmid, designated as pLLS. To prepare the pLLS plasmid, we removed a *bla* gene partial sequence that included KVM175 from pBR 322 and then inserted the A2704-12 and *Le1* segments (Fig. 4). Figure 5 shows the amplification plots of the pLLS plasmids diluted to a concentration ranging from 20 to 250,000 copies per reaction (Fig. 5A and B). The *R*

^{2} values from both A2704-12 and *Le1* were above 0.999 (Figs. 5C and D), which is an acceptable level (R^2 should be above 0.990)[8]. The slopes, representing the amplification efficiencies, were −3.44 and −3.32 for A2704-12 and *Le1* standard curves, respectively. PCR efficiency was calculated by use of the following equation: PCR efficiency $=10^{(-1/\text{slope})}$[19]. The theoretical value with an efficiency of 100% in each cycle is 2.00, and, in this study, PCR efficiencies of 1.95 and 1.99 were obtained for A2704-12 and *Le1*, respectively.

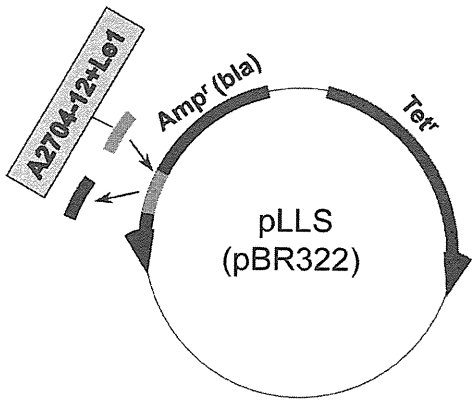

Fig. 4. Schematic diagram of the construction of pLLS plasmid
The region corresponding to positions 3613–3843 in pBR322 was removed, and the specific sequences of A2704-12 and the *Le1* were inserted in tandem. The nucleotide sequence of pBR322 is available under accession number J01749.

Determination of the C_f value for A2704-12

To determine the experimental C_f value for A2704-12, we measured the copy numbers of *Le1* and A2704-12 in the extracted DNA from A2704-12 seeds. The C_f value was determined with two real-time PCR instruments (the AB 7900 and the AB 7500) independently, from the results of 5 laboratories for the AB 7900 and 3 laboratories for the AB 7500. The measurement was repeated twice, and the C_f value was determined as the mean of values measured by these laboratories. The determined C_f values with the AB 7900 and the AB 7500 were similar; in fact, they took the same value of

Table 1. Experimental conversion factor for A2704-12

7900			7500		
Mean	SD	RSD	Mean	SD	RSD
0.98	0.0423	4.31	0.98	0.0232	2.36

SD: Standard deviation
RSD: Relative standard deviation

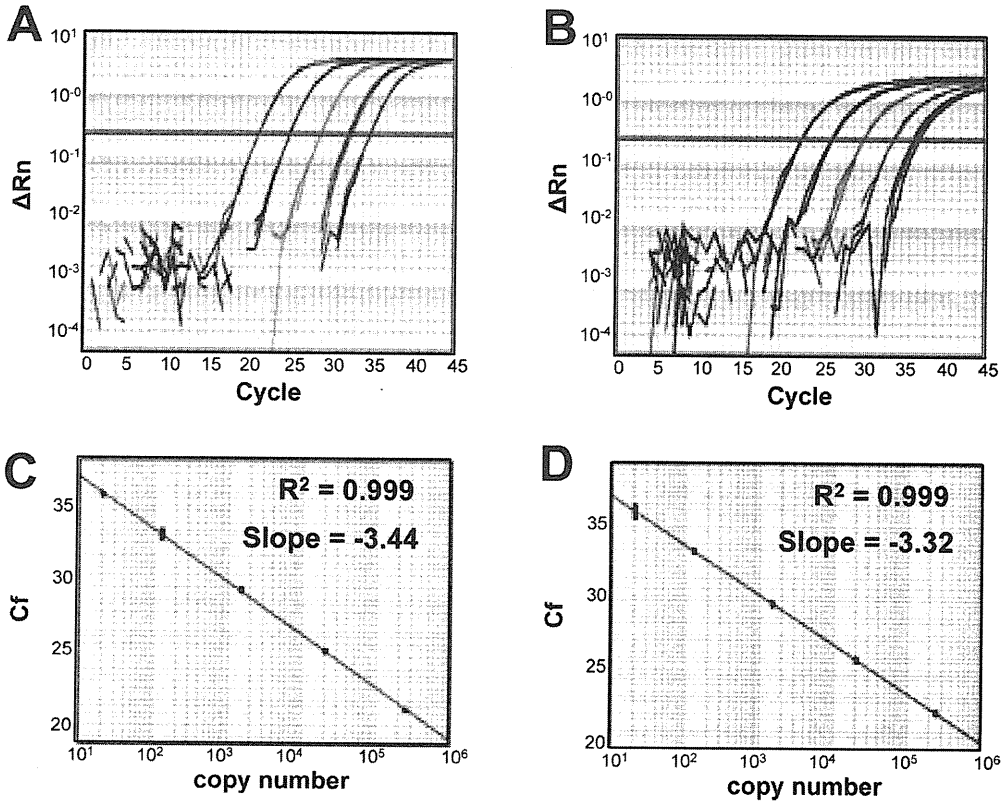

Fig. 5. Amplification plots for dilution series of pLLS plasmid and corresponding curves
Typical amplifications of A2704-12 and *Le1* segment are shown in A and B, respectively. The respective standard curves are shown in the panel below, and the R^2 and slope values of each standard curve are indicated at the upper-right corners. The dilutions contained 250,000, 20,000, 1,500, 125, and 20 initial template copies per reaction, respectively. These analyses were performed with the AB 7900.