

Detection of GM Papaya Event 55-1 in Fresh and Processed Papaya Using Duplex PCR

Sun-A Kim¹, Mikyoung Lee¹, Sang-Jin Yoo¹, Jae-Hwan Kim¹, Hyungjae Lee¹, Kyoung Sik Park², Jayoung Jeong², and Hae-Yeong Kim^{1*}

¹Institute of Life Sciences & Resources and Graduate School of Biotechnology, Kyung Hee University, Yongin 446-701, Republic of Korea

²Nutrition & Functional Food Research Team, National Institute of Food and Drug Safety Evaluation, Seoul 122-704, Republic of Korea

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Duplex PCR analysis was developed to detect a genetically modified (GM) papaya event 55-1 in both fresh and processed papaya fruit. GM papaya event 55-1 is a genetically modified organism (GMO) not currently approved for food in Korea. Using a primer set specific to *papain*, an endogenous papaya gene, we obtained a PCR product only from papaya genomic DNA, thus defining the primer's specificity. In another qualitative PCR, we used an event-specific primer pair to amplify and distinguish the target region in GM papaya event 55-1 among fourteen different GM events. Combining the two primer pairs, we developed a duplex PCR and used it to detect GM papaya event 55-1 in eleven fresh and five processed food samples. This new duplex PCR method meets the need to monitor GM papaya event 55-1 in both fresh papaya and processed foods, such as dried or canned papaya.

Key words: duplex PCR, papaya, unapproved GMO

Papaya (*Carica papaya* L.), a tropical or subtropical fruit, contains high levels of vitamin A and C [Xu *et al.*, 2008], as well as papain, an enzyme similar to pepsin that may aid in digestion. Most countries that produce papaya suffer heavy economic losses to papaya ringspot virus (PRSV). Spread by aphids, this infection inhibits photosynthesis and, consequently, plant growth, which reduces fruit quality and quantity [Wall *et al.*, 2004]. To control the disease, PRSV resistance genes are introduced into the plant. Genetically modified (GM) varieties, such as 55-1 and 63-1, have enabled many farmers to recover and increase their papaya production. The United States and Canada have approved these genetically modified organisms (GMOs) [Suzuki *et al.*, 2008], and introduction of the GM papaya event 55-1 variety has played a large role in the recovery of Hawaii as a major papaya-producing area [Wall *et al.*, 2004; Suzuki *et al.*, 2008].

As GM crop cultivation increases, concern rises in Korea and other countries for the safety of GMOs. While

each country implements labeling regulations, technology to detect GMOs develops in parallel [Iida *et al.*, 2005; Yang *et al.*, 2005a; Yang *et al.*, 2005b; Michelini *et al.*, 2008; Oguchi *et al.*, 2008; Shrestha *et al.*, 2008; Mano *et al.*, 2009]. Korean demands for papaya have increased sharply, which has led to an increase in papaya imports from one ton in 2003 to 184 tons in 2007. Korea has not yet approved GM papaya, yet no detection method has been developed for GM papaya that might be possibly mixed with non-GM papaya during importation or distribution. An effective monitoring method is clearly required in Korea to meet growing concerns for GMOs. Canada, China, Japan, and Taiwan have all reported methods to detect GM events in papaya [Davis *et al.*, 2004; Wall *et al.*, 2004; Yamaguchi *et al.*, 2006; Ming *et al.*, 2008; Suzuki *et al.*, 2008; Xu *et al.*, 2008; Fan *et al.*, 2009; Guo *et al.*, 2009]. However, these methods are limited to detection of GM events in the fresh fruit, whereas Korea imports mostly processed fruit. Thus arises the need for a GM detection method applicable to both fresh and processed papaya.

PCR is among the most widely used technique for the detection of GM crops and their food products. Especially, an event-specific detection method is favored because it

*Corresponding author

Phone: +82-31-201-2660; Fax: +82-31-204-8116

E-mail: hykim@khu.ac.kr

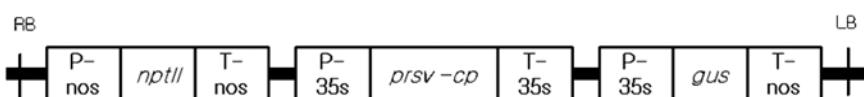


Fig. 1. Schematic diagram of GM papaya event 55-1. *nptII*, neomycin phosphotransferase gene; *papaya ringspot virus (PRSV)* coat protein gene; *gus*, β-D-glucuronidase gene

is able to differentiate GM crops containing the same traits, contrary to gene- and construct-specific detection methods confirming coding genes, promoters, and terminators [Holst-Jensen *et al.*, 2003]. Less than 50% of the more than 130 GMOs developed in other countries have been approved in Korea [Kim *et al.*, 2010]. Therefore, it is necessary to develop effective GMO detection methods to monitor and identify approved as well as unapproved GMOs.

In this study, we designed a set of primers to amplify an endogenous papaya gene in genomic DNA from both fresh and processed papaya. A set of event-specific primers, based on papaya genomic sequences and transferred DNA (T-DNA) flanking regions, was used to amplify the GM event-integrated region and thereby distinguish GM papaya event 55-1 from others. Combined in a duplex PCR, these two primer sets provide a qualitative PCR analysis to simultaneously identify non-GM and GM papaya. This duplex PCR was employed to detect a GM event in fresh and processed papaya and to evaluate the efficiency of the analysis.

Materials and Methods

Samples. Eleven fresh papayas used in this study were purchased from food markets in Korea, the United States, and China. Processed papaya products, including three brands of dried papaya and two brands of canned papaya, were obtained from food markets in Korea. GM papaya event 55-1, supplied by the Korea Food and Drug Administration (KFDA), was used as a standard GM papaya. A schematic diagram of GM papaya event 55-1 is shown in Fig. 1. Twelve fruits and vegetables, including watermelon (*Citrullus vulgaris Schrad*), plum (*Prunus salicina*), kiwi (*Actinidia deliciosa*), pineapple (*Ananas comosus*), soybean (*Glycine max*), canola (*Brassica napus*), cotton (*Gossypium hirsutum*), wheat (*Triticum aestivum*), mung bean (*Vigna radiata*), red bean (*Phaseolus angularis*), cabbage (*Brassica campestris*), and radish (*Raphanus sativus*), were purchased from local markets in Korea to extract their genomic DNA. The thirteen GM crops selected for this study were two GM soybeans (Round-up Ready soybean, A2704-12), nine GM maizes (Bt176, Bt11, MON810, MON863, NK603, GA21, T25, TC1507, MON89034), one GM canola (GT73), and one GM cotton (MON1445).

Genomic DNA extraction. Genomic papaya DNA was extracted from both flesh and seeds. Sliced papaya flesh (4 g) or papaya seed (0.2 g) was ground in the presence of liquid nitrogen using a mortar and pestle. Dried papaya was cut into small pieces and washed with sterile distilled water at least 10 times to remove carbohydrates. Canned papaya slices were selected, chopped, and washed with sterile distilled water at least 7 times. After removal of as much carbohydrate as possible, the processed papaya samples were dried at 65°C overnight. The dried papaya (2 g) was ground under nitrogen gas using a mortar and pestle, and 100–150 µL aliquots of the preparation were transferred to 2 mL tubes for DNA extraction. The cetyltrimethylammonium bromide (CTAB) methods reported by several research groups [Kim *et al.*, 2003; Min *et al.*, 2004; Yamamoto *et al.*, 2006] were modified to extract genomic DNA from the prepared papaya samples. One gram of the dried, ground sample was mixed with 5 mL extraction buffer [1.5% CTAB, 5 M NaCl, 100 mM EDTA, 75 mM Tris-Cl (pH 8.0)] and incubated at 55°C for 30 min. Phenol: chloroform:isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) were used to separate genomic DNA from proteins and other contaminating materials, and 100% isopropanol was added to the resulting solution to precipitate genomic DNA. The pellet was dissolved in sterile deionized water and quantified using a UV-visible spectrophotometer 1700 (Shimadzu, Japan). The integrity

Table 1. Primers used for duplex PCR

GM crops	Primer name	Sequence (5'-3')	Target	Concentration (µM)	Expected size of Amplicon (bp)
papaya	Papain SS11F	TAC GGG TGC AAT GGA GGT TA	<i>papain</i>	3	108
	Papain SS11R	GCG ACA ATA ACG TTG CAC TC			
55-1	55-1 LB SS3F	GGC TCC AGA TTT ATC AGC AA	T-DNA	15	150
	55-1 LB SS3R	CGA TTA ATG CAG CCT TAG AT			

and pattern of the extracted DNA were observed by electrophoresis on 1% agarose, as described by Kim *et al.* [2005].

Primers for PCR. The primers used in this study are showed in Table 1. Based on the sequence of the endogenous papaya gene *papain* (GenBank Accession No. M15203), we designed a set of primers to amplify a 108 bp PCR product. An event-specific primer pair was designed to amplify the region of the papaya genome where GM papaya event 55-1 (Fig. 1) was inserted (the left border) and the T-DNA flanking region. This primer pair produced a 150 bp amplicon.

PCR conditions. Each 25 μ L qualitative PCR mixture contained the following components: 2.5 μ L of 10 \times buffer (Applied Biosystems, Foster City, CA), 200 μ M of each dNTP (Applied Biosystems), 1.5 μ L of 1.5 mM MgCl₂, forward and reverse primers (10 μ M each), 0.8 units of Ampli Gold Taq DNA polymerase (Applied Biosystems), and 50 ng of template. PCR was performed using a Mastercycler machine (Eppendorf, Hamburg, Germany). Conditions for PCR included one denaturation cycle at 94°C for 5 min; 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s; and one final cycle at 72°C for 7 min. The PCR products were stored at 4°C until electrophoresis on 2% agarose gel. A 100-bp DNA ladder (Bioneer, Daejeon, Korea) served as a standard.

Duplex PCR was performed under the same conditions as above, but using 100 ng template DNA and 4% agarose to analyze products.

Limit of detection (LOD). Pure (100%) GM event 55-1 papaya was mixed with non-GM papaya to produce mixtures containing 10, 5, 3, 1, 0.5, 0.1, 0.05, and 0.025% GM papaya. A 100-ng sample of each mixture was used as the template for duplex PCR with both sets of the primers shown in Table 1.

DNA sequencing. PCR amplicons were purified using a PCR purification kit (Qiagen Korea, Ltd., Seoul, Korea). *E. coli* DH5 α was transformed with the purified PCR products, cloned into the pGEM-T easy vector (Promega, Madison, WI). The transformed *E. coli* colonies were selected to extract the recombinant plasmid. The PCR amplicons in the vector were sequenced using ABI PRISM 3700 DNA analyzer (Perkin Elmer, Boston, MA).

Results and Discussion

Analysis of the genomic DNA. One microgram of each genomic DNA extracted from fresh papaya, dried papaya and canned papaya was analyzed on a 1% agarose gel. The band patterns differed depending on the extent of food processing (Fig. 2). As expected, genomic DNA

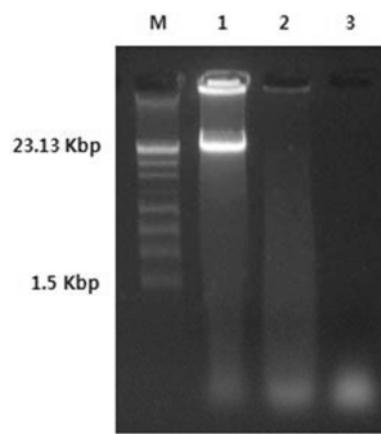


Fig. 2. Integrity of genomic DNA from fresh and processed papaya. Lane M, marker (Lambda DNA/HindIII marker plus); lane 1, fresh papaya; lane 2, dried papaya; lane 3, canned papaya.

from fresh papaya migrated as a large fragment in the gel, apparently intact. However, the DNA extracted from dried papaya showed a smear of small fragments, most likely produced by heat treatment in the presence of sugar. Genomic DNA from canned papaya also showed degradation, probably through cooking and sterilization. These results are consistent with DNA patterns obtained for processed pears using the genomic-tip method of DNA extraction, reported to be the method most suitable for processed fruits [Yamamoto *et al.*, 2006]. We found, however, that the CTAB method produced a sufficiently high yield of DNA from processed papaya for our PCR experiments, at significantly less expense than with the genomic-tip method.

Specificity of a primer pair for *papain*, an endogenous papaya gene. Using a primer pair (*papain* SS11F/R) specific to an endogenous papaya gene with genomic DNA from 13 fresh papayas and 12 varieties of fruits and vegetables, PCR produced a single 108 bp amplicon only from genomic papaya DNA (Fig. 3 (A)). This confirmed the specificity of these primers for the endogenous papaya gene (*papain*) and validates their use in a qualitative PCR assay for GM papaya.

Specificity of event-specific primers for GM event 55-1. We tested the specificity of the primer pair 5-1 LB SS3F/R for GM event 55-1 in a qualitative PCR using 14 templates, i.e., the genomic DNA of GM papaya event 55-1 and 13 GM crops. The assay revealed a 150 bp PCR product only from the DNA of GM event 55-1 papaya (Fig 3 (B)). Fan *et al.* [2009] has also reported on event-specific primers, which identify a GM papaya developed in Taiwan. The primer pair used in the present study may be similarly applied to detect GM papaya event 55-1 and to distinguish it from other GM and non-GM papaya.

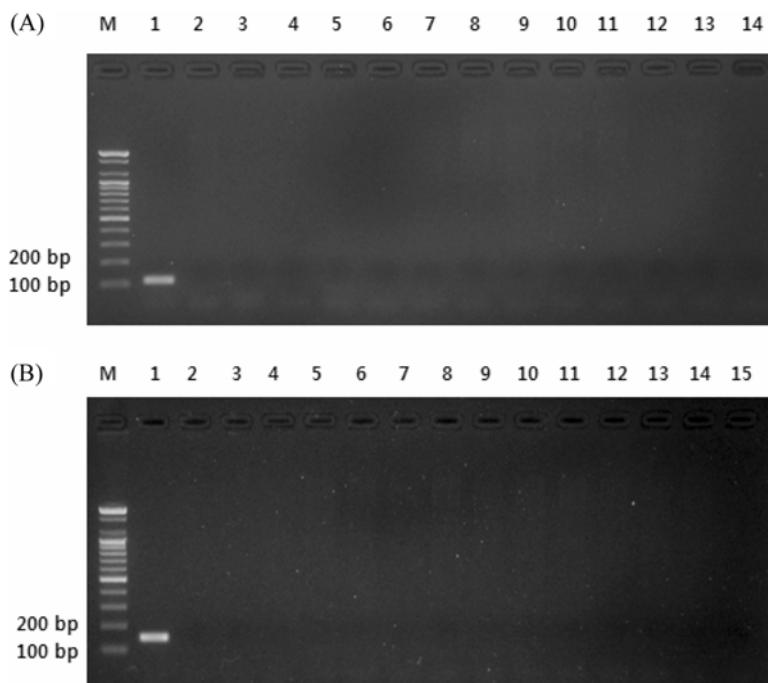


Fig. 3. (A) Analysis of the papaya-specific *papain* gene using specific primer pairs (*papain* SS11-F/R). Lane M, marker (100 bp DNA ladder); lane 1, papaya; lane 2, watermelon; lane 3, plum; lane 4, kiwi; lane 5, pineapple; lane 6, soybean; lane 7, canola; lane 8, cotton; lane 9, wheat; lane 10, mung bean; lane 11, red bean; lane 12, cabbage; lane 13, radish; lane 14, no template. (B) Specificity of the event-specific primer pairs for GM papaya event 55-1. Lane M, marker (100 bp DNA ladder); lane 1, 55-1; lane 2, RRS; lane 3, A2704-12; lane 4, Bt176; lane 5, Bt11; lane 6, MON810; lane 7, MON863; lane 8, NK603; lane 9, GA21; lane 10, T25; lane 11, TC1507; lane 12, MON89034; lane 13, GT73; lane 14, MON1445; lane 15, no template.

LOD of duplex PCR. The LOD is the lowest amount or concentration of a target compound that can be detected using a specified analytical method [Mano *et al.*, 2009]. In a duplex PCR assay for GM papaya event 55-1, we determined 0.5% to be the minimum concentration of GM papaya required to detect the 150 bp amplicon (Fig. 4). This LOD provides an acceptable level of specificity to monitor GM papayas or food mixtures containing GM papaya in commercial distribution.

Detection of GM events using duplex PCR. Duplex PCR is currently used to detect GM papaya with construct-specific primers [Yamaguchi *et al.*, 2006]. Instead, we used event-specific primers, shown to be more specific than gene- and construct-specific primers, to amplify only GM papaya event 55-1. Specific primers, 55-1 LB SS3F (T-DNA flanking region on the LB (left border)), and 55-1 LB SS3R (papaya genome flanking region on the LB of the inserted gene cassette), were synthesized to amplify the cassette region and flanking genome. Our duplex PCR also included primers (*papain* SS11F/R) specific for an endogenous papaya gene (*papain*). With this duplex PCR, we tested 11 fresh papaya bought from markets in Korea, the United States and China, and detected GM papaya event 55-1 in 4 of them (samples 3, 4, 6, and 9;

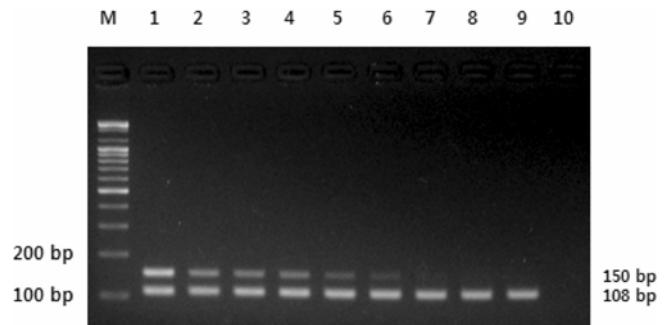


Fig. 4. Limit of detection in a duplex PCR assay for GM papaya event 55-1. Lane M, marker (100 bp DNA ladder); lanes 1-9: 100, 10, 5, 3, 1, 0.5, 0.1, 0.05, and 0.025% of GM papaya; lane 10, no template.

Fig. 5 (A)). We also tested 5 processed forms of papaya from local markets in Korea but detected no GM papaya by this method (Fig. 5 (B)). This finding indicates that the processed foods contained no GM papaya or contained an amount less than 0.5% of total fruit used in processing.

In this study, we developed a qualitative duplex PCR analysis to detect GM papaya event 55-1. We validated the method in assays of fresh papaya and papaya-containing processed foods. The duplex PCR developed in this study

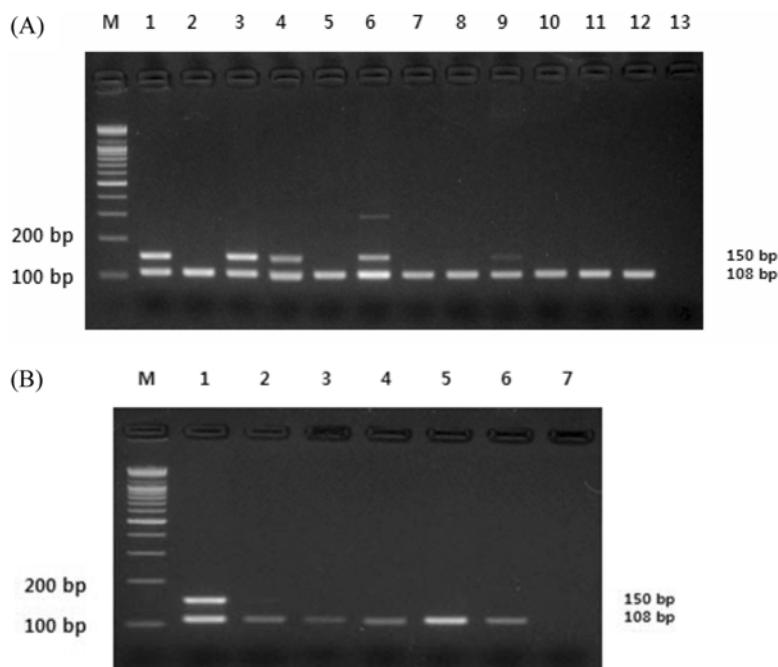


Fig. 5. (A) Detection of GM papaya in various samples using duplex PCR. Lane M, marker (100 bp DNA ladder); lane 1, positive control; lanes 2-9, fresh papaya fruit (purchased from the United States); lanes 10-11, fresh papaya (China); lane 12, fresh papaya (Korea); lane 13, no template. **(B)** Monitoring of GM papaya in processed foods using duplex PCR. Lane M, marker (100 bp DNA ladder); lane 1, positive control; lanes 2-4, dried papaya; lanes 5-6, canned papaya; lane 7, no template.

fulfills an existing need for methods to detect GM papaya not currently approved in Korea, which might be distributed in domestic markets. Our methodology may be extended to monitor not only fresh GM fruits mixed with non-GM fruits (including papaya), but also to monitor processed foods for their presence. This developed event-specific PCR method can be registered to Food Code in KFDA for detection of unapproved GM papaya.

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