

A Novel PCR Method for Quantification of Buckwheat by Using a Unique Internal Standard Material

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ABSTRACT

A novel quantitative and specific method for detection of buckwheat, a known food allergen, in diverse food materials was developed by using a unique internal standard to compensate for the variability in DNA extraction and amplification efficiencies. The method was based on a real-time PCR targeting the internal transcribed spacer region of *Fagopyrum* spp. and was designed to detect both cultivated and wild buckwheat, because wild buckwheat might be potentially allergenic. As the internal standard material, ground seeds of statice (*Limonium sinuatum*) were added to food samples prior to DNA extraction, and the amount of statice DNA measured by real-time PCR was used to standardize the buckwheat content. Statice, an ornamental plant, was chosen as the internal standard material because it was readily available and was inferred to be least likely to be commingled in foods. The specificity of the PCR system was tested against commonly used food materials of plant origin. Quantitative results expressed in buckwheat protein concentrations (mean $\pm$ standard deviation) for various food samples prepared to contain 10 ppm (wt/wt) of buckwheat flour (corresponding to 1.2- μ g/g [ppm] buckwheat protein) ranged from 0.7 ± 0.2 (rice) to 0.9 ± 0.4 (wheat) and for 100-ppm (wt/wt) samples (12- μ g/g [ppm] buckwheat protein) from 7.7 ± 1.0 (pepper) to 9.8 ± 0.5 (wheat) μ g/g (ppm). The method's accuracy, sensitivity, and specificity were considered sufficient for detection of buckwheat contamination at the level required for compliance with the Japanese Food Allergen Labeling Regulation.

Various allergen detection methods based on enzyme-linked immunosorbent assay (ELISA) or PCR have been developed to ensure compliance with food-labeling regulations and to protect allergic consumers from undeclared allergens (14). The quantitative values obtained by using those methods are affected by the presence of various interfering substances for extraction or detection in food matrices and denaturation or degradation of the target protein or DNA that may occur during processing of foods. For detection of genetically modified (GM) maize (*Zea mays*), a maize endogenous DNA sequence was used as an internal standard in quantitative real-time PCR to compensate for such variability (10). The method is used for determination of GM maize contamination in maize matrices. Therefore, an endogenous gene specific to the maize species is a logical choice for the internal standard. For detection of allergenic substances, such as peanut and wheat, by real-time PCR, however, an internal standard has not been used, and it is hard to calculate the quantitative value from their analyses (5, 15, 18). The quantitative analyses of allergenic substances differ from those of GM crops in that the quantities in diverse food matrices must be determined. Therefore, it is practically impossible to find an endogenous DNA sequence that can be used as an internal standard that is applicable to all food materials concerned. For this reason, we sought a material that can be added as an internal standard

to food samples prior to DNA extraction, to compensate for the influences of any substances that interfere with DNA extraction or *Taq* DNA polymerase activity.

Our previous article described a sensitive and specific qualitative PCR method that detects buckwheat (4). Buckwheat is known to be a life-threatening food allergen in Asian, European, and North and South American countries, as well as in Russia and Australia (21, 22). Food-labeling regulations in Japan require that buckwheat must be declared on the food label if its protein is present at concentrations that are higher than a few micrograms per gram (9). Our qualitative method showed that the internal transcribed spacer (ITS) region is a useful DNA sequence for detection of both cultivated buckwheat (*Fagopyrum esculentum* and *Fagopyrum tataricum*) and wild species of *Fagopyrum* that are thought to be potentially allergenic and may contaminate field-grown crops during harvest (4). In this article, we present a novel quantitative PCR-based protocol for buckwheat detection. The method uses finely ground seeds of statice (*Limonium sinuatum*), an ornamental plant, as a unique internal standard material, because its seeds are readily available from seed stores and are thought to be least likely to contaminate foods. The specificity and the accuracy of quantitation are also reported.

MATERIALS AND METHODS

Plant and food materials. Seeds and flours of three cultivars of common buckwheat (*F. esculentum* Moench), namely, Shinano no. 1 (diploid), Takane-ruby (diploid), and Great-ruby (tetraploid),

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and those of Tartarian buckwheat (*F. tataricum* Gaertn., diploid) were purchased from Takano Co., Ltd. (Nagano, Japan). Seeds of wheat (*Triticum aestivum*), peanut (*Arachis hypogaea*), soybean (*Glycine max*), corn (*Zea mays*), mustard (*Sinapis alba*), pepper (*Piper nigrum*), and rice (*Oryza sativa*) were purchased from a local supplier. Seeds of wheat, soybean, corn, mustard, and *Fallopia convolvulus* (a volunteer weed of *Fagopyrum*-related species) were sown to obtain leaf samples. Pepper powder and salt were also obtained from a local grocery store. For the internal standard material, a single lot of statice seeds (Excellent light blue statice, *L. sinuatum*) were purchased from Sakata Seed Co. (Kanagawa, Japan) and were ground to fine powder by using an Ultra Centrifugal Mill ZM1 (Retsch GmbH & Co. KG, Haan, Germany) with a rotor (24 teeth) and a ring sieve (0.20 mm).

Test sample preparation. All test samples were prepared by using buckwheat flour of Shinano no. 1 as a spiking material. Wheat grains and brown rice were ground separately by using the Ultra Centrifugal Mill ZM1 (Retsch GmbH & Co. KG) as described above to prepare wheat and rice flours, respectively. Five types of test samples were prepared for evaluation of our method. (i) Wheat flour samples containing 10 and 100 ppm (wt/wt) of buckwheat flour were prepared by diluting 5.00 g of buckwheat flour with 45.00 g of wheat flour and repeating 10-fold dilutions until 10- and 100-ppm (wt/wt) samples were obtained. (ii) A rice flour sample containing 10 ppm (wt/wt) of buckwheat flour was prepared by serial dilutions of buckwheat flour with rice flour in the same manner as described above. (iii) A wheat and rice flour sample containing 10 ppm (wt/wt) of buckwheat flour was prepared by thoroughly mixing a 12.50-g portion of wheat flour containing 10 ppm (wt/wt) of buckwheat flour prepared as described in part i and a 12.50-g portion of rice flour containing 10 ppm (wt/wt) of buckwheat flour prepared as described in part ii. (iv) A pepper powder sample containing 100 ppm (wt/wt) of buckwheat flour was prepared by mixing 3.00 g of the rice flour containing 1,000 ppm (wt/wt) of buckwheat flour prepared as described in part ii with 27.00 g of pepper powder. (v) A salt and pepper sample containing 100 ppm (wt/wt) of buckwheat flour was prepared by mixing 3.00 g of rice flour containing 1,000 ppm (wt/wt) of buckwheat flour prepared as described in part ii, 12.00 g of pepper powder, and 15.00 g of salt. All mixing was carried out in antistatic plastic bags (Seibou OP type PZ; Fukusuke Kogyo Co., Ltd., Ehime, Japan). In accordance with the protein content of buckwheat flour of Shinano no. 1 (12.3%), which was quantified by using the Kjeldahl method, these test samples containing 10 and 100 ppm (wt/wt) of buckwheat flour were estimated to contain, respectively, about 1.2 and 12 μ g of buckwheat protein per g (ppm).

DNA extraction. Buckwheat and pepper DNAs were isolated from well-ground seeds by using Carlson lysis buffer (2) and Genomic-tip 20/G (Qiagen GmbH, Hilden, Germany), as described previously (4). Peanut DNA was isolated from well-ground seeds by using buffer G2 (Qiagen GmbH) and Genomic-tip 20/G (Qiagen GmbH); it was further purified by using a NucleoSpin Extract column (Machery-Nagel GmbH & Co., Düren, Germany). By use of a DNeasy Plant Mini Kit (Qiagen GmbH), wheat, soybean, corn, mustard, and *F. convolvulus* DNAs were isolated from leaf sections, and rice and statice DNAs were isolated from ground seeds. For specificity studies, leaf sections were used to lessen the probability of foreign DNAs affecting the results, except for pepper, peanuts, and rice, from which leaves were not obtainable. The specificity of buckwheat PCR against statice was tested by using DNA extracted from ground seeds to ensure absence of amplifiable DNA in the sample, because the ground seed itself was used

as the internal standard material. For quantitative analyses, 5.00 g of the test sample and 1.00 g of ground statice seeds were placed in a 50-ml centrifuge tube, to which were added 30 ml of Carlson lysis buffer, 60 μ l of RNase A (100 mg/ml), 75 μ l of 2-mercaptoethanol, 600 μ l of proteinase K (20 mg/ml), and three zirconia balls (YTZ ball, 7-mm diameter; Nikkato Corp., Osaka, Japan) to aid dispersion of the powdery sample. The contents were mixed well by using a shaker (KM Shaker V-DX; Iwaki Sangyo Co., Ltd, Tokyo, Japan) for 10 min or longer; they were then incubated for 20 min at 74°C. After cooling to room temperature and transfer of 4 ml of the mixture to a 15-ml tube, phenol–chloroform–isoamyl alcohol (25/24/1) extraction (once) and chloroform–isoamyl alcohol (24/1) extractions (twice) were carried out. We subjected 150 μ l of the water layer to isopropanol precipitation. The precipitate was dissolved in 100 μ l of sterilized ultrapure water and 900 μ l of Buffer QBT (Qiagen GmbH); the DNA was then purified by using Genomic-tip 20/G (Qiagen GmbH) according to the manufacturer's instructions. The DNA concentration was determined by measuring absorbance at 260 nm. Buckwheat standard DNA was extracted from 5.00 g of buckwheat flour with 1.00 g of ground statice seeds by using the same protocol as that used for test samples, in order to determine the coefficient value, which is used for calculations of the test samples' buckwheat content.

The necessary sample size for DNA isolation was determined as 5.00 g from the particle size and bulk density analyses of the buckwheat flour of Shinano no. 1. The particle size (median size) was measured by using laser diffraction and the scattering method under dry conditions. The bulk density was measured by using mercury porosimetry. Both analyses were performed by Seishin Enterprise Co., Ltd. (Tokyo, Japan).

Oligonucleotide PCR primers and probes. Two sets of primer pairs and fluorescent dye-labeled probes were designed to determine starting copy numbers of buckwheat and statice ITS-1 sequences by using real-time PCR. For buckwheat PCR, Fago-453 primer (5'-CGC CAA GGA CCA CGA ACA GAA G-3'), Fago-261 primer (5'-CGT TGC CGA GAG TCG TTC TGT TT-3'), and Fago-probe (5'-FAM-CGG GAC GCG CTT C-MGB-3') were used. Fago-261 primer was newly designed for this work to eliminate false-positive results that had been predicted previously for *Fallopia* spp. by PCR simulation and had been observed in the qualitative PCR on *F. convolvulus* (4). The PCR simulation was performed by using Amplify 1.0 software (Bill Engels) (3) to predict whether the Fago-453 and Fago-261 primer pair would give PCR products of the target size from the DNA sequences of the ITS-1 and ITS-2 regions and the 5.8S rRNA gene reported for plants listed in Table 1. For statice PCR, Limo-62 primer (5'-TTG GAC GTG TAT CCC TTG TGG TTC-3'), Limo-162 primer (5'-CAC GAA GGT GAA AGT TGC GTT CAT-3'), and Limo-probe (5'-FAM-TGT GCG ACG CGG AAT G-MGB-3') were designed from the sequence reported for the ITS-1 region of *L. sinuatum* (GenBank AJ222860). The primers were synthesized and purified on reverse-phase columns by Qiagen K. K. (Tokyo, Japan). The FAM dye-labeled TaqMan MGB probes were synthesized by Applied Biosystems Japan (Tokyo, Japan). The Plant-1 and Plant-2 primer pair designed to amplify a region of plant chloroplast DNA (16) was used to validate the quality of the template DNA.

Qualitative PCR. For qualitative detection of buckwheat or statice, PCR was performed in 0.2-ml reaction tubes in a final volume of 25 μ l, containing 12.5 μ l of 2 $\times$ HotStarTaq Master Mix (HotStarTaq DNA Polymerase, PCR buffer with 3 mM MgCl₂, and a 400 μ M concentration of each deoxynucleoside triphosphate [dNTP]; Qiagen GmbH), a 0.5 μ M concentration of

TABLE 1. List of plant DNA sequences (ITS-1 and ITS-2 regions and the 5.8S rRNA gene) used for PCR simulation

Taxonomic name of plant (common name)	GenBank accession no.
<i>Fagopyrum</i> spp. (Poygonaceae) ^a	
* <i>Fagopyrum esculentum</i> (common buckwheat)	AB000330
* <i>F. esculentum</i> subsp. <i>ancestrale</i>	AB000331
* <i>F. tataricum</i> (Tartarian buckwheat)	AB000339
* <i>F. tataricum</i> subsp. <i>potanini</i>	AB000340
* <i>F. callianthum</i>	AB000322
* <i>F. capillatum</i>	AB000323
* <i>F. cymosum</i>	AB000324
* <i>F. cymosum</i>	AB000325
* <i>F. cymosum</i>	AB000326
* <i>F. cymosum</i>	AB000327
* <i>F. cymosum</i>	AB000328
* <i>F. cymosum</i>	AB000329
* <i>F. gracilipes</i>	AB000332
* <i>F. homotropicum</i>	AB000333
* <i>F. leptopodium</i>	AB000334
* <i>F. lineare</i>	AB000335
* <i>F. pleioramosum</i>	AB000336
* <i>F. statice</i>	AB000337
* <i>F. statice</i>	AB000338
* <i>F. urophyllum</i>	AB000341
* <i>F. urophyllum</i>	AB000342
Internal standard	
<i>Limonium sinuatum</i> (statice)	AJ222860
Cultivated plants	
<i>Arachis hypogaea</i> (peanut)	AF156675
<i>Triticum aestivum</i> (wheat)	AJ301799
<i>Glycine max</i> (soybean)	U60551
<i>Juglans regia</i> (walnut)	AF303809
<i>Tricholoma matsutake</i> (matsutake mushroom)	U62964
<i>Zea mays</i> (corn)	U46648
<i>Oryza sativa</i> (rice)	AF169230
<i>Piper nigrum</i> (pepper)	AF275197
<i>Sinapis alba</i> (mustard)	X15915
<i>Prunus persica</i> (peach)	AF185621
<i>Malus domestica</i> (apple)	AF186484
<i>Citrus</i> sp. (Valencia orange)	E08821
<i>Fagopyrum</i> -related species ^b	
Polygonaceae	
<i>Aconogonum</i> sp. Won 152	AF189731
<i>Fallopia scandens</i>	AF040069
<i>Polygonum virginianum</i>	U51274
<i>Rumex acetosella</i>	AF189730
Others	
<i>Talinum paraguayense</i>	L78056
<i>Bruinsmia styracoides</i>	AF396438
<i>Talinella pachypoda</i>	L78054
<i>Rehderodendron kwangtungense</i>	AF396448
<i>Pterostyrax corymbosus</i>	AF396445
<i>Anredera cordifolia</i>	L78086
<i>Cistanthe quadripetala</i>	L78062
<i>Xenia vulcanensis</i>	L78060
<i>Talinopsis frutescens</i>	L78058
<i>Talinaria palmeri</i>	L78052
<i>Protulaca</i> sp.	L78049
<i>Phemerantus confertiflorus</i>	L78039
<i>Montiopsis umbellata</i>	L78033

TABLE 1. Continued

Taxonomic name of plant (common name)	GenBank accession no.
<i>Grahamia bracteata</i>	L78028
<i>Herniaria glabra</i>	AJ310965
<i>Alluaudia dumosa</i>	L78011
<i>Sinojackia xylocarpa</i>	AF396451
<i>Halesia macgregori</i>	AF396442
<i>Changios tyrax dolichocarpa</i>	AF396439
<i>Alectryon subdentatus</i>	AF314765
<i>Anacampseros recurvata</i>	L78014
<i>Weinmannia racemosa</i>	AF485597
<i>Bursera tecomaca</i>	AF080029

^a * Plant species that are predicted to yield a PCR product of target size with Fago-453 and Fago-261 primer pair.
^b Plant species with ITS-1 sequences that are homologous to *F. esculentum* (GenBank accession no. AB000330).

each of the primers Fago-453 and Fago-261 or a 0.2 μM concentration each of Limo-62 and Limo-162, 50 ng to 500 fg of template DNA, and sterilized ultrapure water. Amplification was carried out in a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, Calif.) by using the following program: preincubation at 95°C for 15 min and then 45 cycles consisting of denaturation at 95°C for 1 min, annealing at 66°C for 2 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 4 min. The PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide and were analyzed by using a Fluor-Imager 595 (Amersham Biosciences Corp., Piscataway, N.J.). The DNA sequences of the PCR products from common buckwheat (Shinano no. 1) and statice were determined by the direct sequencing method. The PCR with the Plant-1 and Plant-2 primer pair and the program described previously (4) yielded the expected products from all DNA samples.

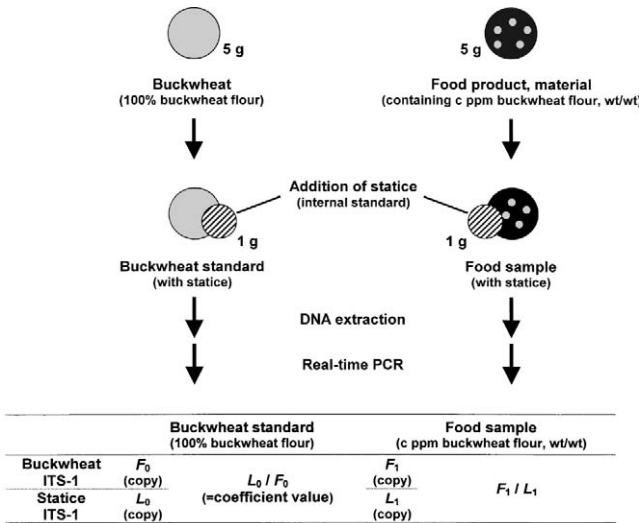
Reference molecule for constructing the standard curve. Amplicons of buckwheat and statice obtained from the respective qualitative PCR were connected into one molecule by using outer primers A (5'-TCT AGA CGC CAA GGA CCA CGA ACA GAA G-3') and D (5'-GGA TCC CAC GAA GGT GAA AGT TGC GTT CAT-3') and bridging primers B (5'-CAA AAG CTT CGT TGC CGA GAG TCG TTC TGT TT-3') and C (5'-ACG AAG CTT TTG GAC GTG TAT CCC TTG TGG TTC-3'), relying on a method adapted from Jayaraman and Puccini (8) and Kuribara et al. (10) with some modifications. Primers A and B, respectively, contain Fago-453 and Fago-261 sequences. Primers C and D, respectively, contain Limo-62 and Limo-162 sequences. These primers also contain sequences for introducing restriction enzyme recognition sites into the reference molecule. To connect the two amplicons, PCR was performed in a final volume of 50 μl containing 25 μl of 2× HotStarTaq Master Mix (HotStarTaq DNA polymerase, PCR buffer with 3 mM MgCl₂, and a 400 μM concentration of each dNTP; Qiagen GmbH), 7.5 μl each of 2 mM Gene Amp dNTP Mix (Applied Biosystems) (final concentration of each dNTP was 500 μM), a 1.0 μM concentration each of outer primers (primers A and D), a 25 nM concentration each of bridging primers (primers B and C), amplicons of buckwheat and statice PCR as template DNAs, and sterilized ultrapure water in 0.2-ml reaction tubes. Amplification was carried out in a PTC-200 DNA Engine (MJ Research, Inc., Waltham, Mass.) by using the following PCR program: preincubation at 95°C for 15 min; 15 cycles consisting of denaturation at 95°C for 1 min, annealing

at 40°C for 1 min, and extension at 72°C for 1 min; and then 30 cycles consisting of denaturation at 95°C for 1 min, annealing at 66°C for 1 min, and extension at 72°C for 1 min. The PCR product was electrophoresed on a 2% agarose gel containing ethidium bromide and analyzed by using a FluorImager 595 (Amersham Biosciences Corp.). The PCR product was then ligated into pGEM-T Easy Vector (Promega Corp., Madison, Wis.) and transformed into *Escherichia coli* JM109 (DH5α) by using the pGEM-T Easy Vector System (Promega Corp.). The plasmid was extracted and purified with the HiSpeed Plasmid Midi Kit (Qiagen GmbH). The nucleotide sequence analysis confirmed that the intended DNA fragments were introduced into the plasmid correctly (data not shown). The plasmid solution concentration was calculated and diluted with sonicated salmon testis DNA (deoxyribonucleic acid sodium salt, from salmon spermary, fibrous; Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution (5 ng/μl) to prepare a series of 10-fold dilutions from 10⁹ to 10¹ copies per 2.5 μl.

Real-time PCR. The real-time PCR to quantitate buckwheat DNA or static DNA was performed in a final volume of 25 μl containing 12.5 μl of 2× QuantiTect Probe PCR Master Mix (HotStarTaq DNA polymerase, QuantiTect Probe PCR buffer, a dNTP mix including dUTP, ROX, and 8 mM MgCl₂; Qiagen GmbH), a 0.2 μM concentration of each of the primers Fago-453 and Fago-261 or Limo-62 and Limo-162, 0.2 μM TaqMan MGB probe (Fago-probe or Limo-probe), template DNA, and sterilized ultrapure water in a MicroAmp optical 96-well reaction plate with MicroAmp caps (Applied Biosystems). A no-template control was also prepared as the negative control, in which sterilized ultrapure water was substituted for the template DNA solution. Amplification was carried out in an ABI PRISM 7700 (Applied Biosystems) by using the following PCR program: preincubation at 50°C for 2 min and 95°C for 15 min and then 45 cycles consisting of denaturation at 95°C for 1 min, annealing at 66°C for 2 min, and extension at 72°C for 1 min. For each run, a standard curve was created by using nine 10-fold dilutions of the reference molecule. Fluorescence data measured at the extension steps were analyzed. The threshold line was drawn according to a previously published method used for quantification of GM maize and soybean (10). Within each run, individual samples were amplified in two wells; the means of the results were used for determinations of the starting copy numbers.

Calculation of coefficient value of buckwheat. Flours of four buckwheat varieties were tested to determine coefficient values. We subjected 50 ng of DNA extracted from the mixture of 5.00 g of buckwheat flour and 1.00 g of ground static seeds to both buckwheat and static real-time PCR. The ratios of static and buckwheat ITS-1 sequences (L_0/F_0) in the DNA extract were calculated by using formula 1 in Figure 1 and denoted as coefficient values. The coefficient value of buckwheat flour of Shinano no. 1 was determined from six DNA extracts, with each being run twice. The coefficient values of other buckwheat varieties were determined from three DNA extracts, also run twice.

Calculation of buckwheat content. Six test samples were analyzed by using PCR to determine the buckwheat content. We subjected 50 ng of DNA extracted from the mixture of 5.00 g of test sample and 1.00 g of ground static seeds to both buckwheat and static real-time PCR. Buckwheat content of each test sample was calculated from the PCR results for three DNA extracts. The ratio of the buckwheat and the static ITS-1 sequences (F_1/L_1) in the DNA extract was substituted into formula 2 in Figure 1, and the concentration of buckwheat flour in the test sample was calculated. A schematic of the quantitative analysis is shown in Fig-



Formula 1.

$$\text{Coefficient value} = \frac{L_0 \text{ (static ITS-1 starting copy number of buckwheat standard)}}{F_0 \text{ (buckwheat ITS-1 starting copy number of buckwheat standard)}}$$

Formula 2.

$$\text{Content of buckwheat flour (c ppm, wt/wt)} = \frac{F_1 \text{ (buckwheat ITS-1 starting copy number of food sample)}}{L_1 \text{ (static ITS-1 starting copy number of food sample)}} \times \text{Coefficient value} \times 10^5$$

FIGURE 1. Schematic diagram of the quantitative analysis of buckwheat content in food products. First, DNA extraction and real-time PCR were performed by using buckwheat flour (100% buckwheat) spiked with ground static seeds. The coefficient value was determined with formula 1 by using the starting copy numbers of buckwheat ITS-1 sequences (F_0) and static ITS-1 sequences (L_0) measured by using real-time PCR. Next, DNA was extracted from food product (containing c ppm buckwheat flour [wt/wt]) that had been spiked with ground static seeds, while the ratio of buckwheat ITS-1 sequences and static ITS-1 sequences, F_1/L_1 , was determined by using real-time PCR. The coefficient value and F_1/L_1 were used to calculate the buckwheat flour content of food product with formula 2.

ure 1. The concentration of buckwheat flour was converted to that of buckwheat protein based on the protein content of common buckwheat (Shinano no. 1) flour (12.3%), as determined by using the Kjeldahl method.

ELISA. Three test samples containing 10-ppm (wt/wt) buckwheat flour were also analyzed with two quantitative ELISA kits, i.e., the Buckwheat Protein ELISA Kit (Morinaga Institute of Biological Science, Inc., Kanagawa, Japan) and FASTKIT Buckwheat ELISA Kit (Nippon Meat Packers, Inc., Osaka, Japan), according to the manufacturers' instructions. Protein was extracted from 2 g of each test sample with 38 ml of the respective extraction buffer supplied by the manufacturer in triplicate. In the case of the Buckwheat Protein ELISA Kit, the extract was diluted 1:3 and the mean of the results from two wells was calculated. In the case of FASTKIT Buckwheat ELISA Kit, the extract was diluted 1:10 and the mean of the results from three wells was obtained.

RESULTS

Specificity of buckwheat primer pair predicted by PCR simulation software. Our previous buckwheat primer pair (Fago-453 and UN-1 primer) gave a PCR product of the target size from *Fallopia convolvulus* (volunteer weed of the *Fagopyrum*-related species), which was distinguish-

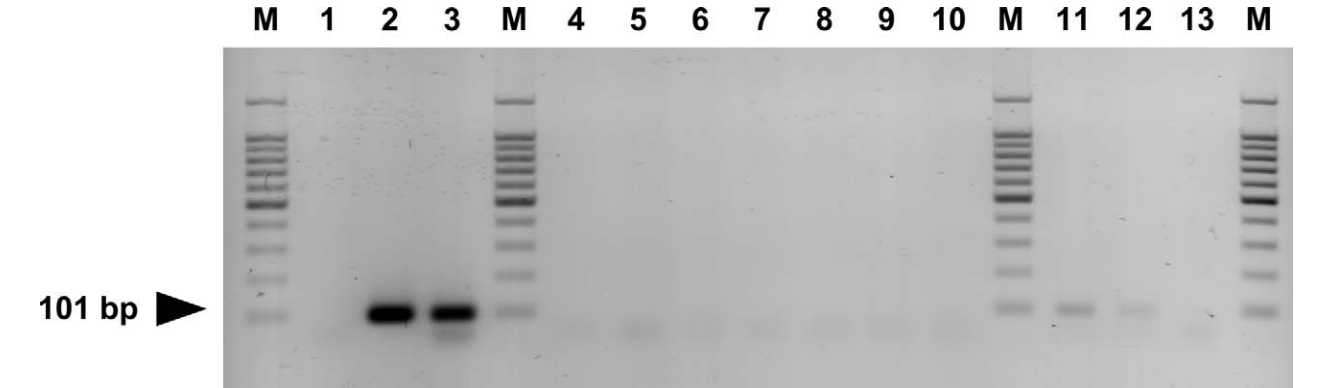


FIGURE 2. Specificity of the Fago-453 and Fago-261 primer pair for buckwheat. PCR was performed by using the Fago-453 and Fago-261 primer pair. The arrowhead indicates the expected PCR product. Lane 1, no-template control; lane 2, amplification of 500 fg of common buckwheat (*F. esculentum* Moench, cv. Shinano no. 1) genomic DNA; lane 3, amplification of 500 fg of Tartarian buckwheat (*F. tataricum* Gaertn.) genomic DNA; lanes 4 to 10, amplification of 50 ng of wheat (lane 4), peanut (lane 5), soybean (lane 6), corn (lane 7), mustard (lane 8), pepper (lane 9), and rice (lane 10) genomic DNAs; lanes 11 to 13, amplification of 50 ng (lane 11), 5 ng (lane 12), and 500 pg (lane 13) of *F. convolvulus* genomic DNA; and M, 100-bp ladder size standard.

able from that of buckwheat only by DNA sequencing (4). Therefore, a new buckwheat primer pair (Fago-453 and Fago-261 primer) was designed and tested for its specificity by PCR simulations against the sequences listed in Table 1. Products of 101 bp were predicted from all 21 sequences of the genus *Fagopyrum* including both cultivated and wild buckwheat. The amplicon size reported here, 101 bp, is 2 bp shorter than that predicted by Amplify 1.0, which takes into account the terminal transferase activity shown by most thermostable DNA polymerases. No PCR products were predicted from sequences of 12 commonly cultivated plants, 27 *Fagopyrum*-related species, and *L. sinuatum* (statice) used as the internal standard material. The 27 *Fagopyrum*-related species in Table 1 were tested here by using only PCR simulation, because they were not readily available from the market.

Specificity of buckwheat PCR. The specificity of the buckwheat PCR was confirmed by using both qualitative and real-time PCR. The result of qualitative PCR with the Fago-453 and Fago-261 primer pair is shown in Fig. 2.

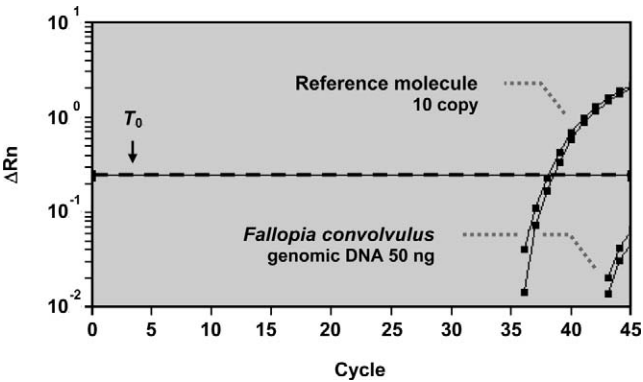
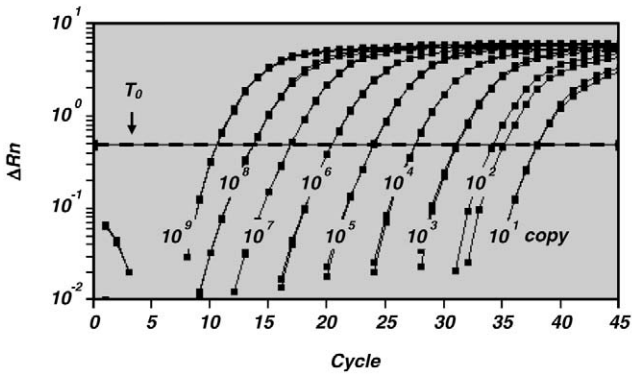


FIGURE 3. Specificity and sensitivity of the Fago-453 and Fago-261 primer pair and Fago-probe for buckwheat. Real-time PCR was performed by using the Fago-453 and Fago-261 primer pair and Fago-probe. Amplification plots were generated for 10 copies of the reference molecule and 50 ng of *F. convolvulus* genomic DNA. The horizontal line (T_0) indicates the threshold line.

From 500 fg of common buckwheat (*F. esculentum* Moench, cv. Shinano no. 1) and Tartarian buckwheat (*F. tataricum* Gaertn.) DNAs, PCR products at about 100 bp were obtained (lanes 2 and 3, respectively). A nucleotide sequence analysis of the PCR product from common buckwheat (Shinano no. 1) confirmed that the 101-bp sequence of the ITS-1 region–5.8S rRNA gene of the *Fagopyrum* spp. was amplified with this primer pair (data not shown). Although very weak PCR products of approximately 100 bp were produced from 5-ng and 50-ng DNA samples of *F. convolvulus*, no detectable PCR products were found when the template DNA was 500 pg or less. Figure 2 shows that, with this exception, amplification of a 101-bp product did not occur in plants other than buckwheat. Furthermore, the use of the newly designed primer pair (Fago-453 and Fago-261) eliminated the nonspecific product from wheat (*T. aestivum*) observed previously (4). The specificity of the real-time PCR was confirmed by using the same primer pair and Fago-probe. The amplification signal was observed from 500 fg of common buckwheat (Shinano no. 1) DNA (data not shown). Although a very weak amplification signal was observed from 50 ng of *F. convolvulus* DNA, its amplification curve did not cross the threshold line (Fig. 3). No amplification signal was observed from 50 ng of wheat, peanut, soybean, corn, mustard, pepper, rice, or statice DNA (data not shown).

Specificity of statice PCR. The specificity of the statice PCR was also confirmed by using both qualitative and real-time PCR. Qualitative PCR with the Limo-62 and Limo-162 primer pair yielded a product of 101 bp from 50 ng of statice DNA but none from 50 ng of common buckwheat (Shinano no. 1), Tartarian buckwheat, wheat, peanut, soybean, corn, mustard, pepper, rice, or *F. convolvulus* DNA (data not shown). The nucleotide sequence of the PCR product obtained from statice matched the corresponding sequence of *L. sinuatum* (GenBank accession no. AJ222860) (data not shown). The result of real-time PCR with the same primer pair and Limo-probe also confirmed the specificity of the primer-probe combination; an ampli-

A. Amplification plots of real-time PCR for buckwheat



B. Standard curve of real-time PCR for buckwheat

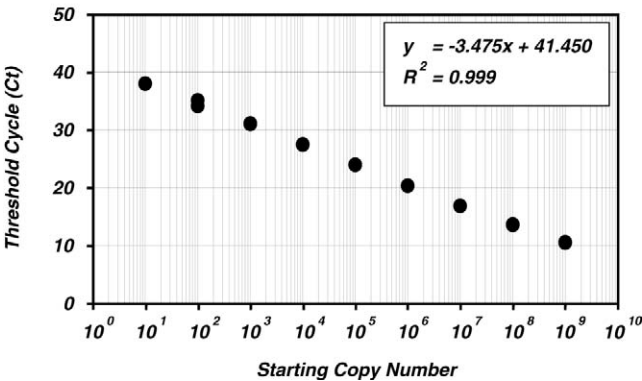


FIGURE 4. Amplification plots and standard curve of real-time PCR for buckwheat. Real-time PCR was performed by using the Fago-453 and Fago-261 primer pair and Fago-probe. (A) Amplification plots were generated for 10^1 to 10^9 copies of the reference molecule. The horizontal line (T_0) indicates the threshold line. (B) Standard curve was generated from the amplification plots shown in panel A.

fication signal was observed only from static DNA (data not shown).

Standard curves of real-time PCR. The linearity of standard curves for buckwheat and static DNA was demonstrated by real-time PCR with the reference molecule described in “Materials and Methods.” Figure 4 shows typical amplification plots and a standard curve for buckwheat. For a wide range of starting copy numbers, i.e., 10^1 to 10^9 copies of the reference molecule per reaction well, the correlation coefficients of the standard curves (buckwheat: $R^2 = 0.999$; static: $R^2 = 0.999$) were greater than 0.99, and the respective reaction efficiencies calculated from the slopes (buckwheat: $y = -3.475x + 41.450$, static: $y = -3.385x + 41.855$) were 94 and 97%, suggesting nearly 100% doubling of copy numbers in each cycle for both PCR systems.

Coefficient value of buckwheat. Calculated coefficient values are shown in Table 2. Mean coefficient values for the four cultivated varieties of buckwheat were 2.36 to 4.75.

TABLE 2. Coefficient values of buckwheat

Buckwheat flour	Coefficient values	
	Mean	SD ^a
Common buckwheat (Shinano no. 1) ^a		
<i>F. esculentum</i> Moench, diploid	2.36	0.26
Common buckwheat (Takane-ruby) ^b		
<i>F. esculentum</i> Moench, diploid	3.25	0.61
Common buckwheat (Great-ruby) ^b		
<i>F. esculentum</i> Moench, tetraploid	2.70	0.25
Tartarian buckwheat ^b		
<i>F. tataricum</i> Gaertn., diploid	4.75	0.52

^a Experiment was performed by using six DNA extracts; an average of duplicate PCR results for each DNA extract was used for calculation of the mean and the standard deviation.

^b Experiment was performed by using three DNA extracts; an average of duplicate PCR results for each DNA extract was used for calculation of the mean and the standard deviation.

Buckwheat protein content of test samples. Buckwheat flour and the corresponding protein concentrations of the six test samples determined with our method are summarized in Table 3. The protein concentrations were calculated from buckwheat flour concentrations by using the protein content (12.3%) of the flour of common buckwheat (Shinano no. 1). The calculated protein concentrations (mean $\pm$ standard deviation) for samples prepared to contain 10 ppm (wt/wt) of buckwheat flour (1.2- μ g/g [ppm] protein) ranged from 0.7 ± 0.2 (rice) to 0.9 ± 0.4 (wheat) and for 100-ppm (wt/wt) samples (12- μ g/g [ppm] protein) from 7.7 ± 1.0 (pepper) to 9.8 ± 0.5 (wheat) μ g/g (ppm). The protein concentrations of the 10-ppm (wt/wt) flour samples were determined by using two officially recognized commercial ELISA kits. They ranged from 1.6 ± 0.6 (rice) to 1.9 ± 1.0 (wheat and rice) for the Buckwheat Protein ELISA Kit (Morinaga Institute of Biological Science, Inc.) and from 2.0 ± 0.3 (wheat and rice) to 2.5 ± 1.4 (rice) μ g/g (ppm) for the FASTKIT Buckwheat ELISA Kit (Nippon Meat Packers, Inc.).

DISCUSSION

Sensitive, specific, and accurate methods for detection and quantitative analysis of allergen in foods are indispensable for providing consumers with accurate information about the presence of allergens in foods and, through labeling, for preventing incidences of food allergy. Quantitative methods that are presently available for allergenic plants are based on ELISA (6, 7, 11, 17). In these methods no consideration is made for internal standards for addressing the variability that is inherent in analyses of diverse food samples. If food samples contain substances that interfere with extraction of the target molecular species or with the reactions of the assay procedures, the obtained values for allergen content might be either lower than the actual concentration or even falsely negative, unless a proper recovery test is performed on each sample. Therefore, it is essential to introduce a pertinent internal standard and thereby standardize protein-based or DNA-based assays (15). Kuribara et al. (10) used a single copy gene in the

TABLE 3. Buckwheat content analysis of test samples

Test sample	Concn of buckwheat ^a			
	Flour (ppm, wt/wt)	Protein (μg/g, ppm)		
	PCR ^b	PCR ^c	ELISA (A) ^d	ELISA (B) ^d
10-ppm buckwheat flour				
In wheat	7.7 ± 3.1	0.9 ± 0.4	1.8 ± 0.3	2.1 ± 0.4
In wheat and rice	6.4 ± 1.7	0.8 ± 0.2	1.9 ± 1.0	2.0 ± 0.3
In rice	5.7 ± 1.5	0.7 ± 0.2	1.6 ± 0.6	2.5 ± 1.4
100-ppm buckwheat flour				
In wheat	80.0 ± 4.0	9.8 ± 0.5	NT	NT
In pepper	62.4 ± 7.9	7.7 ± 1.0	NT	NT
In salt and pepper	72.7 ± 20.7	8.9 ± 2.5	NT	NT

^a Data are means ± standard deviations. NT, not tested.

^b Measured concentration of buckwheat flour (parts per million [wt/wt]) with PCR. PCR was performed on triplicate DNA extracts; the result for each DNA extract was used for calculation of the mean and the standard deviation.

^c Converted concentration of buckwheat protein (number of micrograms per gram [parts per million]) from the PCR data (parts per million [wt/wt]) obtained by using the result of protein content analysis of buckwheat flour (12.3%).

^d Measured concentration of buckwheat protein (number of micrograms per gram [parts per million]) with ELISA. ELISAs were performed on triplicate protein extracts; the result for each protein extract was used for calculation of the mean and the standard deviation. ELISA (A), Buckwheat Protein ELISA Kit (Morinaga Institute of Biologica Science, Inc.). ELISA (B), FASTKIT Buckwheat ELISA Kit (Nippon Meat Packers, Inc.).

maize endogenous DNA as an internal standard in developing the quantitative PCR methods for analyses of GM maize. The targets of their real-time PCR analyses involved both a specific region of GM maize DNA and the maize endogenous gene, by which they calculated the percentage of GM maize in maize matrices. As a practical matter, it is impossible to find a DNA sequence that is common to all food samples and that can therefore serve as an internal standard. In this study, we selected ground statice seeds as an internal standard material that can be added to food samples prior to DNA extraction. The DNA sequence to be amplified was selected from the ITS-1 region of the statice DNA found in GenBank. Statice is an ornamental plant belonging to the genus *Limonium* and is unlikely to be present as a voluntary weed in or near the field where food crops are grown; the probability of the food crops being contaminated with statice or related species during harvesting or postharvest processing is expected to be negligible. Indeed, statice DNA was not detected from any food materials that we tested. In addition, statice seeds are available at local seed stores. These features render ground statice seeds an excellent candidate for an internal standard material to be used in PCR for quantitative analyses of allergenic materials and other biological food contaminants.

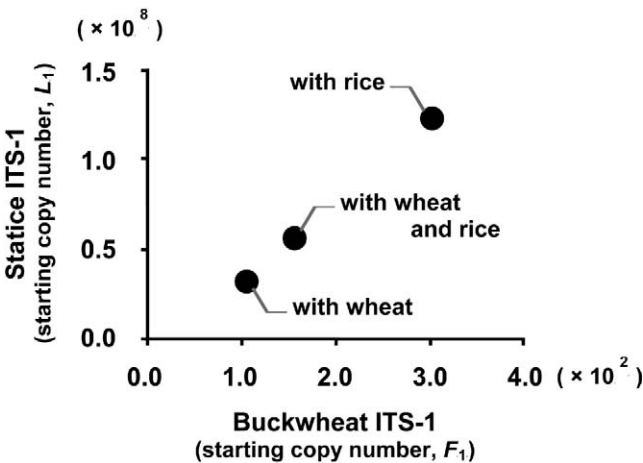
Japanese food-labeling regulations require that an allergen must be declared on a product label if its protein concentration in a food product is greater than 10 μg/g or 10 μg/ml (10 ppm), as measured with one of the two officially recognized ELISA kits (11, 17) used in this study. Table 3 shows that the buckwheat protein concentrations determined with our method for various test samples prepared to contain 10- and 100-ppm (wt/wt) flour were reasonably close to the calculated values of 1.2 and 12 μg/g (ppm) based on the Kjeldahl analysis of Shinano no. 1 flour.

The protein concentrations determined for 10-ppm (wt/wt) flour were also comparable with those obtained by using the two officially recognized ELISA kits (11, 17). Our method was considered sufficiently sensitive for use in quantitation of buckwheat protein contaminations at 10 μg/g (ppm) or less. The coefficients of variance determined for our PCR method by using results of 10-ppm (wt/wt) flour samples were 26 to 40% and were also considered comparable with 17 to 56% for the Buckwheat Protein ELISA Kit (Morinaga Institute of Biological Science, Inc.) and 14 to 58% for the FASTKIT Buckwheat ELISA Kit (Nippon Meat Packers, Inc.). Our method is useful for detecting buckwheat contamination to ensure compliance with allergen-labeling requirements.

The starting copy numbers of buckwheat ITS-1 sequences determined for test samples containing 10 ppm (wt/wt) of buckwheat flour (1.2-μg/g [ppm] protein) were more than 100 copies, which is more than 10 times larger than the minimum starting copy numbers required for real-time PCR to yield reliable results. The salient implication is that quantitative detection of 1-ppm (wt/wt) buckwheat flour (0.12-μg/g [ppm] protein) might be feasible with our method. Calculations based on the average particle size and bulk density of the buckwheat flour reveal that, at a concentration of 1 ppm (wt/wt), only about 15 particles of buckwheat flour would be present in 5 g of test samples. As a rule of thumb, at least 100 particles per sample would be necessary to ensure uniform distribution of the particles in the powdery samples. Consequently, we concluded that the lower limit of quantitation for buckwheat flour in powdery samples, such as those used in the present work, was 10-ppm (wt/wt) flour or ca. 1-μg/g (ppm) protein.

Figure 5A and 5B shows correlations between starting copy numbers of the target sequences (ITS regions) of

A. 10 ppm buckwheat flour



B. 100 ppm buckwheat flour

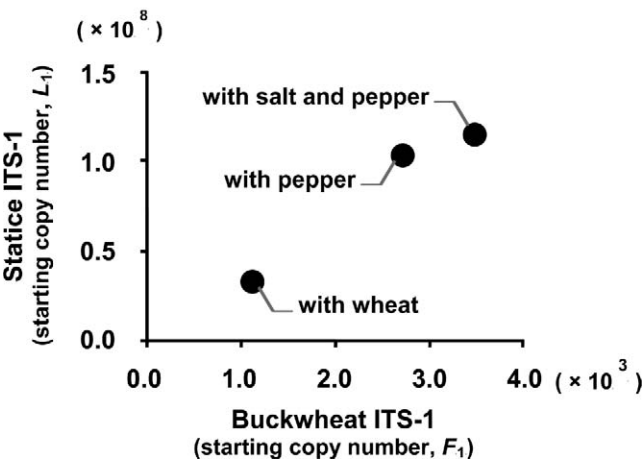


FIGURE 5. Relationship of starting copy numbers of test samples made with various diluent materials. Test samples were analyzed by using real-time PCR for buckwheat and statice. Plots were generated by starting copy numbers of the ITS-1 region of the test samples containing 10 ppm (wt/wt) of buckwheat flour (A) and 100 ppm (wt/wt) of buckwheat flour (B).

buckwheat and of statice DNAs determined for various test samples containing buckwheat flour at concentrations of 10 and 100 ppm (wt/wt). Although the ratios of measured buckwheat and statice DNAs were nearly constant for test samples containing the same amount of buckwheat flour, amounts of measured DNA varied widely depending on the diluent materials. For example, the starting copy number determined for the wheat sample containing 10 ppm (wt/wt) of buckwheat flour was found to be much smaller than the starting copy number determined for the rice sample containing the same amount of buckwheat (Fig. 5A). It was also found that the total amount of DNA extracted from the wheat sample was larger than the amount extracted from the rice sample (data not shown); this difference may be attributable to the difference in the genome sizes of

wheat and rice, the former being about 40 times larger than the latter (1). A similar difference in the starting copy numbers was observed between the pepper sample and the salt and pepper sample, both containing 100 ppm (wt/wt) of buckwheat flour (Fig. 5B). The larger starting copy number of buckwheat DNA determined for the salt and pepper sample was attributed to a smaller amount of total DNA extracted from the salt and pepper sample and further to the absence of DNA in the salt used as part of the diluent materials. Since an excess DNA in a reaction mixture would interfere with amplification of the target sequence, the total amount of DNA added to a reaction well was set at 50 ng for all samples. Therefore, starting copy numbers of buckwheat DNA in the reaction mixture were inversely proportional to the amount of DNA extracted from the food matrices, if buckwheat concentrations were the same. The same was true for statice DNA, because the amount of statice added to food samples was always the same (1 to 5 g of sample).

Although the starting copy numbers determined for individual samples may vary depending on many factors, of which genome size of the diluent materials is one, the nearly constant ratios of the starting copy numbers of buckwheat to the corresponding starting copy numbers of statice within samples of the same buckwheat flour content (10 or 100 ppm [wt/wt]) suggest that, if a known amount of statice is added to each sample and is used as the internal standard, the amount of buckwheat content can be determined accurately in various food matrices by compensating for variability in the total amount of DNA extracted from the sample and for other possible variations arising from the presence of various factors interfering with the PCR process.

The coefficient values, which are required in the calculations of buckwheat content, were determined for only three cultivars of common buckwheat (*F. esculentum*) and Tartarian buckwheat (*F. tataricum*). A rather large difference was observed between the species. It may be attributable to the difference in the ITS target copy numbers among the *Fagopyrum* spp., the amplification efficiency resulting from the nucleotide sequence in the target region, or some other unknown reasons.

In this study the average coefficient value of 2.36 determined for common buckwheat (*F. esculentum* Moench, cv. Shinano no. 1) was used for calculations of buckwheat flour content in the test samples, because they were spiked with the flour of Shinano no. 1. The more conservative value of 4.75 for Tartarian buckwheat should be used for analyses if information about which buckwheat species is present in the suspected food is available a priori. Further research is necessary to establish the most appropriate coefficient value by evaluating more buckwheat species and cultivars.

Nair and Adachi (13) reported presence of an allergenic 22-kDa buckwheat protein (12) in at least 11 *Fagopyrum* spp., including both cultivated and wild species. Wang et al. (20) reported that a 24-kDa protein, which was considered to be allergenic (19), was present in Tartarian buckwheat (*F. tataricum*) and in common buckwheat (*F. esculentum*). Because wild buckwheat growing as voluntary

weeds in or near the field of food crops may be inadvertently included in the crops during harvesting or postharvest processing, our real-time PCR protocol was designed to detect all *Fagopyrum* spp. by applying PCR simulation software to yield a product of expected size through use of the DNA sequences of the ITS-1 and ITS-2 regions and the 5.8S rRNA gene of all the *Fagopyrum* spp. available from the GenBank database.

The buckwheat primer pair and probe combination used in this work was considered to be specific enough to avoid most false-positive results. A weak amplification signal that was observed when more than 5 ng of *Fallopia convolvulus* DNA was present in the sample would not pose a problem in analyses of food samples, because it is very unlikely that a volunteer weed, such as *F. convolvulus*, would contaminate food materials at concentrations as high as 5 ng of DNA, which corresponds to ca. 10% (wt/wt) of the plant material in the sample.

It should be noted that the results of our specificity study by PCR simulations are not definitive, because it remains possible that amplification might occur from other parts of the genome that are not listed in the GenBank accessions. It is not practical, however, to test all possibilities of cross-reactivity by actual PCR experiments. On the other hand, the ITS sequences are frequently used as evidence of phylogenetic relationships. In addition, the ITS sequences of the *Fagopyrum*-related species, against which we evaluated specificity by using the PCR simulations, were those identified to be most highly homologous to the target sequence of our buckwheat PCR. For these reasons, it was considered that the PCR simulations reported here should give us a good indication of the specificity of our PCR systems.

Our method could be implemented more efficiently if different fluorescent dyes were used to label the buckwheat and static probes (Fago-probe and Limo-probe) so that the two reactions might be carried out simultaneously in the same PCR well or tube. Work is under way to test applicability of our current method to cooked foods. Preliminary results suggests that quantitative determinations of approximately 10- μ g/g (ppm) buckwheat protein in baked, boiled, deep-fried, and steamed wheat dough might be feasible.

In conclusion, a useful protocol for the quantitative PCR method for buckwheat detection was established. It has two technical features: the use of ground static seeds as a unique internal standard material and the use of an ITS region as the PCR target sequence. Because of these features, the method offers some advantages over other presently available methods; the variability in extraction and amplification efficiencies that arises from the diversity of sample matrices can be compensated for; also potentially allergenic *Fagopyrum* spp. can be detected in an intragenetically specific manner with sufficiently high sensitivity and a wide dynamic range. Examples of promising applications of the method include quality control of food ingredients and products for proper labeling and etiological investigations upon incidence of allergic reactions.

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