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# Application of a qualitative and quantitative real-time polymerase chain reaction method for detecting genetically modified papaya line 55-1 in papaya products

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ABSTRACT

Genetically modified (GM) papaya (*Carica papaya* L.) line 55-1 (55-1), which is resistant to papaya ringspot virus infection, has been marketed internationally. Many countries have mandatory labeling regulations for GM foods, and there is a need for specific methods for detecting 55-1. Here, an event- and construct-specific real-time polymerase chain reaction (PCR) method was developed for detecting 55-1 in papaya products. Quantitative detection was possible for fresh papaya fruit up to dilutions of 0.001% and 0.01% (weight per weight [w/w]) for homozygous SunUp and heterozygous Rainbow cultivars, respectively, in non-GM papaya. The limit of detection and quantification was as low as 250 copies of the haploid genome according to a standard reference plasmid. The method was applicable to qualitative detection of 55-1 in eight types of processed products (canned papaya, pickled papaya, dried fruit, papaya-leaf tea, jam, puree, juice, and frozen dessert) containing papaya as a main ingredient.


## 1. Introduction

Genetically modified (GM) papaya (*Carica papaya* L.) line 55-1 (55-1), which is resistant to papaya ringspot virus (PRSV) infection, was developed in Hawaii in 1998 and has been cultivated for food in the United States for more than a decade (Fermín et al., 2011). 55-1 is a GM fruit that is commercially available worldwide in various types of food products, such as fresh papaya fruit, canned papaya, pickled papaya, dried fruit, papaya-leaf tea, jam, puree, juice and frozen dessert.

Today, the acceptance of GM foods by consumers remains controversial, and concerns about their safety persist among the public (Akiyama et al., 2005). Therefore, GM labeling on food products containing GM material is mandatory in many countries, such as the European Union, Japan and Korea (Leimanis et al., 2006; Sheldon, 2002). The enforcement of regulations on GM labeling for foods has created a demand for the development of reliable GM papaya detection methods. As the basis for labeling, the European Union, Japan and Korea have set threshold values of 0.9%, 5% and 3%, respectively, of GM foodstuffs in a non-GM background (Commission Regulation (EC) 49/2000, 2000; Notification 2000-31, 2000; Notification 79, 2000). However, the threshold values are inapplicable to processed papaya products because of complex ingredients and food processing. To ensure reliability of the GM labeling system for processed papaya products, an accurate qualitative detection method was needed.

To assist with a labeling system for GM papaya, we previously developed methods to detect 55-1 in fresh papaya fruit using qualitative polymerase chain reaction (PCR) assay with DNA purified from fresh papaya fruit, and using a histochemical assay with fresh papaya seeds (Goda, Asano, Shibuya, Hino, & Toyoda, 2001; Wakui et al., 2004; Yamaguchi et al., 2006). These detection methods were not applicable to processed papaya products since high detection sensitivity was not achieved due to fragmentation of the targeted DNA and enzyme denaturation or the disposal of papaya seeds during processing (Greiner, Konietzny, & Jany, 1997; Gryson, 2010; Nakamura et al., 2010). To our knowledge, there is no detailed study on qualitative and quantitative real-time PCR detection method for detecting 55-1 in papaya products.

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In the present study, we developed a novel 55-1 real-time PCR detection method applicable to GM labeling for a variety of papaya products produced worldwide.

## 2. Materials and methods

### 2.1. Samples

Hawaiian non-GM (Sunset) and two cultivars of 55-1 (SunUp and Rainbow) were purchased from a trade agency via the Hawaii Papaya Industry Association. SunUp is a homozygote generated by transforming the non-transgenic inbred cultivar Sunset, whereas Rainbow is a first filial generation ($F_1$) hybrid from a cross between SunUp and non-transgenic Kapoho (Fermín et al., 2011). Thirty-eight commodities from eight types of processed papaya products (canned papaya, pickled papaya, dried fruit, papaya-leaf tea, jam, puree, juice, frozen dessert) were purchased online (Supplementary Table 1).

### 2.2. DNA extraction and purification

For juice and frozen dessert, samples were pre-lyophilized before use. Briefly, 10 g fresh fruit, canned papaya, pickled papaya, dried fruit, jam, puree, and frozen dessert, 2 g papaya-leaf tea, or 30 g juice was ground and treated using an ion-exchange resin-type DNA extraction and purification kit (Genomic-tip; Qiagen, Hilden, Germany). The protocol provided by the manufacturer was modified as follows: 30 ml G2 buffer (Qiagen) containing 800 mM guanidine HCl, 30 mM Tris HCl (pH 8.0), 30 mM EDTA (pH 8.0), 5% Tween-20 and 0.5% Triton X-100, 20 μl of 100 mg/ml RNase A (Qiagen), and 500 μl cellulase (Sigma–Aldrich, St. Louis, MO, USA) were added to the ground sample, mixed thoroughly with a vortex mixer, and incubated at 50 °C for 1 h. Proteinase K (200 μl; Promega, Madison, WI, USA) was added, and the mixture was incubated at 50 °C for another 1 h, during which time the tubes were regularly inverted to mix the contents. Samples were then centrifuged at 3000*g* for 20 min at 4 °C. The supernatant was applied to a Genomic-tip 100/G column (Qiagen) pre-equilibrated with 4 ml QBT buffer (Qiagen) containing 750 mM NaCl, 50 mM 3-(N-morpholino)propanesulfonic acid (pH 7.0), 15% isopropanol, 0.15% Triton X-100. The tip was washed three times with 7.5 ml QC buffer (Qiagen) containing 1.0 M NaCl, 50 mM MOPS (pH 7.0) and 15% isopropanol, transferred to a fresh centrifuge tube, and 3 ml pre-warmed (50 °C) QF buffer (Qiagen) containing 1.25 M NaCl, 50 mM Tris HCl (pH 8.5) and 15% isopropanol was added to elute the DNA. The DNA sample was transferred to a centrifuge tube, an equal volume of isopropyl alcohol was added, and the sample was mixed thoroughly. DNA was collected by centrifugation at 12,000*g* for 15 min. The pellet was rinsed with 1 ml 70% (volume per volume [v/v]) ethanol and centrifuged at 12,000*g* for 5 min. The supernatant was discarded, and the precipitate was dried with an aspirator. The DNA was dissolved in 50 μl deionized water for use in analyses. DNA samples were quantified by measuring the ultraviolet (UV) absorption at 260 nm ($A_{260}$) using an ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The quality of the samples was estimated from the UV absorption ratios at 260 and 280 nm ($A_{260}/A_{280}$) and 260 and 230 nm ($A_{260}/A_{230}$). Samples were diluted to 10 ng/μl using water, whereas those at lower concentrations were used directly in experiments.

### 2.3. Real-time PCR assay

Real-time PCR assays were performed using an ABI PRISM™ 7900 Sequence Detection System (Life Technologies, Carlsbad, CA, USA). Sample DNA solution (2.5 μl) was mixed with 12.5 μl Gene Expression Master Mix® (Life Technologies), 0.8 μM of each primer, and 0.1 μM probe in a final volume of 25 μl. The PCR conditions were as follows: 2 min at 50 °C, 95 °C for 10 min, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C.

### 2.4. Oligonucleotide primers and probe for real-time PCR detection

The oligonucleotide sequences of primer pairs (Q-Chy-1F2 and Q-Chy-2R) and probe (Q-Chy-P′) for detecting the papaya endogenous internal control gene *chymopapain* (*Chy*; GenBank ID: AY803756) were consistent with those described previously (Guo et al., 2009; Nakamura et al., 2011). The Q-Chy-P′ probes with an internal quencher (black hole quencher 1 [BHQ-1]) were synthesized with a 3′ phosphate, to eliminate extension of the reverse primer and probe during PCR amplification. For detection of 55-1, the transformation vector sequence (GenBank ID: FJ467933.1) (Suzuki et al., 2008) and the genomic sequence of SunUp (GenBank ID: ABIM00000000.1) (Ming et al., 2008) were used to design real-time PCR oligonucleotide primers and probes. The event-specific set of primers and probe (55-1 primer1, 55-1 primer2 and 55-1 probe) was designed around the transgenic insert flanking sequence (Fig. 1). The 55-1 probe was labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethyl-rhodamine (TAMRA) at the 5′ and 3′ ends, respectively. The amplicon sizes of the event-specific (71 base pair [bp]) and *Chy* (72 bp) detection methods were also designed to be of approximately equal length, in order to obtain a more reliable result targeting DNA fragments in the DNA sample purified from processed foods. The construct-specific detection method was designed to confirm the result of the event-specific real-time PCR method. Specificity of the construct-specific detection method was considered for 55-1 among the other developed GM papayas lines reported. GM papaya lines 63-1 and X17-2, which have been approved for food consumption in the United States, do not carry the reporter gene β-glucuronidase (GUS) from *Escherichia coli* in the transgenic genome (USDA-APHIS, 1996; USDA-APHIS, 2008). Similarly, Huanong No. 1, which was approved for commercialization in China in 1996 (Guo et al., 2009), and PRSV-YK, which is an unapproved GM papaya strain that has a similar transgenic construct to the GM papaya line 16-0-1/17-0-5 (Fan et al., 2009) and has been detected in processed papaya-leaf tea products in Japan (Nakamura et al., 2011), lack GUS in the transgenic insert sequence. Accordingly, the construct-specific set of primers and probe (GUS primer, P35S primer and GUS-P35S probe) was designed around the border sequence of *GUS* and Cauliflower Mosaic Virus 35S promoter (P35S) in the transgenic construct structure (Fig. 1). The GUS-P35S probe was labeled with FAM at the 5′ end and a Minor-Groove Binder (MGB) at the 3′ end. Sets of primer pairs and probes for detection of P35S, 35S-F and 35S-R with 35S-P, and nos-terminator from *Agrobacterium tumefaciens* (T-nos), 180-F and 180-R with TM-180, were used for *cis*-element-specific detection method. The probes 35S-P and TM-180 were labeled with FAM and TAMRA at the 5′ and 3′ ends, respectively. A construct-specific detection method for detecting GM papaya PRSV-YK was used according to a previously described paper (Nakamura et al., 2011).

All primers and probes were diluted with an appropriate volume of distilled water, and stored at −20 °C until use. Results were analyzed using SDS 2.1 sequence detection software (Life Technologies) on the ABI PRISM™ 7900 Sequence Detection System.

### 2.5. Real-time quantitative PCR assay

For evaluation of an event- and construct-specific real-time PCR method for detecting 55-1, a positive control plasmid was generated by inserting amplicons into the pGEM-T Easy vector