Detection of Walnut Residues in Processed Foods by Polymerase Chain Reaction

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A sensitive qualitative detection method for walnut (Juglans regia) using polymerase chain reaction (PCR) was developed. For detection of walnuts with high specificity, the primer pair WAL-F/WAL-R was designed based on walnut matK genes. Trace amounts of walnuts in commercial food products can be qualitatively detected using this method.

Key words: allergen; walnut; pecan nut; PCR; restriction enzyme

Botanical food allergies, including allergy to walnuts (Juglans regia), represent a crucial and growing health problem in industrialized countries.1–4) In Japan, it has become clear from epidemiological investigations that the number of patients with walnut allergy has increased.5,6) The Ministry of Health of the Japanese government has enforced a labeling system for allergenic food materials since April 2002.5) In this system, labeling of five food products (egg, milk, wheat, buckwheat, and peanuts) is mandatory, while it is recommended for 20 other food materials, including walnuts. Therefore, the development of a rapid detection method for hidden walnut residues in foods is desirable. Recently, a specific detection method for walnuts using real-time PCR equipment was reported by European researchers.7) While the method is rapid, it also requires expensive real-time PCR instruments and specific probes. PCR methods for detecting wheat, peanuts, soybeans, and kiwifruit have already been established using conventional PCR and electrophoresis.8–12) In the present study, we developed a detection method for walnuts with sensitivity and specificity using conventional PCR. We show that the method established can be applied to processed food products, and that even trace amounts of walnut contained in a commercial food product can easily be detected by it.

Walnuts and samples of kiwifruit, apple, yam, banana, and soybean were also purchased by our laboratory. Eight kinds of nuts (almond, cashew, macadamia, pistachio, hazelnut, Brazil nut, pecan nut, and walnut) were kindly provided by Morinaga Institute of Biological Science, Inc. (Yokohama, Japan).

Genomic DNA was extracted from each plant material, including walnuts, using a silica-gel membrane-type kit (DNeasy Plant Mini; Qiagen, Hilden, Germany) according to the procedure described in a previous study, with some modifications.13) Genomic DNA was extracted from each walnut-derived food material and commercial food product using an anion exchange-type kit (Genomic-tip 20/G; Qiagen), according to the procedure described in a previous study.10) The extracted DNA was diluted with the appropriate volume of distilled water (DW) to a final concentration of 20 ng/μl, and stored at −20°C until needed. When the concentration of extracted DNA was less than 20 ng/μl, an undiluted DNA extract was used for the subsequent PCR analysis. The primers were synthesized and purified in a reversed-phase column by Operon Biotechnologies (Tokyo), diluted with the appropriate volume of DW to a final

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To detect walnuts specifically using PCR, we investigated gene sequences specific to walnuts. We designed primer pairs for various walnut-specific gene sequences, and examined PCR amplification using the designed primer pairs on walnuts and the other nut and food samples. We found that the walnut matK gene was the most specific for amplifying the gene sequence. Hence, we chose the gene encoding walnut matK as a walnut-specific gene, and designed primer pair WAL-F/WAL-R by referring to GeneBank accession no. AF118027. In addition, the primer pair CP 03-5’/CP 03-3’, for the universal detection of DNA derived from plants, was used to verify the extracted DNAs. The primer pair CP 03-5’/CP 03-3’ generated a 123-bp amplified fragment. The sequences of the designed oligonucleotides in this study are listed in Table 1.

As shown in Fig. 1, a fragment (120 bp) amplified using primer pair WAL-F/WAL-R was specifically detected in walnut genomic DNA. In contrast, no amplified fragment was detected when the DNAs extracted from other foods, such as kiwifruit and soybean, were used, as shown in Fig. 1B. In addition, eight other nut species (almond, cashew, macadamia, pistachio, hazelnut, brazil nut, pecan nut, and walnut) were used as template DNA (Fig. 1C and D). The results suggest that only the genomic DNAs from walnuts and pecan nuts are detected using the WAL-F/WAL-R primer pair. In addition, we confirmed that the detection limit of this PCR for both walnut and pecan nut was 0.5 pg (10 ppm equivalency) when we tested salmon genome DNA samples containing walnut DNA or pecan nut DNA (data not shown). However, according to the current Japanese article standard classification, pecan nuts are not classified as part of the walnut family, and hence, it is necessary to distinguish walnuts and pecan nuts. The matK gene, which is the target gene of this PCR, is known to exist in many edible kinds of walnut, but the gene sequence is unknown for Juglandaceae, Carya illinoensis, which is a food pecan nut. Hence, for the first time, we identified the gene sequence of matK for pecan nuts using a TA cloning technique. For insertion into the pCR®II-TOPO® vector (TOPO TA cloning® kit, Invitrogen Japan), a partial pecan nut matK gene was obtained by PCR amplification from pecan nut genome DNA with a sense primer (walnut-378F, 5’-CGG ACG AGA ATA AAG ATA GAG T-3’), antisense primer (walnut-1295R, 5’-GAT CTA TAT TGT TGG AAA ATG TAG C-3’), under the reaction conditions described above. The 1.0-kb PCR product was cloned into pCR®II-TOPO® vector by standard procedures, and its sequence was analyzed by Hokkaido System Science (Sapporo, Japan). The pecan nut matK gene of the 148 bp fragment, including a domain amplified using the walnut PCR method, is shown in Fig. 2A.

By sequence analysis, we found that the products amplified from walnut and pecan nuts both involve the restriction enzyme site AclI, and we further determined that the products amplified from the walnut matK gene but not from the pecan nut matK gene involve a restriction enzyme site, Bfa1 (Fig. 2A). Therefore, to determine whether Bfa1 enzyme digestion discriminates between walnuts and pecan nuts, we examined digestion by the restriction enzyme with an amplification product concentration of 50 μmol/l, and stored at −20 °C until needed.

The reaction mixture for PCR was prepared in a PCR reaction tube. The reaction volume of 25 μl contained 50 ng of genomic DNA, 0.2 mM dNTP, 1.5 mM MgCl₂, 0.2 μM of the 5’ and 3’ primers, and 0.625 units of AmpliTaQ Gold (Applied Biosystems, Foster City, CA), topped up with DW. When the concentration of the extracted DNA was less than 20 ng/μl, 2.5 μl of an undiluted DNA extract was added to a PCR reaction tube. The reaction was buffered with PCR buffer II (Applied Biosystems), and amplified in a thermal cycler (PTC-200 DNA Engine DYAD; Bio-Rad Laboratories, Hercules, CA). The PCR step-cycle condition was as follows: pre-incubation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 64 °C for 0.5 min, and extension at 72 °C for 0.5 min, followed by a final extension at 72 °C for 7 min. When the CP 03-5’/CP 03-3’ primer pair was used, the annealing temperature was changed to 60 °C. After PCR amplification, agarose gel electrophoresis of the PCR product was carried out following previous studies.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Specificity</th>
<th>Amplicon</th>
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<td>A:</td>
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<td></td>
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<tr>
<td>CP 03-5’</td>
<td>5’-CGG ACG AGA ATA AAG ATA GAG T-3’</td>
<td>Chloroplast DNA /sense</td>
<td>Plants</td>
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<tr>
<td>WAL-F</td>
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<td>Chloroplast maturase (matK) gene/sense</td>
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<td>Chloroplast maturase (matK) gene/antisense</td>
<td></td>
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A: For confirmation of validity of the DNA extracted from plants for polymerase chain reaction.
B: For specific detection of walnut.

Table 1. List of Designed Primers for Detection of Walnut
Fig. 1.  Specificity of the PCR Method Using Walnut Primers (WAL-F/WAL-R).

A and B, Amplification of DNA of various plants species with primers for plants (A) or walnuts (B). Genome DNAs were obtained from kiwifruit (lane 1), walnut (lane 2), apple (lane 3), yam (lane 4), banana (lane 5), and soybean (lane 6). NC is the no-template control. Amplicons were electrophoresed in a 1.5% agarose gel. M, 100-bp ladder size standard. C and D, Amplification of DNA of various nut species with primers for plants (C) or walnuts (D). Genome DNAs were obtained from almond (lane 1), cashew (lane 2), macadamia (lane 3), pistachio (lane 4), hazelnut (lane 5), Brazil nut (lane 6), pecan nut (lane 7), and walnut (lane 8). NC is the no-template control. Amplicons were electrophoresed in a 2.5% agarose gel (plant) or a 1.5% agarose gel (walnut). M, 100-bp ladder size standard.

Walnut :TTATGATAATAAATCTAGTTTACTGATTGTAAAACGTTTAATTACGCGAATGTATCAACAGAATCATTTG
Pecan nut:TTATGATAATAAATA
Hickory :TTATGACAATAAATCCCGTTTACTAATTGTAAAACGGTTAATTACTCGAATGTATCAACAGAATCATTTT

Fig. 2.  Comparison of matK Genes of Walnut, Pecan Nut and Hickory (A) and Electrophoresis Analysis in 2.5% Agarose Gels of the Products Obtained with Endonuclease (B).

Primer WAL-F and WAL-R are underlined. GenBank accession nos.: walnut, AF118036, AF118027; hickory, AF118039. B, Odd numbers, walnut; even numbers, pecan nut; lanes 1 and 2, no endonuclease control; lanes 3 and 4, digestion with Acl I; lanes 5 and 6, digestion with Bfa I; lanes 7 and 8, digestion with Acl I and Bfa I; M, 100-bp ladder size standard.
of walnut DNA and one of pecan nut DNA. As shown in Fig. 2B, we confirmed that the PCR products of the walnut matK gene were exactly digested with the restriction enzyme Bfa1 and were divided into two fragments while the PCR products of the pecan nut matK gene were not. These results suggest that Bfa1 enzyme digestion can discriminate between the PCR products of walnuts and pecan nuts.

To investigate the applicability of the walnut DNA detection method to commercial food products and food materials, we purchased 10 processed food samples and tested them for the presence of walnut DNA using our method. In two food product samples of mixed nuts and a bean-jam bun which included walnuts on the lists of ingredients, walnut DNA was clearly detected by the PCR method with the WAL-F/WAL-R primer pair. In the other eight processed food samples, such as snacks and cookies which did not include walnuts on the list of ingredients, no walnut DNA was detected using the PCR method with the WAL-F/WAL-R primer pair.

These results suggest that the proposed PCR method using the WAL-F/WAL-R primer pair is applicable in identifying walnuts in processed food products.

In conclusion, we developed a detection method for walnuts and pecan nuts using conventional PCR without expensive real-time PCR instruments. In addition, we discovered how restriction enzyme digestion of the PCR products can discriminate between walnuts and pecan nuts. To minimize the risk of false negative or positive results in the inspection for walnut contamination, we believe that the PCR method can be used to complement protein-based detection methods such as ELISA.

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References