Simultaneous detection of fruit allergen-coding genes in tomato, apple, peach and kiwi through multiplex PCR

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Abstract Fruit allergies have become more common in recent years, and are now a serious health problem. In this study, a multiplex PCR assay was used to detect potential fruit allergens causing food allergy labeling in Korea. For the detection of these allergens, specific primer pairs were designed to amplify the allergen-coding genes *Cyclophilin* (tomato), *Md11* (apple), *Pru p 2.01A* (peach) and *Pectin methylesterase inhibitor* (kiwi), and primer pair targeting the 18S ribosomal RNA gene was additionally used as an endogenous control. Primer specificity was assessed with 23 plant species. A mixture of DNA from the four fruits was serially diluted and used to determine the sensitivity of the multiplex PCR assay, which was approximately 0.08 ng. Eleven commercial fruit products were evaluated to verify the applicability of the multiplex PCR assay. This assay is expected to be a specific and efficient method for detecting fruit allergens in foods.

Keywords Food allergy · Fruit allergen · Multiplex PCR · Peach · Tomato · Apple · Kiwi

Introduction

Food allergies are occurred when the immune system recognizes a specific protein of food as an antigen and causes an excessive antigen–antibody reaction. These allergic reactions may cause cutaneous, gastrointestinal or respiratory symptoms, as well as angioedema or anaphylaxis (Son et al., 2002). The prevalence of food allergies has been increasing in the last 30 years, with 6–8% of children being affected worldwide (Prescott et al., 2013). The incidence of food allergies is higher infants and young children than adults because children have lower immunity (Wen et al., 2007).

However, some allergies such as oral allergy syndrome (OAS) caused by fruits and vegetables, have been observed in older children and adolescents (Fernandez-Rivas et al., 2008; Lee et al., 2016). Allergies caused by fruits and vegetables comprise 7.4% and 7% of all food allergies, respectively, and mainly result in OAS, but have also been reported to cause systemic symptoms in 8.7% and anaphylaxis in 1.7% (Christopher et al., 2010). Fruit allergies often occur in people over 5 years old, mainly due to fruits of the Rosaceae family (apples, peaches, etc.) (Fernandez-Rivas et al., 2008). Allergies caused Rosaceae family are known to cross-react with pollen allergies containing Actinidiaceae family, Solanaceae family etc. (Garcia and Lizaso, 2011; Kondo and Urisu, 2009). The only way to prevent allergic reactions is to avoid allergy-inducing ingredients. For this reason, allergy labeling systems have been adopted globally according to differences in dietary culture (Marchisotto et al., 2016; Park et al., 2013).

The Ministry of Food and Drug Safety of Korea has been implementing an allergy labeling system for the following 21 foods: eggs, milk, buckwheat, peanuts, soybeans, wheat, mackerel, crab, shrimp, pork, peaches,

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tomatoes, sulfites, walnuts, chicken, beef, squid and shellfish (clam, mussel, abalone, and oyster). This system is being expanded to include processed foods and foods sold in vulnerable open markets (e.g., school meals and fast foods) (Lee and Ryu, 2016). Recently, allergies to apples and kiwis have been reported for children in Korea, in addition to allergies to peaches and tomatoes, so it is highly likely that apples and kiwis will be included in food allergy labeling (Choi et al., 2017; Lee, 2017).

Enzyme-linked immunosorbent assays (ELISAs) to detect proteins and polymerase chain reactions (PCR) are the main methods used to determine whether allergens are present in foods. In the case of ELISA, proteins are extracted from the food, and the presence of a specific antigen is detected through an antigen–antibody reaction. ELISAs are advantageous in that they directly react with allergens in foods and are easy to use, but have the drawback of low sensitivity in processed foods due to protein denaturation (Park et al., 2013). PCR is a powerful technique for the detection of pathogens and GMOs (Kim et al., 2017; Song et al., 2017) and effective for processed foods because DNA is heat-stable rather than protein (Graziano et al., 2018; Reisch et al., 2015). A specific primer pair can be used to amplify the part of the DNA sequence encoding an allergen protein (Pafundo et al., 2010).

In a multiplex PCR assay, several primer pairs are used to detect multiple targets from a single reaction, thereby reducing the cost and time (Zhang, 2013). Due to its ability to detect several species that can cause cross-reactivity in allergic reactions simultaneously, multiplex PCR can be a useful tool for the simultaneous identification of four fruit allergens. Since the primers used for multiplex PCR compete with each other and may have different amplification efficiencies (Cheng et al., 2016), it is important to optimize multiplex PCR condition assay and verify the applicability of this assay in processed foods.

The aim of this study was to develop the multiplex PCR assay for the detection four fruit allergens in tomato, apple, peach, and kiwi and monitor these allergens in commercial food products.

Materials and methods

Samples

Tomato (*Solanum lycopersicum*), apple (*Malus domestica*), peach (*Prunus persica*) and kiwi (*Actinidia deliciosa*) were obtained from the National Institute of Horticultural and Herbal Science (NIHHS) in Korea. Nineteen non-target plant species, including wheat, soybean, buckwheat, pine nut, walnut, peanut, sesame, maize, rice, barley, potato, hot pepper, cherry, plum, pistachio, almond, cashew nut,

hazelnut and perilla, were purchased from a local market in Korea. All samples were ground in liquid nitrogen with a mortar and pestle, and were immediately stored at -20°C until DNA extraction. The applicability of this method to processed food products was evaluated using 11 commercial samples containing juice, dried fruit, and fruit powder, which were purchased at markets in Korea.

DNA extraction

One gram of each sample was used for DNA extraction. Wheat, soybean, buckwheat, corn, rice, barley, sesame and perilla samples were extracted with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Tomato, apple, peach, kiwi, cherry and plum samples were extracted with the cetyl trimethylammonium bromide (CTAB) method. The remaining samples were extracted with a modified Qiagen kit. For the modified Qiagen kit method, 500 μL of chloroform was added, the sample was centrifuged at 13,000 rpm for 15 min, and the supernatant was applied to a DNeasy Mini Spin Column. All processed foods samples were extracted with the CTAB method. Especially, the liquid samples were centrifuged to concentrate the solids and used to DNA extraction. The various extraction methods were used to obtain DNAs with good purity. The DNA concentration and purity were measured with a UV/VIS Spectrophotometer (Mecasys, Daejeon, Korea).

Primer design

Gene sequences of tomato, apple, peach and kiwi were obtained from GenBank and aligned with the Clustal Omega program to find the specific region of target species against different species. Specific primers for DNA sequences encoding allergy-inducing proteins were designed in the Primer Designer program version 3.0 (Scientific and Educational Software, Durham, NC, USA). Primer pairs Sola 5-F/R, Mal 2-F/R, Pru-F/R and Act 6-F/R were designed to amplify *Cyclophilin* from tomato, *Md1* from apple, *Pru p 2.01A* from peach and *Pectin methylesterase inhibitor* from kiwi, respectively. Oligonucleotides were synthesized by the Bionics Company (Bionics, Seoul, Korea). The primer sequences and information are shown in Table 1.

Sequencing of PCR products

PCR products amplified by tomato, apple, peach and kiwi primers were purified from a 1% agarose gel with a QIA-Quick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and cloned into a pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer's protocol. The

Table 1 Primers used in the multiplex PCR

Food name	Target gene	Primer name	Sequence (5'-3')	Product size (bp)	Concentration (nM)	Accession No.
Peach	<i>Pru p 2.01A</i>	Pru-F	GCAACCGGAATTAGCAAC	209	3200	EU424257.1
		Pru-R	AAATCTTGACCCCCGTTCTC			
Plant	<i>18S rRNA</i>	18S-F	CGAAAGCATTTGCCAAGGAT	172	200	AJ272181.1
		18S-R	CCGGAACCCAAAGACTTTGA			
Tomato	<i>Cyclophilin</i>	Sola 5-F	GGAGCCAAATTCAACGATG	146	400	M55019.1
		Sola 5-R	ACGACGTGCTTTCCGTTGA			
Kiwi	<i>Pectin methylesterase inhibitor</i>	Act 6-F	AAATCTGTCCCAAACTCGC	127	400	AB091088.1
		Act 6-R	TTAGCACTGGCCTGAGCTAT			
Apple	<i>Md11</i>	Mal 2-F	CTTGCCCTTGCCTTTGGTGAT	105	400	AF090143
		Mal 2-R	GGCACTGCTTCTCAAAGATCTCA			

^aF: forward primer, R: reverse primer

^bAccession numbers were collected from Genbank of the NCBI (<http://www.ncbi.nlm.nih.gov>)

cloned plasmids were isolated from bacterial cultures with a DNA-spinTM Plasmid DNA Purification Kit (Intron Biotechnology, Seongnam, Korea) and confirmed by sequence analysis on an ABI PRISM 3700 DNA analyzer (Perkin Elmer, Boston, MA, USA) at GenoTech (Daejeon, Korea). The results were checked by a BLAST search of the NCBI database.

Single PCR and multiplex PCR

Single PCR amplifications were performed in a final volume of 25 µL, containing 2.5 µL of 10X Ex *Taq* buffer, 200 µM of dNTP mix, 0.5 U of Ex *Taq* polymerase (TaKaRa, Shiga, Japan), 400 nM forward and reverse primers, and 50 ng of template DNA. Amplification was performed in a thermal cycler (PC808; ASTEC, Kyoto, Japan) under the following conditions: pre-incubation for 5 min at 94 °C; 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 62 °C, and extension for 30 s at

72 °C; and a final extension for 7 min at 72 °C. A no-template control was used as a negative control. The amplified PCR products were analyzed on a 2% agarose gel in 0.5X Tris–acetate-EDTA buffer stained with 0.5 mg/mL ethidium bromide.

For multiplex PCR, the primer concentration and annealing temperature were optimized. The information of the five primer pairs are shown in Table 1. Other conditions were similar to those of the single PCR assay, except that annealing was performed for 20 s at 62 °C.

Results and discussion

Specificity tests for the single PCR assays

All the fruit primers were designed to amplify products below about ~ 200 bp for effective application to processed foods (Mafra et al., 2008). Primers for 18S rRNA

Fig. 1 Specificity of the designed primer pairs for fruit allergen genes in single PCR. Lane M, 100-bp DNA ladder; lanes 1–23, wheat, soybean, buckwheat, tomato, apple, peach, kiwi, pine nut, walnut,

peanut, sesame, maize, rice, barley, potato, hot pepper, cherry, plum, pistachio, almond, cashew nut, hazelnut, perilla; lane N, no template

Fig. 2 Sensitivity of the single PCR assay with genomic DNA as a template. Lane M, 100-bp DNA ladder; lanes 1–6, positive gDNA 50, 5, 0.5, 0.05, 0.005, 0.0005 ng; lane N, no template

were used as an internal control to confirm the failure of the PCR (Kim and Kim, 2017; Pafundo et al., 2011). The specificity of each primer pair was confirmed by single PCR. The fruit (tomato, apple, peach and kiwi) primer pairs exhibited no cross-reactivity in 23 plant species, including wheat, soybean, buckwheat, tomato, apple, peach, kiwi, pine nut, walnut, peanut, sesame, maize, rice, barley, potato, hot pepper, cherry, plum, pistachio, almond, cashew nut, hazelnut and perilla (Fig. 1). The results of the sequencing matched the expected product sizes for the tomato, apple, peach, kiwi and endogenous control specific primer pairs (146, 105, 209, 127 and 172 bp, respectively).

In addition, as a test of the sensitivity of each primer pair, DNA from each target species was serially diluted in tenfold from 50 to 0.0005 ng. The sensitivities of tomato, apple and peach primers were 0.005 ng, while that of kiwi primers was 0.05 ng (Fig. 2). These results are similar to

Table 2 Analysis of 11 commercial food products using the developed multiplex PCR assay

Declared allergenic ingredients	Food product	Test results
Tomato	Fruit juice	Tomato
	Dried fruit	Tomato
Apple	Fruit juice	Apple
	Dried fruit	Apple
	Dried fruit	Apple
	Fruit powder	Apple
Peach	Fruit juice	Peach
	Dried fruit	Peach
Kiwi	Fruit juice	Kiwi
Kiwi	Dried fruit	Kiwi
Kiwi	Dried fruit	Kiwi

those of previously reported allergen detection methods using gene analysis. Han et al. (2012) reported detection limits of 1–10 pg for 7 fruits, and Shang et al. (2014) reported a detection limit of 5 pg for peach. In other allergen-containing foods, Garino et al. (2016) reported a detection limit of 1 pg for pine nut, and Linacero et al. (2016) reported a detection limit of 2.5 pg for walnut.

Specificity and sensitivity test of the multiplex PCR assay

We developed a multiplex PCR assay to simultaneously detect DNA from tomato, apple, peach and kiwi fruits, taking into account the PCR product size, annealing temperature and cross-reactivity of the primer pairs. The

Fig. 3 Specificity of the multiplex PCR assay. Lane M, 100-bp DNA ladder; lanes 1–23, wheat, soybean, buckwheat, tomato, apple, peach, kiwi, pine nut, walnut, peanut, sesame, maize, rice, barley, potato, hot

pepper, cherry, plum, pistachio, almond, cashew nut, hazelnut, perilla; lane N, no template

Fig. 4 Sensitivity of the multiplex PCR assay with gDNA as a template. Lane M, 100-bp DNA ladder; lanes 1–7, positive gDNA 50, 10, 2, 0.4, 0.08, 0.016, 0.0032 ng; lane N, no template

Fig. 5 Analysis of 8 commercial food products using the developed pentaplex PCR assay. Lane M, 100 bp DNA ladder; lane 1, positive control; lanes 2–3, tomato processed foods; lanes 4–7, apple

processed foods; lanes 8–9, peach processed foods; lanes 10–12, Kiwi processed foods; lane N, no template

optimized conditions were used to confirm the specificity of the multiplex PCR. In the multiplex PCR assay, tomato, apple, peach and kiwi were amplified specifically at 146, 105, 209 and 127 bp, respectively, and there was no cross-reactivity in 23 plant species, including wheat, soybean, buckwheat, tomato, apple, peach, kiwi, pine nut, walnut, peanut, sesame, maize, rice, barley, potato, hot pepper, cherry, plum, pistachio, almond, cashew nut, hazelnut and perilla (Fig. 3). The primer pair for 18S rRNA used in the reaction as a positive control amplified its target sequence in all 23 food species with a product size of 172 bp.

A sensitivity test for the multiplex PCR was performed with a mixture of genomic DNA from tomato, apple, peach and kiwi. For this test, the DNA mixture was serially diluted in fivefold from 10 to 0.0032 ng. The sensitivity of the multiplex PCR assay was approximately 0.08 ng (Fig. 4).

The sensitivity of our multiplex PCR assay was comparable with allergen reported previously. Hubalkova and Rencova (2011) reported detection limits of 100 pg for pecan and Brazil nut allergy. Zhang et al. (2018) reported detection limits of 10–100 pg for peanut, soybean and sesame allergy. The sensitivity of our assay has better than or similar to the sensitivities and diagnostic efficiencies described in previous reports. Although the reaction limit is unknown because of different sensitivity rate of allergy every peoples, it is used to be sufficient to detect allergens that cause allergies.

The multiplex PCR assay developed in this study was applied to 11 commercial food samples (4 juices, 6 dried fruits and 1 fruit powder) (Table 2). As a result, the targets (tomato, apple, peach, and kiwi) including 18S rRNA were amplified in all the samples used (Fig. 5).

As food allergens, tomato, apple, peach and kiwi fruits are a growing concern for many people, and their allergenicity is an emerging problem. Several countries, including Korea, have implemented allergy labels for these fruits. No previous reports have described the simultaneous detection of fruit allergens from tomato, apple, peach and kiwi through multiplex PCR in processed fruit products. Therefore, the multiplex PCR method developed in this study could be a useful tool for the detection of these four fruit allergens in various commercial fruit products.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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