



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 ring-spot 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.

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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 μ l of 100 mg/ml RNase A (Qiagen), and 500 μ l 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 μ l; 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 3000g 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 12,000g for 15 min. The pellet was rinsed with 1 ml 70% (volume per volume [v/v]) ethanol and centrifuged at 12,000g for 5 min. The supernatant was discarded, and the precipitate was dried with an aspirator. The DNA was dissolved in 50 μ l 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/ μ l 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 μ l) was mixed with 12.5 μ l Gene Expression Master Mix® (Life Technologies), 0.8 μ M of each primer, and 0.1 μ M probe in a final volume of 25 μ l. 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

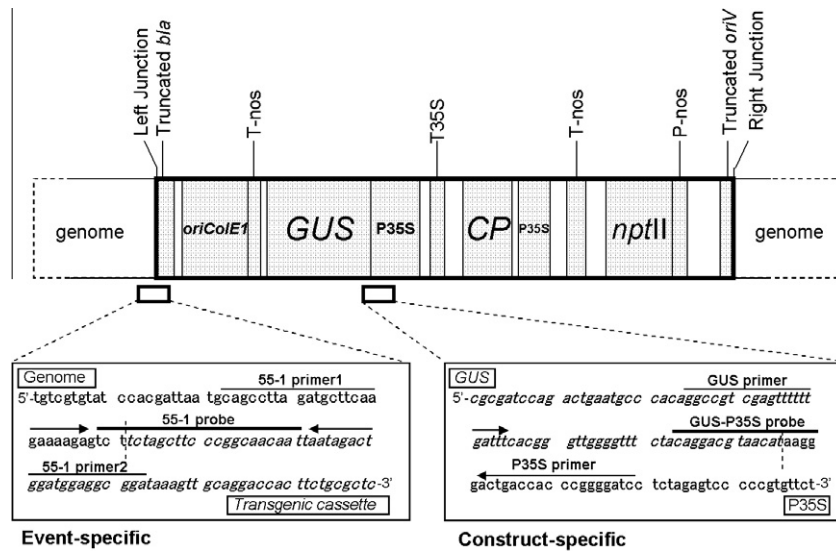


Fig. 1. Transgenic construct in 55-1. Positions of real-time PCR primers and probe for event- and construct-specific detection are depicted as two arrows and a bar, respectively. Oligonucleotide sequences of the primers and probe are shown underneath. The transgenic construct contained oriColE1 (origin of replication site for plasmid pColE1 derived from pBR322), *GUS* (β -glucuronidase), *CP* (PRSV coat protein), *nptII* (neomycin phosphotransferase II), P35S, T35S (Cauliflower Mosaic Virus 35S terminator), T-nos, P-nos (nopaline synthase promoter), truncated *bla* (β -lactamase) at the left junction, and truncated *oriV* (origin of vector replication) at the right junction. Vertical broken-lines in the oligonucleotide sequences indicate the border sequence between the genome and transgenic cassette (italics) for event-specific detection, and *GUS* (italics) and P35S for construct-specific detection.

(Promega) according to the manufacturer's instructions. A series of dilutions (2.5, 25, 250, 2500, 25,000, 250,000, 2,500,000, and 25,000,000 copies) of the linearised control plasmid was spiked in DNA purified from non-GM papaya Sunset. The sequence of the insert was verified using pUC/M13 forward and reverse sequencing primers.

The detection limit for 55-1 in non-GM papaya was evaluated from a series of dilutions (0.000001%, 0.00001%, 0.0001%, 0.001%, 0.01%, 0.1%, 1%, and 10%) of DNA purified from 55-1 using an equal concentration (10 ng/ μ l) of DNA purified from non-GM papaya Sunset. In each reaction, a 25-ng template DNA sample was used for analysis. All reactions were performed in duplicate.

2.6. Real-time qualitative PCR data analysis

The baseline was set to cycles 3–15. The normalized reporter signal (ΔR_n) threshold for plotting cycle threshold (Ct) values

was set to 0.2 during exponential amplification. Reactions with a Ct value of <48 and exponential amplification, as judged by visual inspection of the respective ΔR_n plots and multi-component plots, were scored as positive. If a Ct value could not be obtained, the reaction was scored as negative. Reactions with a Ct value of <48, but without exponential amplification, as judged by visual inspection of the respective ΔR_n plots and multi-component plots, were scored as negative.

The difference between the Ct values (ΔCt) obtained using the 55-1 detection method and the *Chy* detection method, as a reference, was calculated to estimate the relative copy number of the transgenic construct in GM papaya cultivars. Differences in ΔCt values ($\Delta \Delta Ct$) between GM papaya cultivars were obtained, and the relative copy number of the transgenic construct was calculated using the following formula:

$$\text{Relative copy number} = 2^{-\Delta \Delta Ct} \quad (1)$$

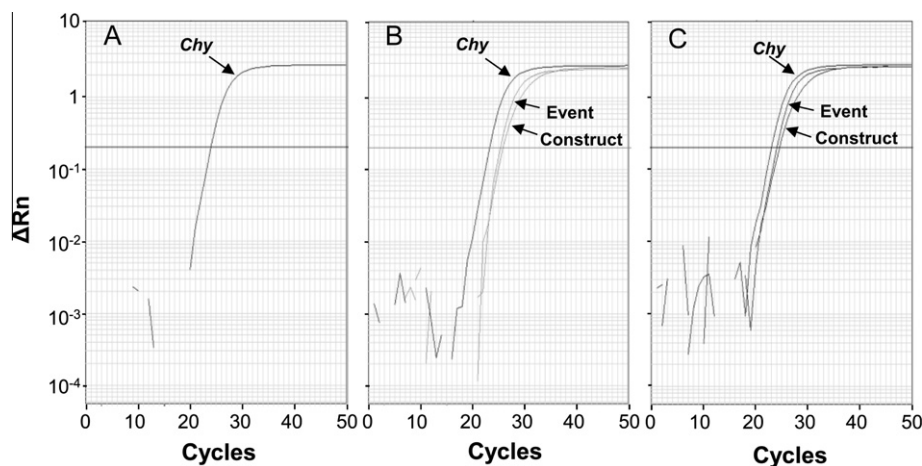


Fig. 2. Amplification plots of real-time PCR. DNA templates purified from Sunset (A), Rainbow (B), or SunUp (C) were used for real-time PCR using a primer and probe set for an endogenous *Chy* and 55-1 detection method.

Table 1
Oligonucleotide primers and probes used in this study.

Target	Primer name	Sequence (5'–3')	Amplicon (bp)	Reference
Endogenous gene (<i>Chy</i>)	Q- <i>Chy</i> -1 F2	CCATGCGATCCTCCCA	72	This study
	Q- <i>Chy</i> -2R	CATCGTAGCCATTGTAACACTAGCTAA		
	Q- <i>Chy</i> -P'	FAM-TTCCTTCAT(BHQ1)CCATCCCCTCTTGAGA- Φ^*		
Event-specific sequence (55-1)	55-1 primer1	CAGCCTTAGATGCTTCAAGAAAAGA	71	This study
	55-1 primer2	TCCGCCTCCATCCAGTCTATT		
	55-1 probe	FAM-TCCTTCTAGCTTCCCGCAACAAT-TAMRA		
Construct-specific sequence (GUS-P35S)	GUS Primer	GGCCGTCGAGTTTTTTGATTT	75	This study
	P35S Primer	GATCCCCGGGTGGTCAGT		
	GUS-P35S probe	FAM-CAGGACGTAACATAAGG-MGB		
Cis-element-specific sequence (P35S)	35S-F	GCCTCTGCCGACAGTGGT	82	Waublinger, Ernst, Anderson, and Pietsch (2008)
	35S-R	AAGACGTGGTTGGAACGTCTTC		
	35S-P	FAM-CAAAGATGGACCCCCACCCACG-TAMRA		
Cis-element-specific sequence (T-nos)	180-F	CATGTAATGCATGACGTTATTTATG	84	Reiting, Broll, Waublinger, and Grohmann (2007)
	180-R	TTGTTTTCTATCGCGTATTAAATGT		
	TM-180	FAM-ATGGGTTTTTATGATTAGAGTCCCGCAA-TAMRA		

* Φ , phosphate.

3. Results and discussion

3.1. Detection of 55-1 using real-time PCR

To test the developed 55-1 real-time PCR detection method, DNA purified from fresh papaya fruit of the homozygous and heterozygous cultivars SunUp and Rainbow, respectively, and the non-GM cultivar Sunset were used as samples. As shown in Fig. 2, papaya endogenous *Chy*-specific real-time PCR using 25 ng template DNA gave an amplification signal at Ct values (threshold value, 0.2) of 22.95, 23.21, and 23.87 for SunUp, Rainbow, and Sunset, respectively. Event- and construct-specific real-time PCR using 25 ng DNA purified from fresh GM papaya fruit gave an exponential amplification signal at Ct values (threshold value, 0.2) of 25.34 and 25.86 (Rainbow; Fig. 2B), and 24.26 and 24.75 (SunUp; Fig. 2C), respectively. No false-positive signal was detected using Sunset template DNA in either the event- or construct-specific detection method (Fig. 2A). Thus, the developed methods detected only GM papayas.

To investigate method specificity, DNA samples purified from representative genetically modified crops and from fruits that are processed together in papaya products (Supplementary Table 1) were tested (crops: rice, soybean, maize, potato, and rapeseed; fruits: pineapple, guava, passion fruit, grape, prune, banana, lemon, apple and mango). No positive signals were observed from any of those samples using event- or construct-specific methods, while the *Chy* detection was positive for non-GM papaya (data not shown). Therefore, the primers and probe sets designed for 55-1 detection method were specific and appropriate for monitoring papaya products.

To confirm the accuracy of the 55-1 detection system, the relative copy numbers of the transgenic construct in SunUp and Rainbow were estimated from the obtained Ct values. Papaya

undergoes diploid inheritance (Ming et al., 2008), and a haploid papaya genome has a single copy of the *Chy* gene (Guo et al., 2009). Therefore, differences in the mean Ct values (Δ Ct at a threshold value of 0.2) obtained using event-/construct-specific methods and the *Chy* detection method were calculated. The Δ Ct values were 1.08 and 1.80 (SunUp), and 2.07 and 2.71 (Rainbow) for event- and construct-specific methods, respectively (Table 2). From three independent experiments (conducted in duplicate), relative standard deviation (RSD) of the experiments was within 5%, indicating the high repeatability and reproducibility of the experiments. According to Eq. (1), the copy number of the targeted sequence in SunUp, relative to that of Rainbow, was calculated from the differences in Δ Ct values ($\Delta\Delta$ Ct) to be closer to 2 with the event-specific method (1.99; RSD, 11.26%) than with the construct-specific method (1.88; RSD, 15.93%). The copy number of the 55-1 targeted sequence in the haploid genome of homozygous SunUp was calculated at an RSD of less than 16% (event-specific, 1.8–2.2 copy; construct-specific, 1.6–2.2 copy) and indicated to be double the number present in heterozygous Rainbow. Homozygous and heterozygous 55-1 cultivars were accurately identified from relative differences in Ct values using the developed 55-1 detection system.

To estimate the sensitivity of the established 55-1 real-time PCR assay, samples were prepared from sequential dilutions (0.000001%, 0.00001%, 0.0001%, 0.001%, 0.01%, 0.1%, 1%, 10%, and 100%) of DNA purified from either SunUp or Rainbow. A 25-ng sample of total papaya genomic DNA was used as a template for each PCR reaction. Both event- and construct-specific detection methods observed parallelism ($r^2 = 0.996$ – 0.998) between the concentration of line 55-1 cultivars and Ct values obtained in amplification plots at concentrations of 0.001–100% for SunUp DNA and 0.01–100% for Rainbow DNA (Fig. 3). Therefore, estimated quantitative amplification limits of diluted 55-1 DNA were 0.001% and 0.01% for SunUp and Rainbow, respectively.

Table 2
Ct values^a obtained for papaya cultivars.

Cultivar	<i>Chy</i>	Event	Construct	Δ Ct _{Event}		$\Delta\Delta$ Ct _{Event}		Relative copy No.	Δ Ct _{Construct}		$\Delta\Delta$ Ct _{Construct}		Relative copy No.
				Mean	RSD (%)	Mean	RSD (%)		Mean	RSD (%)	Mean	RSD (%)	
Sunset	22.76 ± 0.10	ND	ND										
SunUp	22.48 ± 0.10	23.56 ± 0.10	24.28 ± 0.20	1.08	4.86	−0.99	11.26	1.99	1.80	0.78	−0.91	15.93	1.88
Rainbow	22.71 ± 0.05	24.78 ± 0.10	25.42 ± 0.22	2.07	2.87	0		1	2.71	5.86	0		1

^a Ct values were obtained from duplicates of three independent experiments. ND, not detected.

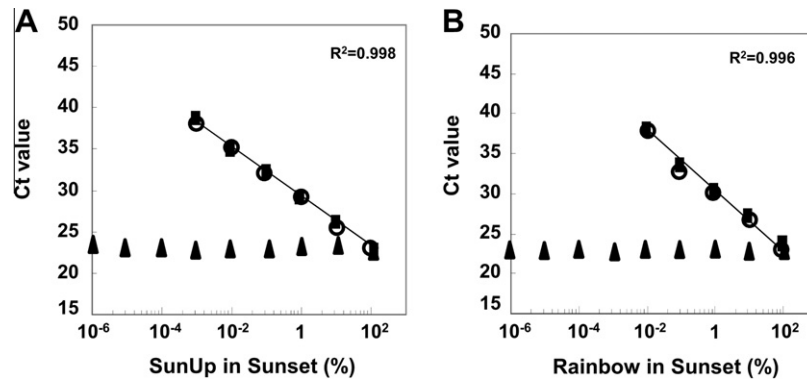


Fig. 3. Detection limits of 55-1. DNA template (25 ng) containing 0.000001%, 0.00001%, 0.0001%, 0.001%, 0.01%, 0.1%, 1%, and 10% (w/w) of DNA purified from SunUp (A) or Rainbow (B) was diluted with Sunset DNA and used for real-time PCR. Event-specific (filled square), construct-specific (open circle) and endogenous *Chy* (triangle).

3.2. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD is generally defined as the smallest amount of analyte that can be reliably detected, whereas the LOQ is the lowest amount of analyte in a sample that can be quantitatively determined with an acceptable level of precision and accuracy (Bertheau, Dioloz, Kobilinsky, & Magin, 2002; ISO 24276:2006). To evaluate in-house LOD and LOQ, a dilution series of positive-control plasmid at 2.5, 25, 250, 2500, 25,000, 250,000, 2,500,000, and 25,000,000 copies/reaction was tested. As shown in Table 3, the results indicated that the LOD was as low as 25 copies for construct-specific and *Chy* detection, and 250 copies for event-specific detection. The in-house LODs had a false-negative rate of $\leq 5\%$ and a RSD of reproducibility of $\leq 33\%$, which are both within ISO 24276 requirements (ISO 24276:2006). To obtain reliable quantification results under ideal conditions, the LOQ was as low as 25 copies for construct-specific and *Chy* detection, and 250 copies for event-specific detection. The observed in-house LOQs had a RSD of reproducibility of $\leq 25\%$, which is within the requirements (ISO 24276:2006). These results indicated that the construct-specific and *Chy* detection methods had higher repeatability, and lower LOD and LOQ, than the event-specific method. Using the developed 55-1 detection system, reliable detection was possible at as low as 250 copies of the haploid genome according to a standard reference plasmid.

3.3. Applicability of 55-1 real-time PCR detection method to processed products

The developed real-time PCR method was tested using thirty-eight samples from eight types of processed papaya product (canned papaya, pickled papaya, dried fruit, papaya-leaf tea, jam, puree, juice, and frozen dessert). To obtain DNA samples for testing, an ion-exchange resin-type DNA extraction and purification

method was used as described in Section 2. The yield and purity of DNA were estimated by measuring A_{230} , A_{260} , and A_{280} (Supplementary Fig. 1). Canned papaya (B), dried fruit (A–E), jam (A, B, D, E, F), puree (A–C), and juice (A, B and D) all showed a low purification efficiency at <20 ng DNA per 1 g of starting material (Supplementary Table 1). As minimal information was available about the processing techniques of these papaya products, it was possible that their low purification efficiency was caused by DNA degradation during processing; however, a low DNA content in the original product could not be ruled out. The observed absorbance ratios varied greatly depending on the product (Supplementary Fig. 1). All dried fruit (A–E; $n = 5$) that had been bleached with sulfurous acid salts showed low DNA purification efficiency. Indeed, *Chy* could not be detected from the five dried fruit samples (Table 4). From our preliminary observation on the optimization of the DNA purification method, highly processed products, such as in the case of dried fruit, juice, jam and pickled products, could not be detected using the *Chy* real-time PCR detection method with the amplicon size of 72 bp due to DNA degradation in the products (Ohmori et al., to be published).

Among 27 products, which were detectable using the papaya endogenous *Chy* detection method, two products (papaya-leaf tea A and jam A), were positive for 55-1 using the construct- and event-specific 55-1 detection methods (Table 4). The Ct values obtained from those products were as follows: papaya-leaf-tea A; *Chy* = 22.90 and Event = 33.13, jam A; *Chy* = 27.20 and Event = 39.72 (Supplementary Table 2). The copy number of the 55-1 targeted sequence relative to that of *Chy* was calculated, and was 0.0027 and 0.0008 for papaya-leaf tea A and jam A, respectively. From this result, papaya-leaf tea was estimated to have a 3.38 times higher content of 55-1 than jam A.

A variety of other PRSV-resistant GM papaya lines has been developed in various places, such as Florida, China, Jamaica, Tai-

Table 3
Detection and quantification limit of GM papaya line 55-1 detection method.

Copy number	Signal rate ^a (false negative rate (%))			Mean Ct value			SD			RSD (%)		
	<i>Chy</i>	Event	Construct	<i>Chy</i>	Event	Construct	<i>Chy</i>	Event	Construct	<i>Chy</i>	Event	Construct
25,000,000	9/9 (0)	9/9 (0)	9/9 (0)	16.48	17.71	18.07	0.41	0.14	0.12	2.49	0.78	0.67
2,500,000	9/9 (0)	9/9 (0)	9/9 (0)	20.64	20.73	20.61	0.85	0.13	0.18	4.10	0.65	0.85
250,000	9/9 (0)	9/9 (0)	9/9 (0)	23.72	24.59	24.17	0.55	0.23	0.34	2.34	0.95	1.42
25,000	9/9 (0)	9/9 (0)	9/9 (0)	27.06	27.75	28.23	0.44	0.16	0.32	1.64	0.59	1.13
2500	9/9 (0)	9/9 (0)	9/9 (0)	30.80	31.02	31.62	0.35	0.18	0.41	1.13	0.58	1.30
250	9/9 (0)	9/9 (0)	9/9 (0)	33.63	35.12	34.28	1.92	0.27	0.35	5.70	0.77	1.01
25	9/9 (0)	8/9 (11)	9/9 (0)	37.22		38.20	0.64		0.67	1.72		1.76
2.5	6/9 (33)	3/9 (67)	3/9 (67)									

^a Signal rate was indicated by number of positive signals out of nine experimental trials. SD, standard deviation.

Table 4

Analysis of the processed papaya products for GM papaya ingredient.

Product	Lot	Real-time PCR Ct value (mean; n = 2)					
		Endogenous Chy	55-1 (Event-specific)	55-1 (Construct-specific)	P35S (Cis-element-specific)	T-nos (Cis-element-specific)	Unapproved GM papaya PRSV-YK (Construct-specific)
Canned	A	25.90	–	–	–	–	–
	B	30.16	–	–	–	–	–
	C	24.48	–	–	–	–	–
Pickled	A	27.68	–	–	37.63	36.54	36.47
	B	21.19	–	–	33.40	33.22	33.24
	C	21.70	–	–	–	–	–
	D	–	–	–	–	–	–
Dried fruit (sulfured)	A	–	–	–	–	–	–
	B	–	–	–	–	–	–
	C	–	–	–	–	–	–
	D	–	–	–	–	–	–
	E	–	–	–	–	–	–
Dried fruit (Unulfured)	F	24.43	–	–	–	–	–
	G	23.50	–	–	–	–	–
	H	23.20	–	–	–	–	–
	I	23.70	–	–	–	–	–
	J	22.10	–	–	–	–	–
	K	25.50	–	–	–	–	–
Papaya-leaf tea	A	22.90	33.13	33.05	24.03	24.69	26.03
	B	23.98	–	–	32.90	32.37	31.60
	C	28.60	–	–	–	–	–
Jam	A	27.20	39.72	39.61	33.57	31.72	30.32
	B	25.73	–	–	–	–	–
	C	32.98	–	–	–	–	–
	D	–	–	–	–	–	–
	E	–	–	–	–	–	–
	F	–	–	–	–	–	–
	G	–	–	–	–	–	–
Puree	A	21.96	–	–	–	–	–
	B	23.99	–	–	–	–	–
	C	23.01	–	–	–	–	–
Juice	A	24.43	–	–	–	–	–
	B	34.95	–	–	–	–	–
	C	–	–	–	–	–	–
	D	43.14	–	–	–	–	–
	E	34.40	–	–	–	–	–
Frozen dessert	A	21.30	–	–	–	–	–
	B	21.60	–	–	–	–	–

wan, Thailand, Australia, Malaysia, the Philippines and Vietnam (Nakamura et al., 2011), including GM papaya lines that have no record of safety assessment with respect to human consumption. P35S and T-nos are the most common promoter and terminator, respectively, used in the transformation of papaya for various GM papaya traits (Tecson Mendoza, Laurena, & Botella, 2008). To monitor papaya products for other GM papaya lines with a different event-specific sequence from that of 55-1, cis-element-specific primers and probe for detecting P35S and T-nos were used (Table 1). As shown in Table 4, GM papaya, which is not 55-1, was found in pickled papaya A and B, papaya-leaf tea A and B and jam A. The GM papaya was identified as PRSV-YK using PRSV-YK's construct-specific real-time PCR detection method. The application of a real-time PCR system using P35S, as well as T-nos, could be used for monitoring papaya products for possible contamination with GM papaya lines other than 55-1.

4. Conclusions

A reliable construct- and event-specific real-time PCR detection method for 55-1 was developed. Although the quality of the purified DNA samples varied greatly among products, our method was found to be useful for detecting 55-1 in fresh fruit and eight types

of processed products containing papaya as a main ingredient. Monitoring papaya products for the presence of GM papaya was demonstrated using a P35S and T-nos real-time PCR detection method. A collaborative variability test using this technique is currently underway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2012.08.088>.

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Supplementary table 1

Estimated purity and yield of DNA purified from processed papaya products.

Product	Lot no.	Absorbance ratio		DNA Conc. (ng/μL)	DNA (ng)/Sample (g)	Processed place	Ingredients indicated on the product
		A_{260}/A_{280}	A_{260}/A_{230}				
Canned	A	1.77	1.60	26.95	72.84	Philippines	pineapple, papaya, nata-de-coco, sugar, citric acid
	B	2.07	0.65	1.51	3.72	Thailand	pineapple, papaya, guava, sugar, passion fruit juice, citric acid
	C	1.84	1.65	47.33	108.22	Thailand	papaya, pineapple, sugar, flavor, calcium lactate
Pickled	A	1.74	1.32	1124.25	1784.52	Japan	papaya, soy sauce, sugar, salt, red pepper
	B	1.80	1.87	513.17	931.34	Japan	papaya, sugar, salt, soy sauce, Miso
	C	1.86	2.15	520.03	922.86	Japan	papaya, soy sauce, sugar, vinegar, salt, seasoning (amino acids), sweetener (stevioside)
	D	1.57	0.93	163.35	271.80	Japan	papaya, seaweed, soy sauce, vinegar, sweet sake, sugar
Dried fruit (sulfured)	A	2.18	0.36	2.21	4.35	Thailand	banana, papaya, raisin, prune, sugar, plant oil, banana flavor, preservative, bleach
	B	1.43	0.41	2.77	5.22	Thailand	sugar, papaya, bleach
	C	1.90	0.45	3.69	12.88	Thailand	papaya, sugar, bleach
	D	1.56	0.42	1.93	4.61	Unites States	sugar, papaya, bleach
	E	ND	ND	ND	ND	Thailand	papaya, sugar, preservative, lime juice, lemon flavor
Dried fruit (Un sulfured)	F	1.85	1.78	1566.32	3098.56	Philippines	papaya, sugar, preservative
	G	1.84	2.29	1621.00	3013.01	Sri Lanka	papaya
	H	1.84	2.14	1083.39	2132.66	Sri Lanka	papaya
	I	1.87	1.88	1475.64	26588.11	Japan	papaya
	J	1.87	2.11	1326.16	13130.30	Japan	papaya
	K	1.78	1.75	8313.34	82719.80	Japan	papaya
Papaya-leaf tea	A	1.79	1.78	2655.83	25173.74	Japan	papaya-leaf
	B	1.84	2.19	161.19	1653.23	Japan	papaya-leaf
	C	1.60	1.28	27.47	271.98	Japan	papaya
Jam	A	1.77	0.60	10.13	13.08	Japan	papaya, flat lemon, sugar, cassava
	B	1.92	0.79	7.82	5.18	Japan	papaya, sugar, pectin, lemon juice
	C	1.94	2.37	314.56	929.73	Japan	papaya, sugarcane vinegar, sugarcane sugar, salt, seasoning
	D	0.92	0.14	2.12	1.39	Japan	papaya, sugar, passion fruit
	E	ND	ND	ND	ND	Kenya	sugar, hibiscus, papaya
	F	1.54	0.36	13.95	9.14	Unknown	papaya, sugar, apple, ginger, lemon juice, pectin, vitamin C
	G	1.49	0.60	32.85	21.58	Japan	papaya, sugar, lemon juice
Puree	A	2.33	0.65	4.42	5.61	Colombia	papaya juice, sugar, citric acid, vitamin C
	B	1.75	0.51	16.33	10.67	France	papaya, sugar
	C	2.53	0.89	12.46	8.30	Unknown	papaya, sugar
Juice	A	1.99	0.13	0.79	0.44	South Africa	papaya, pineapple juice, sugar, citric acid, vitamin C
	B	0.96	0.44	1.39	0.42	Spain	papaya, mango, sugar, vitamin C
	C	1.61	0.83	2170.38	3059.03	Taiwan	papaya juice, powdered milk, sugar, carrot juice, emulsifier
	D	2.52	0.70	1.32	0.14	Japan	papaya, lemon, papaya juice, sugar, vitamin C, vitamin A, seasoning, emulsifier, flavor, sweetener
	E	1.47	0.66	213.47	240.67	Taiwan	papaya juice, powdered milk, sugar, preservative, emulsifier, flavor, carotene
Frozen dessert	A	1.87	2.22	1735.94	3745.29	Unknown	papaya, sugar, preservative, lemon juice
	B	1.87	2.18	1483.75	3187.43	Unknown	papaya, sugar, lemon juice, preservative

ND, not detected

Supplementary table 2

55-1 real-time PCR detection test for monitoring processed papaya products.

Product	Endogenous <i>Chy</i> (72 bp)	Line 55-1		Transgenic construct (copies)/ <i>Chy</i> (copies)	Agreement ^a (%)
		Event (71 bp)	Construct (75 bp)		
Canned	3/3	0/3	0/3	0	100
Pickled	3/4	0/4	0/4	0	100
Dried fruit (sulfured)	0/5	0/3	0/3	0	100
Dried fruit (unsulfured)	6/6	0/10	0/10	0	100
Papaya-leaf tea	3/3	1/3	1/3	0.0027	100
Jam	3/7	1/7	1/7	0.0008	100
Puree	3/3	0/3	0/3	0	100
Juice	4/5	0/5	0/5	0	100
Frozen dessert	2/2	0/2	0/2	0	100

^aAgreement based on duplicate tests using three independently purified DNA templates by event- and construct-specific methods.