JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 8145–8151 8145
DOI:10.1021/jf100675c

Specific Detection of Banana Residue in Processed Foods Using Polymerase Chain Reaction

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Specific polymerase chain reaction (PCR) methods were developed for the detection of banana residue in processed foods. For high banana specificity, the primer set BAN-F/BAN-R was designed on the basis of 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. 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 (w/w) banana DNA spiked in 50 ng of salmon testis DNA, whereas SYBR Green I real-time semiquantitative 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.

KEYWORDS: Allergen; banana; polymerase chain reaction; real-time polymerase chain reaction; ribulose-1,5-bisphosphate carboxylase gene

INTRODUCTION

Banana is a major worldwide fruit that is consumed and produced in Asia, Central and South America, and Africa. The origin of modern bananas derives from two native species of Southeast Asia, *Musa acuminate* and *Musa balbisiana* (*1*). Modern bananas as foods are mostly diploid or triploid cultivars obtained by crossing two wild cultivars. To classify banana genotypes, primers have been developed (*2*); however, studies have yet to determine their cross-reactivity with other biological species and detection sensitivity. Such detection of banana residue in foods needs to be achieved due to its potential allergenicity.

Since April 2002, the Japanese government has enforced a labeling system for allergenic food materials (*3*). In this system, labeling is now mandatory for seven food products (egg, milk, wheat, buckwheat, peanuts, shrimp/prawn, and crab); labeling for shrimp/prawn and crab became mandatory in 2008. In addition to these seven products, 18 additional food materials, including banana, have been recommended for labeling. Banana became recommended for labeling in 2004, based on a 2002/2003 investigation that reported an increase in the number of banana allergy patients in Japan.

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Banana contains food allergens that are common to those in latex or pollens (*4*, *5*). Many clinical studies have reported cross-reactivity of banana and latex, referred to as the latex−fruit syndrome (*6*−*8*). These studies monitored the number of patients with a food allergy in Japan and found that patients with banana allergy comprised the second largest population among those with fruit allergies; kiwifruit allergy was the most prevalent (*9*). The general symptom of the banana allergy is the oral allergy syndrome involving an allergic reaction induced in the mouth and pharynx. The latex−fruit syndrome can also develop as an immediate-type allergic reaction, including generalized urticaria, asthma, and severe anaphylactic reactions, which may have fatal consequences (*6*). Class I chitinases containing the hevein-like domain are major allergens in banana and could be the panallergens responsible for the latex−fruit syndrome (*10*, *11*).

For better consumer protection, specific and sensitive methods are required for the detection of trace amounts of allergens in commercial food products. PCR-based methods can be applied to the detection of allergenic food material contamination. Conventional and real-time PCR methods have already been established for the detection of wheat, buckwheat, peanuts, soybeans, walnuts, pork, and kiwifruit (*12*−*20*).

In the present study, we developed novel conventional and real-time PCR methods with high specificity and sensitivity for the detection of banana in processed foods. The primer set was

Published on Web 07/06/2010

Table 1. Designed Primers Used for Detection of Banana[a]

	name	sequence	specificity	amplicon	
A	CP 03−5′	5′-CGG ACG AGA ATA AAG ATA GAG T-3′	chloroplast DNA/sense	plants	123 bp
	CP 03−3′	5′-TTT TGG GGA TAG AGG GAC TTG A-3′	chloroplast DNA/antisense		
B	BAN-F	5′-TCG TCA CCT ATT GGG ATG C-3′	chloroplast DNA/sense	banana	186 bp
	BAN-R	5′-GCT TT A ATA AGT GCT TCG GTG-3′	chloroplast DNA/antisense		

[a] A, primer set used to confirm the validity of the DNA extracted from plants for PCR; B, primer set used to specifically detect banana DNA.

designed to detect the gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbcL*). The present PCR methods were shown to be applicable for the detection of trace amounts of banana contained in commercially processed food products.

MATERIALS AND METHODS

Samples Used in DNA Isolation. Food products including banana, yam, monkey banana, and eight commercial products containing banana (banana puree, fruit juice, banana juice, soy milk, banana chip, chocolate, soft cookie, and a cereal product) were purchased from a local market in Japan. Twenty-three kinds of fruits (apple, pear, Japanese pear, Japanese persimmon, peach, prune, apricot, Japanese apricot, cherry, orange, Satsuma orange, fig, kiwifruit, grape, blueberry, raspberry, juneberry, strawberry, melon, avocado, papaya, pineapple, and mango) were kindly provided by House Foods, Corp. (Tokyo, Japan). For the examination of sensitivity, we prepared a chocolate sample spiked with 1% (w/w) banana chip and a soy milk sample spiked with 0.01% (w/w) banana puree. We used chocolate and soy milk without the addition of banana as negative controls.

Extraction and Purification of Genomic DNA. Genomic DNA was extracted from plant materials including banana using a silica gel membrane-type kit (DNeasy Plant Mini, Qiagen, Hilden, Germany), according to the procedure described in previous studies (*15*, *19*, *20*). Genomic DNA was extracted from each banana-derived food material and commercial food product using an anion exchange-type kit (Genomic-tip 20/G, Qiagen) as described previously (*14*, *18*). Extracted DNA was diluted with an appropriate volume of distilled water to a final concentration of 20 ng/μL and stored at −20 °C until use in the PCR analyses.

PCR Conditions. The reaction mixture for PCR was prepared in a PCR tube. The reaction volume of 25 μL contained 50 ng of template genomic DNA, 0.2 mM each of dNTP, 1× Ex Taq buffer (Mg^{2+} free), 2.0 mM $MgCl_2$, 0.625 unit of Ex Taq (Takara, Shiga, Japan), and 0.2 μM each primer (**Table 1B**), topped up with distilled water. When the concentration of extracted DNA was < 20 ng/μL, 2.5 μL of undiluted DNA extract was added to the reaction tube for normalizing volume of template DNA. Amplification was performed in a GeneAmp PCR system 9600 (Applied Biosystems, Foster City, CA) as follows: preincubation at 95 °C for 10 min; 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 64 °C (60 °C for CP 03−5′/CP 03−3′ primer set (*14*)) for 0.5 min, and extension at 72 °C for 0.5 min; and final extension at 72 °C for 7 min. After PCR amplification, agarose gel electrophoresis of the PCR products (5 μL) was carried out according to previous studies (*14*, *18*). The amplified fragments generated with the BAN-F/BAN-R primer set were cloned into pCR II-TOPO vector (TOPO TA cloning kit, Invitrogen, Tokyo, Japan), and the sequences were analyzed using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

SYBR Green I Real-Time PCR. For real-time PCR amplification, the reaction mixture (25 μL) contained 50 ng of template genomic DNA, 0.2 μM each of the primer set, and 12.5 μL of *Power* SYBR Green PCR Master mix (Applied Biosystems). Reactions were performed using the Applied Biosystems 7500 Real-Time PCR System, with the following PCR conditions: 50 °C for 2 min, 90 °C for 10 min, followed by 90 °C for 15 s and 64 °C for 1 min, repeated for 45 cycles. For dissociation after PCR amplification, the protocol included a slow heating from 60 to 95 °C at increments of 0.2 °C. T_m curve analysis was performed using SDS software 1.2 (Applied Biosystems).

RESULTS

Primer Design for Banana DNA Detection. To specifically detect banana by PCR, banana-specific genes have been investigated. For various banana-specific gene sequences, a primer set was designed for PCR amplification of DNA extracted from banana and other food samples including fruits. The banana *rbcL* gene was predicted to have the highest specificity for detection of banana DNA in foods on the basis of previous investigations (*21*, *22*). Therefore, in this study we used the gene encoding banana *rbcL* as the banana-specific gene and designed the primer set BAN-F/BAN-R on the basis of its reported sequence (GenBank accession no. AF378770). The primer set CP 03−5′/CP 03−3′ was used for the universal detection of DNA derived from plants to verify DNA extraction (*14*); this primer set generated a 123 bp amplified fragment from genomic DNA extracted from banana. We amplified DNA from banana and other food samples using these primer sets. The PCR results confirmed that products of the target size could be predicted from *Musa* spp. The sequences of the designed primers used in this study are in **Table 1**.

Specificity and Sensitivity of Conventional and Semiquantitative Real-Time PCR. The specificity of the detection method using the BAN-F/BAN-R primer set was confirmed by conventional PCR. The amplification products obtained using the CP 03−5′/CP 03−3′ primer set were detected in all DNA samples (**Figure 1A**), confirming the quality of the template DNA. The conventional PCR fragment (186 bp) amplified using the BAN-F/BAN-R primer set was specifically detected in genomic DNA of banana and monkey banana; no cross-reactivity was detected for DNA extracted from 23 other fruits (**Figure 1B**) and eight crops (wheat, buckwheat, peanut, rice, corn, soybean, walnut, and yam) (data not shown). The sensitivity of conventional PCR was evaluated using from 5000 to 0.005 pg of banana DNA spiked in 50 ng of salmon testis DNA; the detection limit was 0.05 pg of banana genomic DNA, which corresponded to ca. 1 ppm (w/w) banana DNA (**Figure 3A**). The target products were also detected in the chocolate sample spiked with 1% (w/w) banana chip and in the soy milk sample spiked with 0.01% (w/w) banana puree, which corresponded to ca. 380 and 1.1 ppm of banana protein, respectively (**Table 4**, no. 1 and 2).

As the sequences of banana and yam from the GenBank database have similar homology (**Figure 2**), real-time PCR using SYBR Green I was examined for identifying these plants. Nonspecific bands seen in **Figure 1C**,**D** were detected at low levels under the extension temperature of 64 °C compared to 60 °C. As these PCR products have specific melting temperatures (T_m), melting curve analysis could discriminate between the PCR products of banana (T_m = 76.9 °C) and yam (78.3 °C) genomic DNA. There was no cross-reactivity to 30 other fruits and crops except yam using either real-time or conventional PCR. The amplification curve could be consistently obtained from 50000 to 0.5 pg (10 ppm equivalency) of banana DNA (**Figure 3B**); the calculated R^2 value of the standard curve was 0.995 in the range from 50000 to 50 pg (**Figure 3C**). These results show that banana genomic DNA can be specifically detected using the BAN-F/BAN-R primer set.

The allelic variation of the *rbcL* gene among different banana cultivars from Philippines (four cultivars), Taiwan (two),

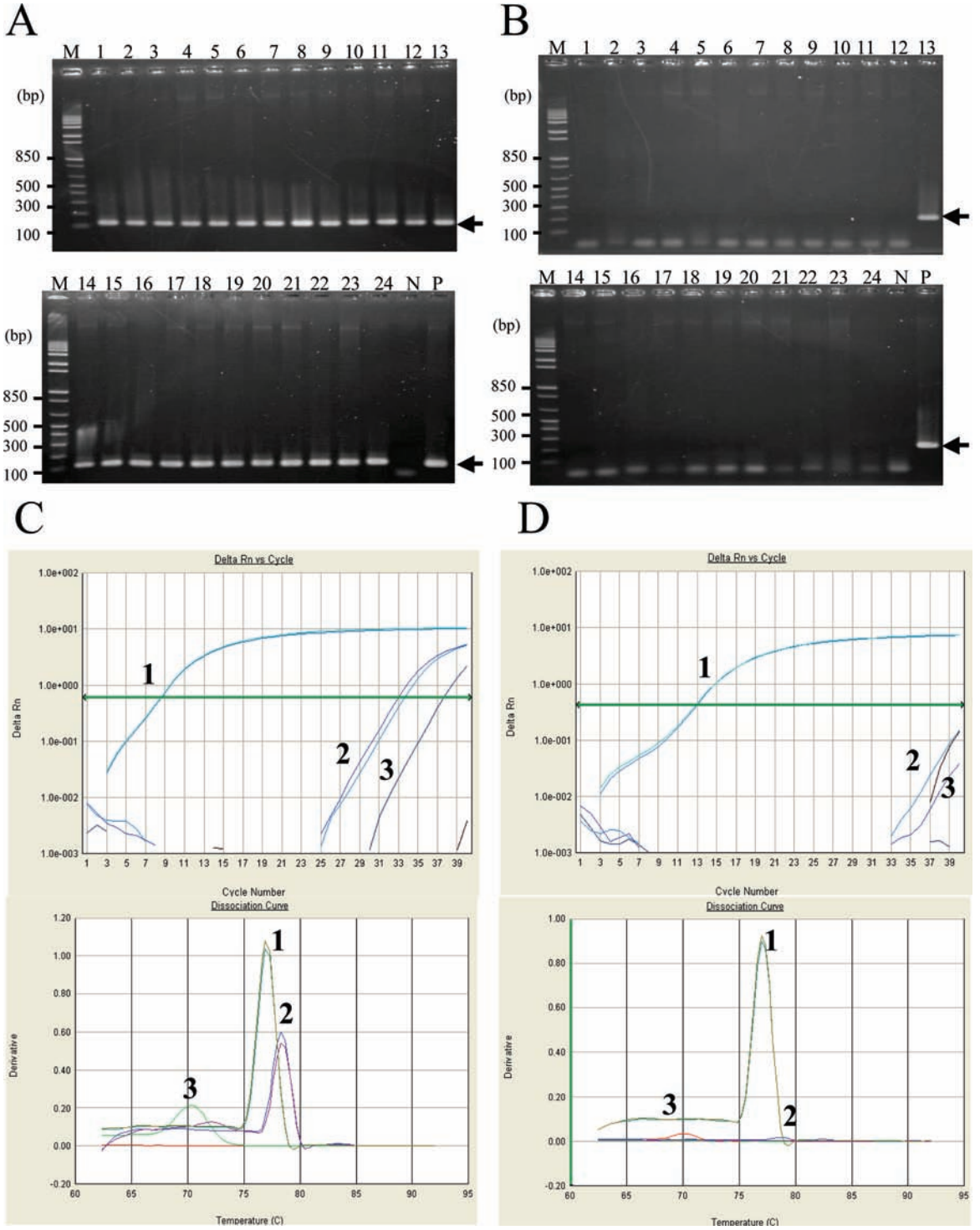

Figure 1. Specificity of PCR using the BAN-F/BAN-R primer set. Amplification of DNA from various plant species was performed with primer sets for plants (**A**) or banana (**B**). The arrowhead indicates the expected PCR product. Lanes: P, amplification of 50 ng of genomic DNA of banana; M, DNA marker (100 bp ladder size standard, Invitrogen); N, nontemplate control; 1−24, amplification of 50 ng of genomic DNA of apple (1), pear (2), Japanese pear (3), Japanese persimmon (4), peach (5), prune (6), apricot (7), Japanese apricot (8), cherry (9), orange (10), Satsuma orange (11), fig (12), monkey banana (13), kiwifruit (14), grape (15), blueberry (16), raspberry (17), juneberry (18), strawberry (19), melon (20), avocado (21), papaya (22), pineapple (23), and mango (24). Amplicons were electrophoresed on a 1.6% agarose gel. Amplification curve and melting curve of banana and yam PCR products were obtained under the extension temperatures of 60 (**C**) and 64 °C (**D**). Curves: 1 and 2, amplifications of 50 ng of banana and yam DNA, respectively; 3, nontemplate control. All reactions were performed in duplicate.

Ecuador (one), Mexico (one), and Colombia (one) was tested using both conventional and real-time PCR. Only the expected PCR products (123 bp in size) with identical size and relative intensity were obtained from these nine cultivars by conventional PCR (**Figure 4**). The melting curve analysis of real-time PCR obtained similar T_m values ranging from 76.2 to 76.7 °C in these nine cultivars using 50 ng of DNA per reaction (**Table 2**). The slight variation in the T_m values might be from the quality of template genomic DNA used. These results suggest that the *rbcL* gene has no allelic variation among the different banana cultivars,

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Banana: TCGTC CCCTA TTGGG ATGCA CTATT AAACC AAAAT TGGGA TTATC TGCAA AAAAC
Yam   : CCGTC CCCTA TTGGG ATGCA CTATT AAACC AAAAT TGGGG TTATC CGCAA AGAAC

Banana: TACGG TAGAG CGGTT TATGA ATGTC TACGT GGTGG ACTTG ATTTT ACCAA AGATG
Yam   : TACGG CAGAG CCGTT TATGA ATGTC TACGT GGTGG ACTTG ATTTT ACCAA GGATG

Banana: ATGAA AACGT AAACT CACAA CCATT TATGC GTTGG AGAGA TCGTT TCTTA TTTTG
Yam   : ATGAA AATGT GAACT CACAA CCATT TATGC GTTGG AGAGA CCGTT TCTTA TTTTG

Banana: CACCG AAGCA CTTTT TAAAG C
Yam   : TGCCG AAGCA CTTTT TAAAG C
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Figure 2. Comparison of gene sequences of banana and yam: partial DNA sequence of the target region on the ribulose-1,5-bisphosphate carboxylase (*rbcL*) gene. The right and left arrows indicate the forward (BAN-F) and reverse (BAN-R) primers, respectively. GenBank accession no.: banana (*Musa acuminata*), AF378770; yam (*Dioscorea japonica*), AF307457.

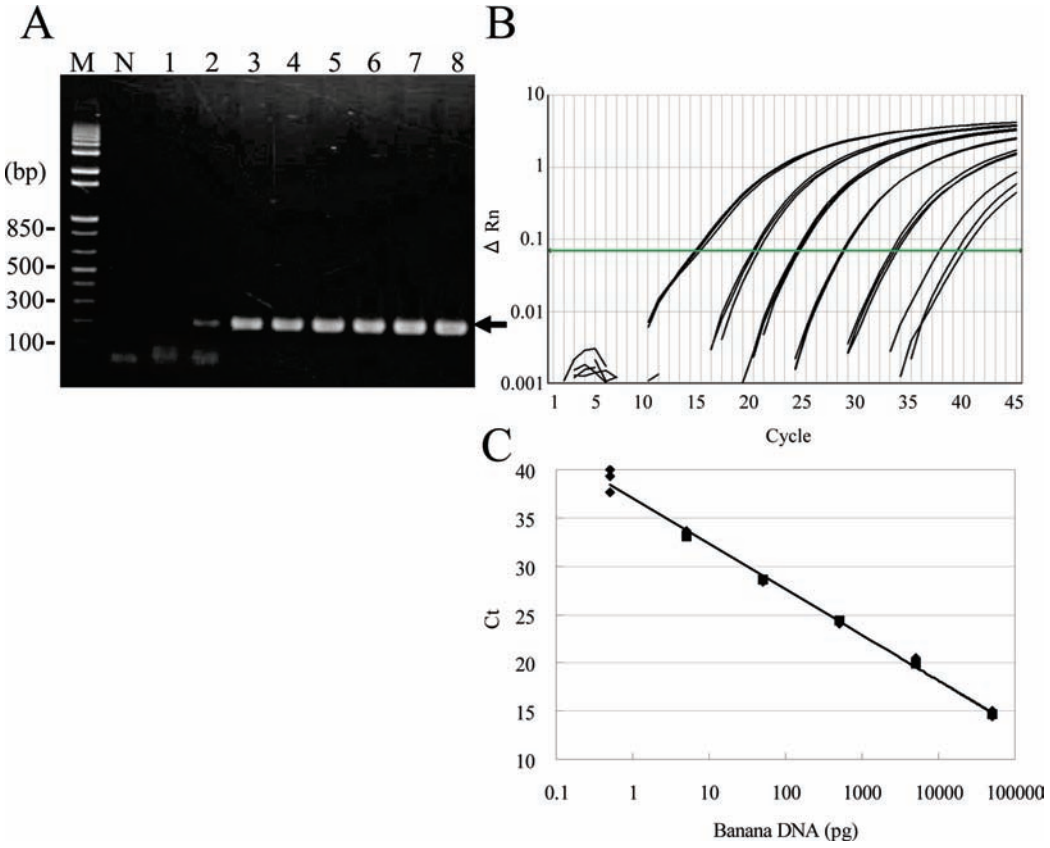

Figure 3. Sensitivity of specific detection method for banana. (**A**) Samples of salmon testis DNA containing various amounts of genomic DNA extracted from banana were used as template DNA. The arrowhead indicates the expected PCR product. Lanes: M, DNA marker (100 bp ladder size standard); N, nontemplate control; 1−8, amplification of genomic DNA samples with banana concentrations of 5 fg (1), 50 fg (2), 500 fg (3), 5 pg (4), 50 pg (5), 500 pg (6), 5 ng (7), and 50 ng (8). Amplicons were electrophoresed on a 1.6% agarose gel. (**B**) Amplification curve generated by serial dilution of banana DNA ranging from 50000 to 0.5 pg. All reactions were performed in triplicate. (**C**) Calibration curve generated from the amplification data in **A**. The relationship between the threshold cycle Ct and log DNA amount was determined to be $y = -4.7273 \log_{10} x + 37.06$ ($R^2 = 0.9945$).

and the copy number of the *rbcL* gene in the tested banana cultivars appears to be consistent.

Repeatability and Reproducibility for Real-Time PCR. Repeatability and reproducibility were evaluated using a dilution of banana genomic DNA (i.e., 50000, 5000, 500, 50, 5, and 0.5 pg). The mean and standard deviation (SD), coefficient of variation (CV %) of the Ct values of repeatability were calculated according to the mean value from three replications performed by one researcher on the same day and on three different days. As seen in **Table 3**, the CV of the Ct values ranged from 0.48 to 3.08% for testing repeatability on one day. For reproducibility, the CV of the Ct values ranged from 2.04 to 5.18% for three different days. All of the results of the repeatability and reproducibility tests indicate that this banana *rbcL* gene quantitative PCR method is reliable.

Application of the Banana DNA Detection Methods in Commercial Products. To investigate the applicability of the banana DNA detection methods for commercial food products, eight food products were purchased from a local market and examined for the presence of banana (**Table 4**, no. 3−10). Labeling indicated that all of the food products contained banana, except for chocolate and soy milk. For PCR, a sufficient amount (20 ng/μL) of extracted DNA was obtained from five processed food products (banana chip, chocolate, soy milk, soft cookie, and cereal product),

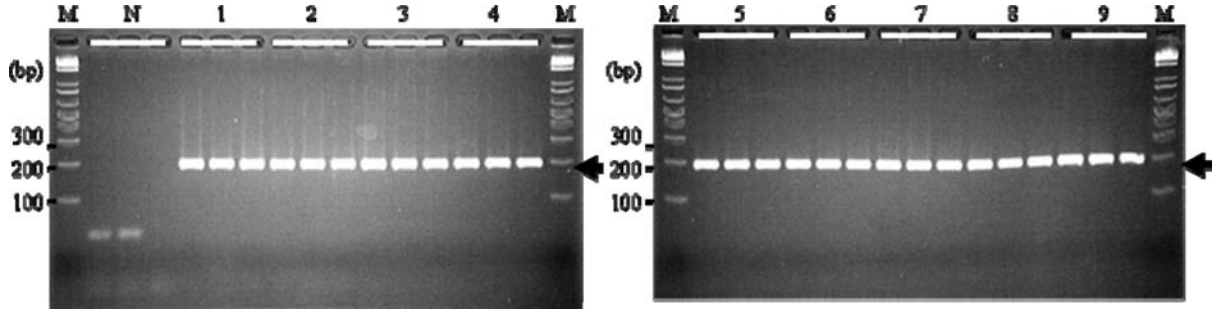

Figure 4. Conventional PCR results. The arrowhead indicates the expected PCR product. Lanes: M, DNA marker (100 bp ladder size standard, Invitrogen); N, nontemplate control; 1−9, amplification of 50 ng of genomic DNA of Philippines-1 (1), Taiwan-1 (2), Taiwan-2 (3), Philippines-2 (4), Philippines-3 (5), Colombia (6), Mexico (7), Ecuador (8), and Philippines-4 (9). Amplicons were electrophoresed on a 2.0% agarose gel. All reactions were performed in triplicate.

Table 2. Melting Curve Analysis Using Real-Time PCR[a]

	real-time PCR	
cultivar	mean T_m	SD
Philippines-1	76.4	0.2
Philippines-2	76.3	0.0
Philippines-3	76.6	0.2
Philippines-4	76.7	0.0
Taiwan-1	76.2	0.2
Taiwan-2	76.7	0.0
Colombia	76.4	0.2
Mexico	76.7	0.0
Ecuador	76.3	0.0

[a] All reactions were performed in triplicate. SD, standard deviation of T_m value

Table 3. Repeatability and Reproducibility of PCR[a]

banana genomic DNA dilution (pg)		same day (n = 3) 1	2	3	3 days (n = 9)
50000	mean Ct	15.04	13.81	14.65	14.73
	SD	0.13	0.38	0.33	0.30
	CV	0.86	2.75	2.25	2.04
5000	mean Ct	21.69	20.10	21.09	21.04
	SD	0.57	0.62	0.29	0.80
	CV	2.63	3.08	1.38	3.80
500	mean Ct	26.35	23.89	24.72	25.47
	SD	0.27	0.50	0.53	1.32
	CV	1.02	2.09	2.14	5.18
50	mean Ct	31.07	27.43	27.52	29.76
	SD	0.71	0.68	0.72	1.16
	CV	2.29	2.48	2.62	3.90
5	mean Ct	36.31	32.13	31.96	34.85
	SD	0.31	0.41	0.34	1.30
	CV	0.85	1.28	1.06	3.73
0.5	mean Ct	40.63	36.19	35.29	39.81
	SD	0.34	0.19	0.17	0.95
	CV	0.84	0.53	0.48	2.39

[a] SD, standard deviation of Ct value; CV, coefficient of variation of Ct value (%).

whereas <20 ng/μL could be obtained from the other products. The universal primer set (CP03−5′/CP03−3′) could generate a specific amplified fragment from all samples (**Table 4**). Banana DNA from all products containing banana was clearly detected by both conventional and real-time PCR using the BAN-F/BAN-R primer set; amplification was not detected for soy milk and chocolate samples lacking banana. The amplified sequence from the processed foods was confirmed to be that for banana reported in the GenBank database (accession no. AF378770). These results suggest that these methods are applicable for the detection of residual banana in a wide variety of processed food products.

DISCUSSION

Bananas are valuable fruits consumed worldwide, but a serious source of fruit allergy. In 1991, cross-reactivity was reported between banana and latex with different kinds of plants (*6*). Half of the patients with latex allergy had simultaneously induced fruit allergies, including those for banana, chestnut, and avocado, a phenomenon termed the latex−fruit syndrome (*7*, *8*). Sanchez-Monge et al. (*4*) reported class I chitinases with an N-terminal hevein-like domain to be one of the major allergens in banana, suggesting their pan-allergenic role in the latex−fruit syndrome. Currently, the only effective way to prevent the life-threatening allergic reactions to banana is to strictly avoid consuming banana, directly or through processed foods. Sufficient information about potentially allergenic ingredients in food products is thus necessary, and a specific and sensitive method of detecting banana residue is required, especially because processed foods contain a combination of many kinds of plant and animal ingredients.

In this study, a primer set was designed that specifically detects banana residue in commercial food products. For specific detection of banana DNA with high sensitivity, the gene encoding banana *rbcL* from chloroplast DNA was used. Improvement in the sensitivity of our detection system for trace amounts of banana residue that contaminate processed foods could be successfully achieved by targeting this multicopy chloroplast gene found in the plant cells. These PCR detection methods were examined using genomic DNA from various fruits including banana and yam and found to be specific for detection of banana genomic DNA. Conventional PCR showed a sensitivity of 1 ppm for banana genomic DNA, thus providing a reliable, specific, and sensitive detection method of banana in processed food products. In addition, real-time PCR using SYBR Green I showed a sensitivity of 10 ppm using the fluorescent amplification signal. This method also proved to have sufficient accuracy, with good repeatability (SD of Ct values = 0.5−3.1%) and reproducibility (2.0−5.2%).

To determine the applicability of our banana DNA detection methods, we tested several commercial food products that are known to contain banana. The BAN-F/BAN-R primer set successfully generated banana-specific fragments from all of the examined food products containing banana. The sensitivity of these

Table 4. PCR Analysis of Commercial Products

no.	sample	concentration of template DNA (ng/μL)	conventional PCR[a]		SYBR Green assay
			CP 03−5′/CP 03−3′	BAN-F/BAN-R	BAN-F/BAN-R[a]
1	chocolate spiked with 1% banana chip	20	+	+	+
2	soy milk spiked with 0.01% banana puree	20	+	+	+
3	chocolate	20	+	−	−
4	soy milk	20	+	−	−
5	banana chip	20	+	+	+
6	banana puree	<10	+	+	+
7	fruit juice	<10	+	+	+
8	banana juice	<10	+	+	+
9	soft cookie with dried banana	20	+	+	+
10	cereal product	20	+	+	+

[a] +, DNA amplification; −, no DNA amplification.

methods was also verified; the specific PCR products could detect approximately 1.1 ppm of banana protein in a spiked soy milk sample. Therefore, trace amounts of banana in processed foods can be detected using these PCR methods, although their applicability may be limited to highly processed foods. Notably, the *rbcL* gene was confirmed to have neither allelic variation among nine different banana cultivars originating from five countries and processed foods containing banana nor variation in the copy number of the *rbcL* gene in these tested samples on the market.

The allergens in banana residue are proteins. The developed methods detect banana DNA. Depending on processing conditions, banana protein and banana genomic DNA could be differentially degraded. We consider that it also would be very important to clarify the correlation between both amounts of banana allergen and banana DNA in a food product. Further studies are underway to develop an ELISA method to detecting banana allergen and investigate the correlation of both amounts of banana allergen and banana DNA in a food product.

In conclusion, we successfully designed a primer set (BAN-F/BAN-R) for the detection of banana DNA using both conventional and real-time PCR. These PCR methods are both specific and useful in detecting trace amounts of banana in processed foods. Real-time PCR, in particular, allows many samples to be analyzed in a short time, with little risk of carry-over contamination from PCR-amplified products, as agarose gel electrophoresis is unnecessary. Thus, these methods can be applied to reliable monitoring and inspections of processed food labeling.

ACKNOWLEDGMENT

We thank Drs. S. Watanabe and T. Hirao of House Foods Corp. for kindly providing the fruit DNAs.

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Received for review February 18, 2010. Revised manuscript received June 16, 2010. Accepted June 21, 2010. This study was supported by Health and Labor Science Research Grants for Research from the Ministry of Health, Labor, and Welfare of Japan.