

PCR Method for Detecting Trace Amounts of Buckwheat (*Fagopyrum* spp.) in Food

Takashi HIRAO,^{1,†} Shinsuke IMAI,¹ Hiroshi SAWADA,¹ Nobuo SHIOMI,^{1,*}
Satoshi HACHIMURA,² and Hisanori KATO²

¹Somatech Center, House Foods Corporation, 1-4 Takanodai, Yotsukaido-shi, Chiba 284-0033, Japan

²Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences,
The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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Buckwheat often causes severe allergic reactions, even when its ingestion level is extremely low. Therefore, buckwheat is listed in several countries as a common food allergen. In addition to common buckwheat and Tartarian buckwheat that are cultivated and consumed widely, wild buckwheat may be potentially allergenic. Food containing undeclared buckwheat poses a risk to patients with the buckwheat allergy. We describe in this report a PCR method to detect buckwheat DNA by using primers corresponding to the internal transcribed spacer region and the 5.8S rRNA gene. The method is buckwheat-specific and compatible with both cultivated and wild buckwheat of the *Fagopyrum* spp. Its sensitivity was sufficient to detect 1 ppm (w/w) of buckwheat DNA spiked in wheat DNA. This method should benefit food manufacturers, clinical doctors, and allergic patients by providing information on the presence of buckwheat contamination in food.

Key words: food allergy; buckwheat; *Fagopyrum*; internal transcribed spacer (ITS); detection

Buckwheat is cultivated worldwide, especially in Asian, Russian, European, South and North American, and Australian countries. The flour of buckwheat seed is an important food material used in buckwheat noodle, jelly, and dumpling in east-Asian countries and in batter for cake, pancake, and crepe in western countries. It is also eaten as a substitute for wheat by some patients with celiac disease. In addition to being a food, buckwheat husk is also used as a stuffing for pillows. On the other hand, buckwheat is a known food allergen, and ingestion of a small amount can cause severe shock in sensitive patients.^{1,2)} Such allergic symptoms have mainly been reported in Asian, European, and North American countries.^{3–7)} Due to the seriousness of the shock and the number of affected people, the Japanese

labeling standard states that buckwheat must be declared on food labels; other substances that must also be specified include peanuts, wheat, eggs, and milk.^{8–10)}

Allergies are generally caused by trace amounts of allergens in food, therefore food manufacturers and patients need to be careful not only about whether food products or materials contain the allergenic ingredients, but also whether unintentional contamination during harvesting, transportation, storage, and manufacturing has occurred. After harvesting, attention must be paid to the contamination by cultivated plant species used for food that are considered to be allergenic. At the stage of harvesting in particular, there is a certain risk of contaminating food materials not only with cultivated but also with wild plant species. The cross-contamination with plants growing as volunteer weeds near the field would occur more unwittingly than with plants cultivated for food in the field. If the allergenicity of wild species is suspected, attention must be paid to both cultivated and wild species. In the case of buckwheat, wild species (*Fagopyrum homotropicum*, *Fagopyrum cymosum*, etc.) belonging to the same genus as the two cultivated species [common buckwheat (*Fagopyrum esculentum*) and Tartarian buckwheat (*Fagopyrum tataricum*)] exist. According to the phylogenetic tree of the *Fagopyrum* spp., some wild buckwheat species are classified at closer positions than the evolutionary distance between the two cultivated types of buckwheat considered to be allergenic.^{11,12)} Besides, it has been reported that some allergenic proteins of cultivated buckwheat might be distributed among the *Fagopyrum* spp.^{13,14)} Thus, the risk of raising the buckwheat allergy due to allergens derived from wild buckwheat may be relatively high. A method to detect the presence of all the *Fagopyrum* spp. in food products would therefore be indispensable for ensuring the safety of buckwheat allergy patients.

We developed in the present study a PCR-based

[†] To whom correspondence should be addressed. Tel: +81-43-237-5211; Fax: +81-43-237-2914; E-mail: t-hirao@housefoods.co.jp

* Present address: House Food Analytical Laboratory Inc., 1-4 Takanodai, Yotsukaido-shi, Chiba 284-0033, Japan

Abbreviation: ITS, internal transcribed spacer

protocol that enabled us to detect both cultivated and wild buckwheat. The chosen target sequence, part of the internal transcribed spacer (ITS) region and the ribosomal RNA (rRNA) gene, rendered this method both specific and sensitive.

Materials and Methods

Samples used in DNA isolation. Seeds of Shirahana soba (common buckwheat, *F. esculentum*) and Dattan soba (Tartarian buckwheat, *F. tataricum*) were purchased from Takano Co., Ltd. (Japan). White pepper was purchased from a local supplier. Commercially available seeds of wheat, soybean, corn, and mustard were germinated to obtain their leaves.

Isolation of DNA from buckwheat and white pepper. Well-ground seeds (1 g) were transferred to a 15-ml centrifuge tube with 4 ml of a Carlson lysis buffer [0.1 M Tris-HCl (pH 9.5), 2% hexadecyltrimethyl-ammonium bromide (CTAB), 1.4 M NaCl, 1% polyethylene glycol 6000, and 20 mM EDTA],¹⁵ 8 µl of RNase A (100 mg/ml), 10 µl of 2-mercaptoethanol, and 80 µl of proteinase K (20 mg/ml). The contents were well mixed and incubated for 20 min at 74 °C. After cooling to room temperature, phenol/chloroform/isoamyl alcohol (25/24/1) extraction (once) and chloroform/isoamyl alcohol (24/1) extraction (twice) were carried out. The resulting aqueous layer was subjected to isopropanol precipitation, and the precipitate was dissolved in 500 µl of Buffer QBT (Qiagen). DNA was then purified by using Genomic-tip 20/G (Qiagen) according to the manufacturer's instructions, but with a slight modification: the tip was washed with 5 ml of Buffer QBT after the sample had been applied. The DNA concentration was determined by measuring the UV absorption at 260 nm.

Isolation of DNA from wheat, soybean, corn, and mustard. DNA was isolated from the leaves by using DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

Dilution of buckwheat DNA. Wheat DNA solutions containing 100,000 ppm, 10,000 ppm, 1,000 ppm, 100 ppm, 10 ppm, 1 ppm, 100 ppb, and 10 ppb of Shirahana soba (common buckwheat) DNA were prepared by stepwise dilution.

DNA sequences. The DNA sequences of the ITS-1 and ITS-2 regions, and the 5.8S rRNA gene were used for primer design. Twenty-one corresponding DNA sequences of the *Fagopyrum* spp. belonging to the family Polygonaceae were selected as cultivated and wild buckwheat. The DNA sequences from peanut (*Arachis hypogaea*), wheat (*Triticum aestivum*), soybean (*Glycine max*), walnut (*Juglans regia*), matsutake mushroom (*Tricholoma matsutake*), corn (*Zea mays*), rice (*Oryza sativa*), white pepper (*Piper nigrum*), mustard

(*Sinapis alba*), peach (*Prunus persica*), apple (*Malus x domestica*), and Valencia orange (*Citrus* sp.) were chosen to represent typical cultivated plant species used for food. Twenty-seven DNA sequences were selected from *Fagopyrum*-related species. Related species were classified according to the sequence of the ITS-1 region: a BLAST homology search using the ITS-1 sequence of *F. esculentum* (GenBank Accession No. AB000330) was performed, and sequences with a score of 60 bits or more and with the highest score in the corresponding genus were selected.

Oligonucleotide primers. Using the ITS-1 region and the 5.8S rRNA gene from *F. esculentum*, primer sequences were designed to be more homologous to the 21 DNA sequences of the *Fagopyrum* spp. than to the corresponding sequences of the other plants just listed. Thus, the Fago-453 sense primer (5'-CGC CAA GGA CCA CGA ACA GAA G-3') was designed, and the specificity for the *Fagopyrum* spp. was given. The antisense UN-1 primer (5'-ATC GCA TTT CGC TAC GTT CTT CAT CG-3') was designed within a conserved region of the 5.8S rRNA gene among plants. Using the sense and antisense primer pair, PCR simulation was performed with Amplify 1.0 (Bill Engels) software¹⁶ to predict whether PCR products of the target size would be obtained from the plants listed in Table 1. The primers were synthesized and purified in reversed-phase columns by Qiagen (Japan) and used for the detection of buckwheat. In addition, Plant-1 primer (5'-CGA AAT CGG TAG ACG CTA CG-3') and Plant-2 primer (5'-GGG GAT AGA GGG ACT TGA AC-3'), designed to amplify a region of plant chloroplast DNA, were used to validate the quality of the template DNA.¹⁷

PCR. PCR to detect buckwheat DNA was performed in a final volume of 25 µl, containing 12.5 µl of 2× HotStarTaq Master Mix (HotStarTaq DNA Polymerase, PCR Buffer with 3 mM MgCl₂, and 400 µM of each dNTP; Qiagen), 0.2 µM of each primer (Fago-453 primer and UN-1 primer), 50 ng–500 ag of template DNA, and sterilized ultrapure water in 0.2-ml reaction tubes. Amplification was carried out with a GeneAmp PCR System 9600 (Applied Biosystems), using the following PCR program: pre-incubation at 95 °C for 15 min; 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; and final extension at 72 °C for 4 min. The PCR products were electrophoresed on 2% agarose gel containing ethidium bromide and analyzed with a FluorImager 595 (Amersham Biosciences Corp.). The DNA sequence of the PCR product from Shirahana soba (common buckwheat) was determined by the direct sequencing method and compared with the deposited sequences of the *Fagopyrum* spp.

To validate the quality of template DNA, PCR was performed by using 0.5 µM of each primer (Plant-1 primer and Plant-2 primer) and 50 ng of template DNA

Table 1. Specificity Prediction of the Fago-453 and UN-1 Primer Pair

Scientific Name of Plant (Common Name)		GenBank Accession No.	Amplicon Size (bp) ^a /Weight Number (W) ^b				
			W6	W5	W4	W3	W2
Genus <i>Fagopyrum</i> (Polygonaceae)	* <i>Fagopyrum esculentum</i> (common buckwheat)	AB000330	146 bp	—	—	—	—
	* <i>Fagopyrum esculentum</i> subsp. <i>ancestrale</i>	AB000331	146 bp	—	—	—	—
	* <i>Fagopyrum tataricum</i> (Tartarian buckwheat)	AB000339	146 bp	—	—	—	—
	* <i>Fagopyrum tataricum</i> subsp. <i>potanini</i>	AB000340	146 bp	—	—	—	—
	* <i>Fagopyrum callianthum</i>	AB000322	146 bp	—	439 bp	—	—
	* <i>Fagopyrum capillatum</i>	AB000323	146 bp	—	—	—	—
	* <i>Fagopyrum cymosum</i>	AB000324	146 bp	—	—	—	—
	* <i>Fagopyrum cymosum</i>	AB000325	146 bp	—	—	—	—
	* <i>Fagopyrum cymosum</i>	AB000326	146 bp	—	—	—	—
	* <i>Fagopyrum cymosum</i>	AB000327	146 bp	—	—	—	—
	* <i>Fagopyrum cymosum</i>	AB000328	146 bp	—	—	—	—
	* <i>Fagopyrum cymosum</i>	AB000329	146 bp	—	—	—	—
	* <i>Fagopyrum gracilipes</i>	AB000332	146 bp	—	—	—	—
	* <i>Fagopyrum homotropicum</i>	AB000333	146 bp	—	—	—	—
	* <i>Fagopyrum leptopodum</i>	AB000334	146 bp	—	—	—	—
	* <i>Fagopyrum lineare</i>	AB000335	—	146 bp	—	—	—
	* <i>Fagopyrum pleioramosum</i>	AB000336	146 bp	—	—	—	—
	* <i>Fagopyrum statice</i>	AB000337	146 bp	—	—	—	—
	* <i>Fagopyrum statice</i>	AB000338	146 bp	—	—	—	—
	* <i>Fagopyrum urophyllum</i>	AB000341	146 bp	—	—	—	—
	* <i>Fagopyrum urophyllum</i>	AB000342	146 bp	—	439 bp	—	—
Cultivated Plant Species Used for Food	<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> (white pepper)	AF275197	—	—	—	—	—
	<i>Sinapis alba</i> (mustard)	X15915	—	—	—	—	—
	<i>Prunus persica</i> (peach)	AF185621	—	—	—	—	—
	<i>Malus x domestica</i> (apple)	AF186484	—	—	—	—	—
	<i>Citrus</i> sp. (Valencia orange)	E08821	—	—	—	—	—

Continued on next page.

with the following program: pre-incubation at 95 °C for 15 min; 35 cycles consisting of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min; and final extension at 72 °C for 2 min. PCR products of the expected size were amplified from all DNA samples.

Real-time PCR. Real-time PCR to detect buckwheat DNA was performed in a final volume of 25 µl, containing 12.5 µl of 2× QuantiTect SYBR Green PCR Master Mix (HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, SYBR Green I, ROX, 5 mM MgCl₂, and dNTP mix including dUTP; Qiagen), 0.2 µM of each primer (Fago-453 primer and UN-1 primer), 50 ng of template DNA, and sterilized ultrapure water in a 96-well reaction plate. A six-dilution series of Shirahana soba (common buckwheat) DNA, containing 1–100,000 ppm diluted with wheat DNA, was used as template DNA. Amplification was carried out in an ABI PRISM 7700 (Applied Biosystems), using the following PCR program: pre-incubation at 50 °C for 2 min and 95 °C for 15 min; 40 cycles consisting of denaturation at 95 °C for 1 min, annealing at 66 °C for 2 min, extension

at 72 °C for 1 min, and data acquisition at 82 °C for 15 sec; and final extension at 72 °C for 4 min. Real-time PCR was performed in duplicate. Fluorescence data measured during the acquisition steps were analyzed, and a standard curve was calibrated by using the 6-dilution series of Shirahana soba (common buckwheat) DNA. The threshold line was drawn according to a previously published method concerning the quantification of genetically modified maize and soybean.¹⁸⁾

Results

Primer design

We designed a primer pair to amplify the region encompassing the boundary between the ITS-1 region and the 5.8S rRNA gene of all the *Fagopyrum* spp. registered in GenBank. The specificity prediction of this primer pair with the PCR simulation software is shown in Table 1. PCR products of 146 bp were predicted from the ITS-1 region and the 5.8S rRNA gene of all the *Fagopyrum* spp. registered in GenBank. Of the 27 *Fagopyrum*-related species, two of the Polygonaceae plants, genera *Fallopia* and *Aconogonum*, were also

Table 1. continued.

Scientific Name of Plant (Common Name)		GenBank Accession No.	Amplicon Size (bp) ^a /Weight Number (W) ^b				
			W6	W5	W4	W3	W2
<i>Fagopyrum</i> -related Species ^c	Family Polygonaceae	* <i>Aconogonum</i> sp. Won 152	—	149 bp	—	—	—
		* <i>Fallopia scandens</i>	—	145 bp	—	—	—
		<i>Polygonum virginianum</i>	—	—	—	—	—
		<i>Rumex acetosella</i>	—	—	—	—	—
	Others	<i>Talinum paraguayense</i>	—	—	—	—	—
		<i>Bruinsmia styracoides</i>	—	—	—	—	—
		<i>Talinella pachypoda</i>	—	—	—	—	—
		<i>Rehderodendron kwangtungense</i>	—	—	—	—	—
		<i>Pterostyrax corymbosus</i>	—	—	—	—	—
		<i>Anredera cordifolia</i>	—	—	—	—	—
		<i>Cistanthe quadripetala</i>	—	—	—	—	—
		<i>Xenia vulcanensis</i>	—	—	—	—	—
		<i>Talinopsis frutescens</i>	—	—	—	—	—
		<i>Talinaria palmeri</i>	—	—	—	—	—
		<i>Portulaca</i> sp.	—	—	—	—	—
		<i>Phemeranthus confertiflorus</i>	—	—	—	—	—
		<i>Montiopsis umbellata</i>	—	—	—	—	—
		<i>Grahamia bracteata</i>	—	—	—	—	—
		<i>Herniaria glabra</i>	—	—	—	—	—
		<i>Alluaudia dumosa</i>	—	—	—	—	—
		<i>Sinojackia xylocarpa</i>	—	—	—	—	—
		<i>Halesia macgregori</i>	—	—	—	—	—
		<i>Changiosyrax dolichocarpa</i>	—	—	—	—	—
		<i>Alectryon subdentatus</i>	—	—	—	—	—
		<i>Anacampseros recurvata</i>	—	—	—	—	—
		<i>Weinmannia racemosa</i>	—	—	—	—	—
		<i>Bursera tecomaca</i>	—	—	—	—	—

The size of PCR products from the ITS-1 and ITS-2 regions, and the 5.8S rRNA gene of plants was predicted by using the PCR simulation software, Amplify 1.0.

*indicates the plant species predicted to yield PCR products of approximately 146 bp.

^aThe amplicon sizes reported in Table 1 are 2 bp shorter than those predicted by Amplify 1.0 which takes into account the terminal transferase activity shown by most thermostable DNA polymerases.

^bAn approximate guide to the quality of the matches and strength of the amplifications. The larger the weight number, the higher the probability of amplification.

^cPlant species with ITS-1 sequences homologous to *F. esculentum*.

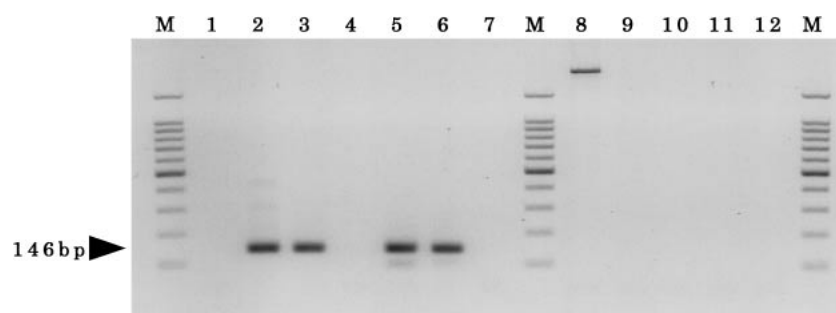


Fig. 1. Specificity of the PCR Method.

PCR was performed by using the Fago-453 primer and UN-1 primer. The arrowhead indicates the expected PCR product. Lane 1, negative control (without DNA); lanes 2–4, amplification of 50 ng (lane 2), 5 fg (lane 3), and 500 ag (lane 4) of Shirahana soba (common buckwheat) genomic DNA; lanes 5–7, amplification of 50 ng (lane 5), 5 fg (lane 6), and 500 ag (lane 7) of Dattan soba (Tartarian buckwheat) genomic DNA; lanes 8–12, amplification of 50 ng of wheat (lane 8), mustard (lane 9), soybean (lane 10), maize (lane 11), and white pepper (lane 12) genomic DNA; M, 100 bp ladder size standard.

predicted to yield PCR products of approximately 146 bp. The amplification products derived from these plants could be distinguished from those derived from the *Fagopyrum* spp. by DNA sequencing. No products were predicted in the other known cultivated plant species used for food.

Specificity

The specificity of this method was confirmed by PCR, and the result is shown in Fig. 1. Five femtograms of Shirahana soba (common buckwheat) and Dattan soba (Tartarian buckwheat) DNA was successfully used to amplify PCR products of 146 bp. The nucleotide sequence analysis of the PCR product obtained from

Shirahana soba (common buckwheat) confirmed that the intended sequence of the ITS-1 region–5.8S rRNA gene of the *Fagopyrum* spp. had been amplified (data not shown). Although a product of approximately 146 bp was amplified from *Fallopia convolvulus* DNA, analysis of its sequence proved that it originated from *Fallopia convolvulus* and not from buckwheat (data not shown). With this exception, no amplification of a 146 bp product occurred in plants other than buckwheat. Wheat DNA yielded a non-specific product of approximately 2,000 bp, which clearly differed in size to the target.

Sensitivity

The occurrence of a non-specific product in wheat DNA concerned us to the extent that this product might affect the sensitivity of buckwheat DNA detection when analyzing samples containing wheat. Therefore, the sensitivity of the method was confirmed in the presence of wheat DNA (Fig. 2). A PCR product of the target size was detected when 5 fg of Shirahana soba (common buckwheat) DNA was intentionally added to 50 ng of wheat DNA. Although a non-specific product from wheat DNA was obtained, it was confirmed that the product did not affect the sensitivity of buckwheat DNA detection. Five–fifty femtograms of buckwheat DNA in 50 ng of wheat DNA converts to 0.1–1 ppm (w/w). This level of sensitivity attained in the presence of wheat DNA may indicate that almost equal sensitivity could be secured for other samples.

Application to quantitative analysis

SYBR Green real-time PCR, using the ITS-1 region–5.8S rRNA gene as a target, was performed to determine whether quantitative PCR would be possible in the future. The amplification plot of fluorescence from

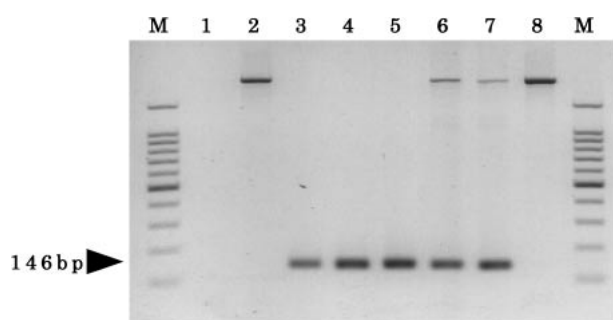


Fig. 2. Sensitivity of the PCR Method.

PCR was performed by using the Fago-453 primer and UN-1 primer. The arrowhead indicates the expected PCR product. Lane 1, negative control (without DNA); lane 2, amplification of 50 ng of wheat genomic DNA; lanes 3–4, amplification of 50 fg (lane 3) and 5 fg (lane 4) of Shirahana soba (common buckwheat) genomic DNA; lanes 5–8, amplification of 50 ng of wheat genomic DNA containing 500 fg (lane 5), 50 fg (lane 6), 5 fg (lane 7), and 500 ag (lane 8) of Shirahana soba (common buckwheat) genomic DNA, corresponding to 10 ppm (lane 5), 1 ppm (lane 6), 100 ppb (lane 7), and 10 ppb (lane 8) as Shirahana soba (common buckwheat) genomic DNA concentration; M, 100 bp ladder size standard.

Shirahana soba (common buckwheat) DNA in the presence of wheat DNA, and the calibration curve are shown in Fig. 3. The correlation coefficient for the calibration curve was higher than 0.99 for the presence of buckwheat DNA of between 1–100,000 ppm (w/w). The curve was highly linear and the slope was theoretically appropriate. The melting curve of the PCR product was a single luminescent peak at the melting temperature of approximately 85.5 °C, and an electrophoretic analysis confirmed no non-specific products to be present (data not shown). It was judged from these results that the luminescence of the aforementioned amplification plot was from the target product.

Discussion

Labeling for food allergens is required in many countries. In the United States, the food industry and consumer associations have jointly prepared voluntary guidelines concerning food allergens. The U. S. Food and Drug Administration (FDA) in the United States exercises regulation to secure food safety, and the following foods are currently considered to be allergenic: peanuts, soybeans, milk, eggs, fish, Crustacea, tree nuts, and wheat.¹⁹⁾ New legislation, the Food Allergen Labeling and Consumer Protection Act (FALCPA),²⁰⁾ was adopted by the US Government in August of 2004 and will go into effect on 1 January 2006. The EU regulation was revised in 2003, making it compulsory to declare celery, cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk and dairy products, mustard, tree-nuts, sesame seeds, and sulphite at concentrations of at least 10 mg/kg.²¹⁾ In Japan, the Ministry of Health, Labour and Welfare made it mandatory to declare five food items (buckwheat, peanuts, wheat, eggs, and milk) and notified their recommendation to declare another 19 items.^{8–10)} In order to provide patients with accurate information on allergens and to prevent food allergy onset through labeling systems, sensitive and specific detection methods are required.

The detection methods presently available for allergenic plants are quantitative ELISA,^{22–26)} qualitative immunoassay,²⁷⁾ qualitative PCR,^{26,28–31)} and qualitative PCR-ELISA.³²⁾ Although these methods can detect the cultivated plant species used for food, it is hard to evaluate whether they can detect wild species of the corresponding genus until each organism has been individually analyzed. Nair and Adachi¹³⁾ have examined the presence of 22 kDa protein known as an allergen of common buckwheat (*F. esculentum*)³³⁾ in 14 different *Fagopyrum* spp. They found the 22 kDa protein in 12 *Fagopyrum* spp., including both cultivated and wild species. In addition, Wang *et al.*¹⁴⁾ have suggested that a 24 kDa protein, which was considered to be allergenic in common buckwheat (*F. esculentum*),³⁴⁾ was also present in Tartarian buckwheat (*F. tataricum*). Since some of the allergenic proteins are likely to be

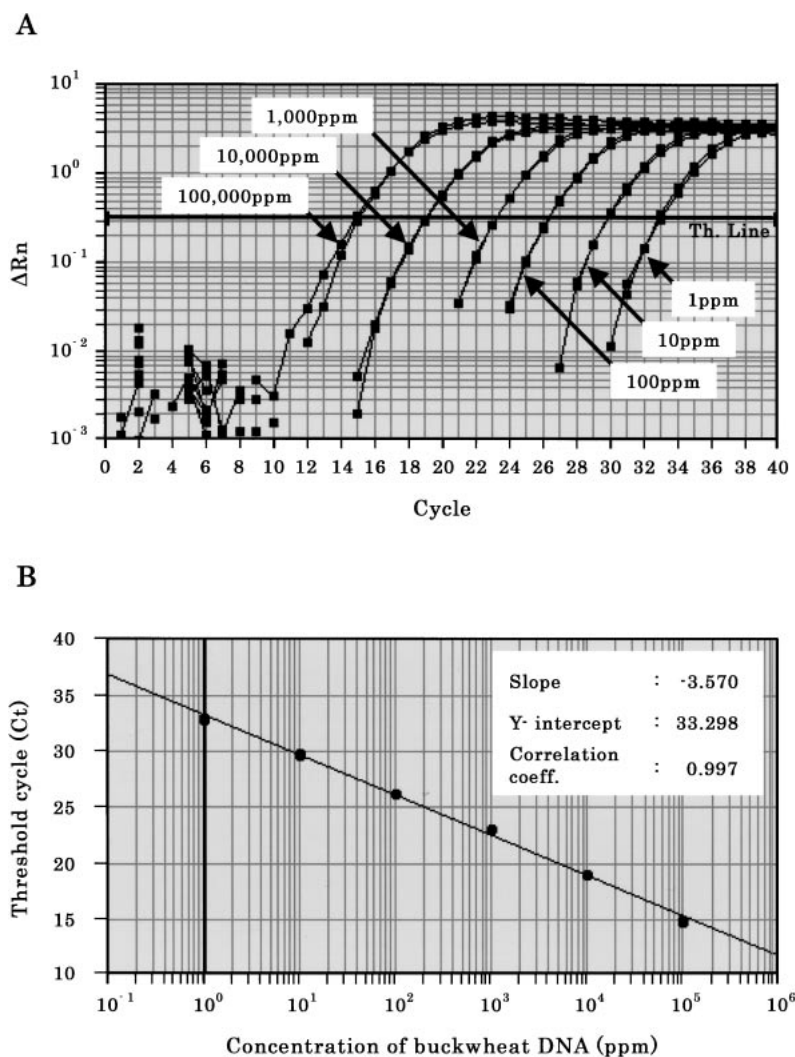


Fig. 3. SYBR Green Real-Time PCR for Serial Dilution of Buckwheat DNA.

(A) Amplification plots of SYBR Green real-time PCR, using the Fago-453 primer and UN-1 primer. Arrows indicate the amplification plots generated by six concentrations of Shirahana soba (common buckwheat) genomic DNA (1, 10, 100, 1,000, 10,000, and 100,000 ppm) diluted with wheat genomic DNA. The total amount of each template DNA was 50 ng. The horizontal line (Th. line) indicates the adopted threshold line. (B) Standard curve for SYBR Green real-time PCR, using the Fago-453 primer and UN-1 primer. The curve was generated from the amplification plots shown in (A).

distributed among the genus *Fagopyrum*, it would be safer to suspect allergenicity if any *Fagopyrum* spp. were found in food. Therefore, a method to detect all the *Fagopyrum* spp., comprising both cultivated and wild buckwheat, is very important when considering the safety of allergic patients.

The method established here has two major advantages over other methods for detecting buckwheat: its high sensitivity, and well-controlled and high specificity. The ITS-1 and ITS-2 regions intervening in the ribosomal RNA gene have been reported as sequences with a large number of copies³⁵⁾ in a wide range of plant species. These regions are known to be characteristic of species and are used in the classification of species.^{11,36)} Accordingly, this region is useful in developing highly sensitive and genus-specific PCR. Indeed, a similar method for detecting peanut, which employs the same

region as a target for PCR, has been also developed (manuscript in preparation). In respect of the sensitivity, this method enabled us to detect 1 ppm of buckwheat DNA present in wheat DNA. This level of sensitivity is sufficiently high and much higher than that of an existing qualitative PCR method²⁸⁾ which could detect 50 ppm of buckwheat DNA present in wheat DNA (Yamakawa, H., personal communication). In respect of the specificity, we chose primer sequences common but characteristic to the ITS regions of the *Fagopyrum* spp. This made it possible to easily predict whether our method could detect wild buckwheat, and it was proved that it could detect all the *Fagopyrum* spp., including both cultivated and wild buckwheat whose ITS-1 region and 5.8S rRNA gene were registered in GenBank. The existing PCR method to detect one of an allergenic protein gene of buckwheat²⁸⁾ is more specific than our

method, since it does not give any PCR products from *Fallopia convolvulus* (Yamakawa, H., personal communication), but it is unknown whether it can detect wild buckwheat because the corresponding target gene has not been widely reported in wild buckwheat. Although our method gave a PCR product from *Fallopia convolvulus*, the product can be distinguished from *Fagopyrum* spp. by its nucleotide sequence. There are also two test kits for buckwheat commercially available based on ELISA.^{22,23)} It is generally difficult to discriminate between true and false positives when using ELISA due to antibody cross reactivity. But PCR could accurately discriminate between true and false positives by nucleotide sequencing.

Actual food samples are made up of diverse organic materials of plant and animal origins, as well as of some inorganics, and may contain substances that would interfere with DNA extraction or inhibit *Taq* DNA polymerase activity. In some cooked foods, heating conditions may affect the integrity of DNA extracted from such foods. Thus, the DNA yield and amplification efficiency of extracts used for PCR often vary from food to food, or from sample to sample. An internal standard, which can be added to those food samples prior to DNA extraction, should help us to compensate for the variabilities by providing reference data for the DNA yield and amplification efficiency of those samples. Such an internal standard would preferably be selected from materials that can be easily obtained and are applicable to the widest possible varieties of food. The results of our preliminary trials indicate that quantitative determination of buckwheat in food samples, raw or cooked, is feasible by using a novel internal standard and by making minor modifications to the PCR protocols described in this paper (manuscript in preparation).

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