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# A Duplex PCR Assay for Differentiating Native Common Buckwheat and Tartarian Buckwheat, and Its Application for the Rapid Detection of Buckwheat Ingredients in Food

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**Abstract** One of the major allergenic proteins in common buckwheat (*Fagopyrum elculentum*) was found to be a BW10KD. In this work, allergenic BW10KD genomic DNAs from the native common buckwheat 'Pyeongchang' and Tartarian buckwheat 'Clfa47' were cloned by polymerase chain reaction (PCR), and their nucleotide sequences were determined. In addition, a novel PCR assay targeting the allergenic BW10KD gene was developed to detect and differentiate both buckwheat species in food. The nucleotide sequences of the BW10KD genomic DNA from 'Pyeongchang' and 'Clfa47' were 94% identical. Base differences in the nucleotide sequences of the BW10KD genes are probably useful as a molecular marker for species-specific identification. The 'Pyeongchang'-specific primer set 154PF/400PR and the 'Clfa47'-specific primer set 154DF/253DR generated 247 and 100 bp fragments in singleplex PCR, respectively. A duplex PCR assay with 2 species-specific primer sets simultaneously differentiated the 'Pyeongchang' and 'Clfa47' in a single reaction. The PCR assay also successfully allowed for the rapid detection of buckwheat ingredients in foods.

**Keywords:** common buckwheat, Tartarian buckwheat, food allergen, BW10KD, polymerase chain reaction (PCR)

## Introduction

Buckwheat is an important agricultural crop of the world. Currently, the most common buckwheat species is *Fagopynum esculentum* (common buckwheat); while some *Fagopynum tataricum* (Tartarian buckwheat) is cultivated in mountainous regions (1). Buckwheat seed tastes harsh for common buckwheat and slight bitter for Tartarian buckwheat (1). The flour of buckwheat seeds is an important food material for the production of buckwheat noodles, jelly, and dumpling in Korea. Buckwheat products are popular as health foods based on their high protein content, abundance of vitamin $B_1$, the flavonoid rutin, dietary fiber, and low fat content (2).

However, serious allergic reactions following the ingestion of buckwheat have been reported in Korea, Japan, and other countries (3-5). Several allergens with different molecular weights have been reported in common buckwheat (6-8). A buckwheat protein (BW10KD) is a member of the 2S-albumin multigene family and is considered one of the major allergens in common buckwheat (6). However, the presence of allergenic BW10KD protein in Tartarian buckwheat has not yet been reported.

The most common reported allergic reactions among sensitized children in Japan were urticaria, wheezing, and anaphylactic shock (5). Even trace amounts of allergens in foods may cause serious allergic symptoms in highly sensitive persons (9). These allergic individuals have to strictly avoid the consumption of the allergenic food to prevent possible life-threatening reactions (10). The Korea Food and Drug Administration stated that the most important food allergens such as buckwheat, eggs, milk, peanuts, soybean, wheat, mackerel, crab, pork, tomato, and peach must be declared on the food label. Although allergenic compounds must be specified on the labels of processed foods, unintentional contamination with these compounds may occur during harvesting, transportation, storage, and food processing procedures (11). Therefore, the development of an accurate, rapid, and highly sensitive detection method for the identification of hidden allergens in processed foods is required for food safety. Currently, 2 rapid detection methods that are increasing in importance are immunoassays for the detection of allergenic proteins, and polymerase chain reaction (PCR) assays for the detection of DNA from the allergen species of interest (12-15).

In this study, BW10KD genomic DNAs from the native common buckwheat 'Pyeongchang' and Tartarian buckwheat 'Clfa47', both buckwheats are consumed in Korea, were cloned and sequenced. We also developed a duplex PCR method for differentiating both buckwheat species by the nucleotide sequence differences of their BW10KD genomic DNAs, as well as to rapidly detect the presence of allergenic buckwheat ingredients in processed food.

## Materials and Methods

**Buckwheat and other samples** The grains of 'Pyeongchang' and 'Clfa47' were obtained from the National Institute of Highland Agriculture. Soybean (*Glycine max*), barley (*Hordeum vulgare* var. *hexastichon*), red bean (*Phaseolus angularis*), black bean (*Castanospermum australe*), German millet (*Setaria italica*), African millet (*Sorghum bicolor*), and wheat (*Triticum aestivum*) flour were purchased from markets in Seoul, Korea.

**DNA isolation** The genomic DNA was purified from 0.1

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g of well-ground seeds using a DNeasy™ Plant DNA Extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The DNA concentration was determined using a spectrophotometer ($A_{260\ nm}$).

**Cloning of BW10KD genomic DNA** The BW10KD cDNA sequence (accession number: AB055892) of common buckwheat was obtained from the NCBI's GenBank database. Genomic DNAs of the 'Pyeongchang' and 'Clfa47' were amplified by high fidelity Taq DNA polymerase (Roche, Indianapolis, IN, USA) with the common buckwheat BW10KD cDNA-specific forward primer FE50F (5'-CATGAAGCTTTTCTTGATCC-3') and reverse primer FE555R (5'-CTTCCTCCCTCTCTAAAA GC-3') by PCR. The amplified 0.5 kb DNA fragment containing the BW10KD gene was inserted into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and transformed into the *Escherichia coli* host Mach1™-T1[R]. The plasmid was purified from a transformant grown in LB plate containing 0.01% ampicillin. The 4.4 kb recombinant plasmid pCR2.1-TOPO containing the BW10KD gene was isolated and confirmed by restriction fragment analysis and DNA sequencing. The resulting plasmids containing the BW10KD gene of 'Pyeongchang' and 'Clfa47' were named pBWPC and pBWDD, respectively. The genomic DNA sequences of the BW10KD gene of 'Pyeongchang' and 'Clfa47' have been deposited in the GenBank under accession numbers EF488807 and EF488808, respectively.

**PCR conditions** The PCR reactions for cloning and detection of BW10KD genes from buckwheats were performed under the same condition except DNA polymerase used. Ex-Taq DNA polymerase (Takara, Shiga, Japan) was used for measuring the sensitivity of PCR system. The PCR reaction was conducted in 25 μL volumes containing an appropriate amount of template DNA (50-100 ng), 20 pmol of each primer, 2.5 mM of dNTPs, 5 μL of 5×reaction buffer, 1 U of Taq DNA polymerase, and deionized water. The PCR reactions were run on an PCR Express thermocycler (Hybaid, Waltham, MA, USA) using the following program: 1 cycle of 3 min at 95°C, 35 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C, and finally 5 min at 72°C. Each reaction was conducted in triplicate. The temperature gradient PCR was performed at different annealing temperature ranging from 55 to 65.2°C to examine the specificity of the primer sets. Five μL of PCR product were loaded onto a 2% agarose gel containing ethidium bromide and visualized under UV illumination.

## Results and Discussion

**Nucleotide sequences of BW10KD genomic DNA** The buckwheat species primarily consumed in Korea are the native common buckwheat 'Pyeongchang' and the Tartarian buckwheat 'Clfa47'. The cDNA sequence of BW10KD gene from common buckwheat was available in GenBank; however, its genomic DNA sequence has not yet been reported. Also, the cDNA sequence of BW10KD didn't show high homology with any other DNA sequences registered in GenBank. Therefore, we cloned the allergenic BW10KD structural gene from both buckwheat genomic DNAs in order to develop a PCR assay for species-specific detection of allergenic buckwheat ingredients in food.

A 0.5 kb DNA fragment was obtained from the genomic DNA of the 2 buckwheat species, respectively, by PCR with the FE50F/FE555R primer set. Amplification of the same size of DNA product from both buckwheat species suggested that Tartarian buckwheat also contains allergenic BW10KD gene like as common buckwheat. The sequencing results revealed that both BW10KD genomic DNAs were composed of 402 base pairs without any introns. Alignment of the BW10KD nucleotide sequences showed that the sequences of common buckwheat and 'Pyeongchang' were exactly the same, except for one base difference (data not shown), while those of common buckwheat and 'Clfa47' were 94% identical (Fig. 1).

**Specificity of PCR assay** Currently, rapid detection methods based on the amplification of specific DNA fragments using PCR are increasingly used for detecting allergenic food ingredients (16-19). In the case of buckwheat, PCR amplification of the boundary region between the internal transcribed spacer (ITS) region and the 5.8S rRNA gene of *Fagopyrum* spp. was first developed for the detection of buckwheat ingredients in food (11). This PCR assay successfully amplified the specific DNA fragment from buckwheat, but not from 12 other plant species tested.

In this study, to detect buckwheat ingredients in food, we developed a PCR assay directly targeting genomic BW10KD DNA, a major allergenic protein gene in buckwheat. Twenty base pair differences between the BW10KD genes allowed for the selection of 2 pairs of oligonucleotide primers for the differentiation of the 'Pyeongchang' and 'Clfa47' (Fig. 1). The 'Pyeongchang'-specific primer set 154PF/400PR and the 'Clfa47'-specific primer set 154DF/253DR, generated 247 and 100 bp fragments, respectively, at various annealing temperatures ranging from 55 to 65.2°C in singleplex PCR (data not shown). To examine whether these species-specific primer sets could discriminate both buckwheat species, the genomic DNA of either 'Pyeongchang' or 'Clfa47' was amplified by duplex PCR with the mixture of 2 primer pairs at the same conditions. As shown in Fig. 2, each species-specific primer set successfully distinguished between cognate and non-cognate template DNA. The above gradient PCR results showed a temperature optimum of 55 to 62.6°C, thus, an annealing temperature of 55°C was used in next experiments. The ability of this PCR assay to correctly identify buckwheat from other cereals and beans was also assessed. We performed duplex PCR with 100 ng of purified DNA from buckwheat and 7 other beans and cereals, including red bean, black bean, German millet, African millet, wheat, barley, and soybean. The buckwheat-specific primer sets generated 247 and 100 bp amplicons from the mixture of 'Pyeongchang' and 'Clfa47' buckwheat DNA only (Fig. 3). This result shows that these 2 primer sets could be specific to buckwheat.

The specificity of these primer sets was utilized to detect the presence of allergenic buckwheat ingredients in foods, including buckwheat jelly, buckwheat flour that was purchased from a market, wheat noodles, brown rice noodles, *misugaru*, breakfast cereal-1, breakfast cereal-2,

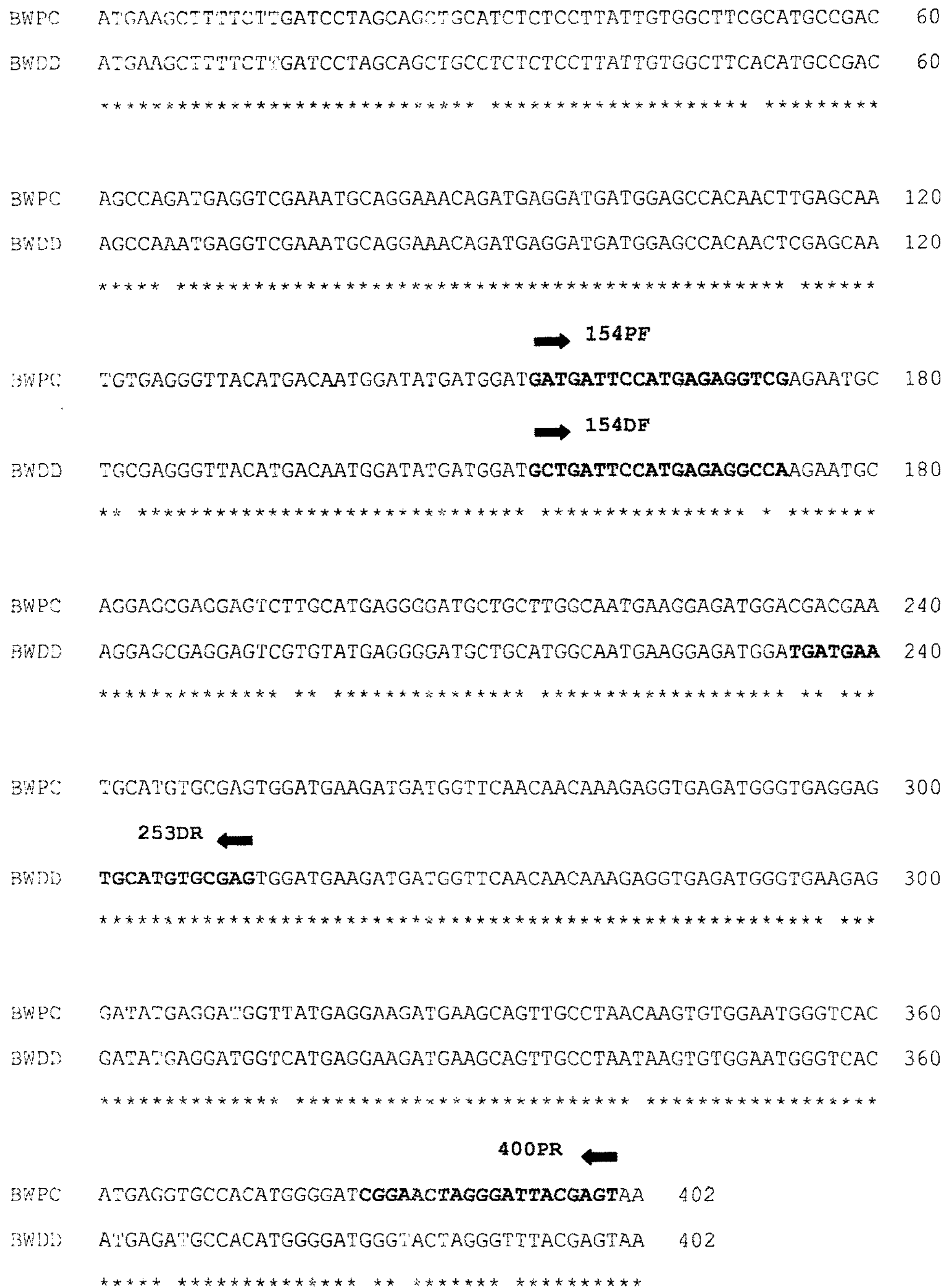


**Fig. 1. Alignment of the nucleotide sequences of buckwheat BW10KDs and positions of the primers used in this study.** BWPC, 'Pyeongchang'; BWDD, 'Clfa47'; 154PF and 400PR, primer set for 'Pyeongchang'; 154DF and 253DR, primer set for 'Clfa47'.

and black sesame gruels. These primer sets enabled PCR amplification of genomic BW10KD DNA from the buckwheat jelly and buckwheat flour, but not from the other processed foods we tested (Fig. 4). The generation of 2 DNA bands from buckwheat jelly and buckwheat flour indicates that both buckwheat products were made of 2 buckwheat species, 'Pyeongchang' and 'Clfa47'. This result suggests that the buckwheat-specific duplex PCR assay could be useful for detecting buckwheat ingredients in processed foods.

**Detection limits of buckwheat-specific duplex PCR** The detection of allergens in processed foods can be very difficult because they are often present in trace amounts, or are masked by the food matrix (20). Therefore, the development of accurate and highly sensitive detection methods is needed to reduce the incidence of buckwheat allergy.

To determine the detection limit of the duplex PCR assay, PCR was performed on increasing amounts of template DNA containing equal amount of 'Pyeongchang' and 'Clfa47' buckwheat DNA (0.01, 0.1, 1, 10, and 100 ng of each DNA per reaction). The results showed that buckwheat-specific duplex PCR was able to detect as little as 0.1 ng of purified template DNA (Fig. 5). The influence of non-specific background DNA on the detection limit

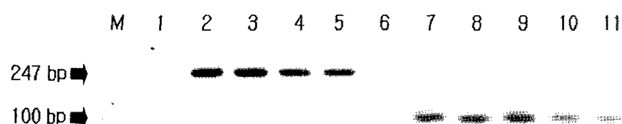


**Fig. 2. PCR amplification of the 'Pyeongchang' and 'Clfa47' buckwheat DNAs with the mixed primer sets (154PF/400PR and 154DF/253DR) at different annealing temperatures.** Lane M, 100 bp ladder; lane 1, no template control (60°C); lane 2 to 6, 'Pyeongchang' buckwheat DNA (55, 57, 60, 62.6, and 65.2°C); lane 7 to 11, 'Clfa47' buckwheat DNA (55, 57, 60, 62.6, and 65.2°C).

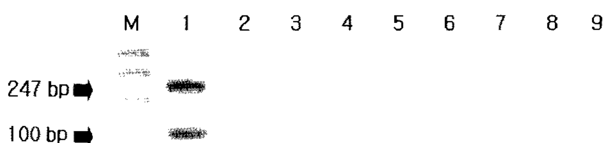


**Fig. 3. Specificity of the buckwheat-specific PCR assay.** Genomic DNA from various cereals and beans was amplified with the mixed primer sets. Lane M, 100 bp ladder; lane 1, mixture of 'Pyeongchang' and 'Clfa47' buckwheats; lane 2, red bean; lane 3, black bean; lane 4, German millet; lane 5, African millet; lane 6, wheat flour; lane 7, barley; lane 8, soy bean; lane 9, no template control.

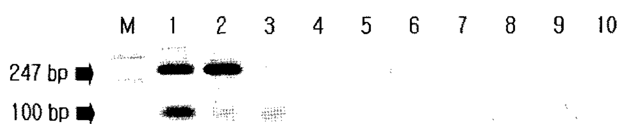


**Fig. 4. PCR amplification of the DNA extracts from various foods with the mixed primer sets.** Lane M, 100 bp ladder; lane 1, mixture of 'Pyeongchang' and 'Clfa47' buckwheat flours; lane 2, buckwheat flour (purchased from a market); lane 3, buckwheat jelly; lane 4, wheat noodles; lane 5, brown rice noodles; lane 6, *misugaru*; lane 7, breakfast cereal-1; lane 8, breakfast cereal-2; lane 9, black sesame gruels; lane 10, no template control.

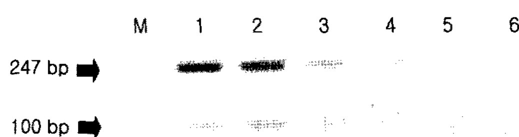


**Fig. 5. Detection limit of the duplex PCR.** Equivalent amounts of purified 'Pyeongchang' and 'Clfa47' DNA were mixed and used as a template DNA. Lane M, 100 bp ladder; lane 1, 100 ng; lane 2, 10 ng; lane 3, 1 ng; lane 4, 0.1 ng; lane 5, 0.01 ng; lane 6, no template control.

was also examined. Purified red bean DNA was mixed with buckwheat DNA before amplification. The concentration of non-target red bean DNA was held constant at 1 μg while increasing the amount of buckwheat DNA (0.01, 0.1, 1, 10, and 100 ng). There were no differences in the performance of the PCR regardless of the presence of non-specific DNA in the reaction mixture (data not shown). The detection limit of duplex PCR method in the presence of high concentration of non-target DNA corresponded to 100 ppm.

The first buckwheat-specific PCR method targeting the rRNA gene (11) was able to detect as little as 5 fg of common buckwheat DNA that had been intentionally mixed with 50 ng of wheat DNA (corresponding to 0.1 ppm). Considering that the average limit of detection of commercially available DNA-based test kits for allergen detection in food products was approximately 10 ppm (21), this sensitivity was extraordinarily high. This high level of sensitivity may originate from high copy numbers of the rRNA genes existing in many plants. In the case of maize, some lines have between 1,650-11,500 copies of rRNA genes per haploid genome (22). Although the detection limit of the buckwheat-specific duplex PCR method established here was not as high, our method was able to detect and discriminate the common buckwheat and Tartarian buckwheat ingredients in food products at the same time.

In summary, we determined and compared the nucleotide sequence of allergenic BW10KD genomic DNA from 'Pyeongchang' and 'Clfa47'. Based on the nucleotide sequence data, we have developed a buckwheat-specific duplex PCR method that allows for the discrimination of both species, and the identification of buckwheat ingredients in food. The buckwheat-specific duplex PCR assay could provide a valuable tool for the rapid detection of hidden buckwheat ingredients in processed foods.

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