Table 2. Results of the analyses of individual kernels in all grains of the non-IP maize samples in 2009.

GM trait	Non-IP maize sample lot										Total	
	1		2		3		4		5			
	Kernel number	Content (%)	Kernel number	Content (%)	Kernel number	Content (%)	Kernel number	Content (%)	Kernel number	Content (%)	Kernel number	Content (%)
Bt11	3	1.0	2	0.8	5	2.4	8	3.6	2	0.9	20	1.7
TC1507	4	1.4	16	6.8	15	7.1	7	3.2	13	5.7	55	4.6
MON810	11	3.8	8	3.4	40	18.9	19	8.6	11	4.8	89	7.5
MON863	8	2.8	4	1.7	2	0.9	1	0.5	8	3.5	23	1.9
MON88017	97	33.6	28	11.8	8	3.8	26	11.8	60	26.2	219	18.4
GA21	1	0.3	0	0	1	0.5	2	0.9	0	0	4	0.3
NK603	18	6.2	21	8.9	22	10.4	24	10.9	18	7.9	103	8.7
DAS59122	11	3.8	8	3.4	1	0.5	2	0.9	7	3.1	29	2.4
MIR604	6	2.1	1	0.4	1	0.5	1	0.5	1	0.4	10	0.8
T25	2	0.7	0	0	1	0.5	1	0.5	1	0.4	5	0.4
Single GM	161	55.7	88	37.1	96	45.3	91	41.2	121	52.8	557	46.9
Bt11×MIR604	0	0	0	0	0	0	4	1.8	3	1.3	7	0.6
Bt11×GA21	2	0.7	0	0	1	0.5	2	0.9	0	0	5	0.4
TC1507×MON88017	2	0.7	8	3.4	9	4.2	2	0.9	4	1.7	25	2.1
TC1507×NK603	4	1.4	4	1.7	6	2.8	2	0.9	0	0	16	1.3
TC1507×DAS59122	21	7.3	17	7.2	0	0	8	3.6	16	7.0	62	5.2
MON810×MON863	1	0.3	4	1.7	0	0	4	1.8	1	0.4	10	0.8
MON810×MON88017	42	14.5	54	22.8	24	11.3	52	23.5	26	11.4	198	16.7
MON810×NK603	7	2.4	8	3.4	26	12.3	16	7.2	3	1.3	60	5.1
MON863×NK603	0	0	0	0	2	0.9	0	0	0	0	2	0.2
MON88017×DAS59122	0	0	0	0	0	0	0	0	2	0.9	2	0.2
NK603×DAS59122	0	0	1	0.4	2	0.9	0	0	2	0.9	5	0.4
Bt11×MIR604×GA21	0	0	0	0	0	0	2	0.9	0	0	2	0.2
TC1507×MON810×NK603	0	0	0	0	0	0	0	0	0	0	0	0
TC1507×MON88017×DAS59122	1	0.3	2	0.8	0	0	1	0.5	0	0	4	0.3
TC1507×NK603×DAS59122	0	0	1	0.4	2	0.9	3	1.4	0	0	6	0.5
MON810×MON863×NK603	2	0.7	4	1.7	1	0.5	2	0.9	3	1.3	12	1.0
Stacked GM	82	28.4	103	43.5	73	34.4	98	44.3	60	26.2	416	35.0
GM	243	84.1	191	80.6	169	79.7	189	85.5	181	79.0	973	81.9
Non-GM	46	15.9	46	19.4	43	20.3	32	14.5	48	21.0	215	18.1
Total	289	100	237	100	212	100	221	100	229	100	1,188	100

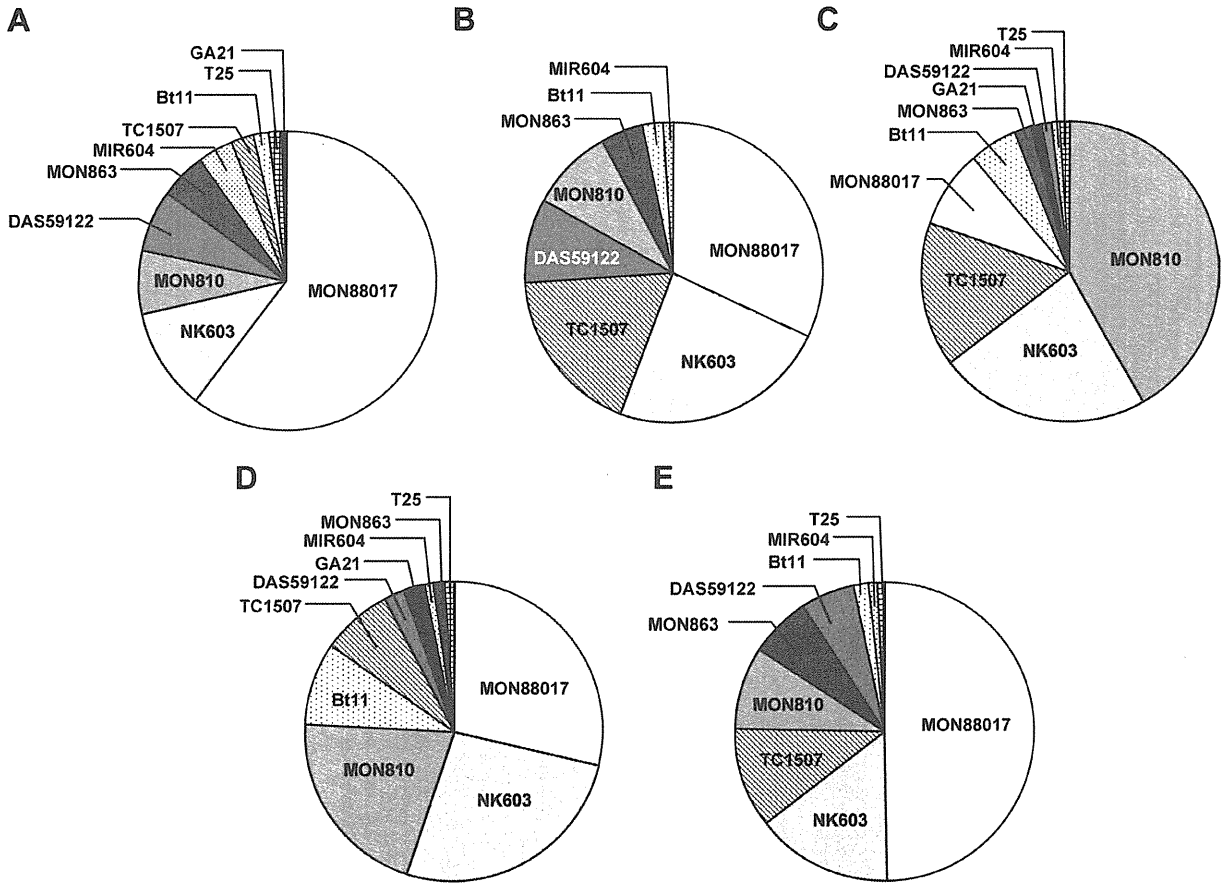

Fig. 1. Pie chart representing the single GM event grain population in five non-IP maize samples in 2009
A–E show the results for sample, No. 1–5, respectively.

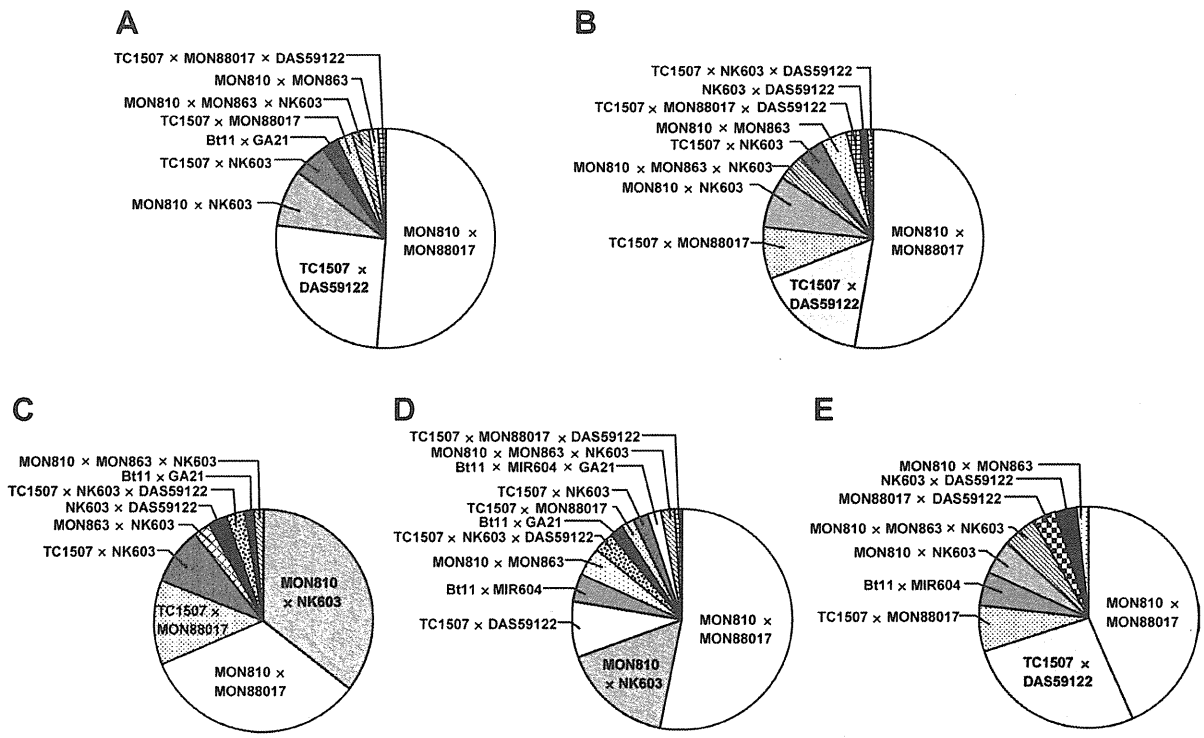

Fig. 2. Pie chart representing the stacked GM event grain population in five non-IP maize samples in 2009
A–E show the results for sample, No. 1–5, respectively.

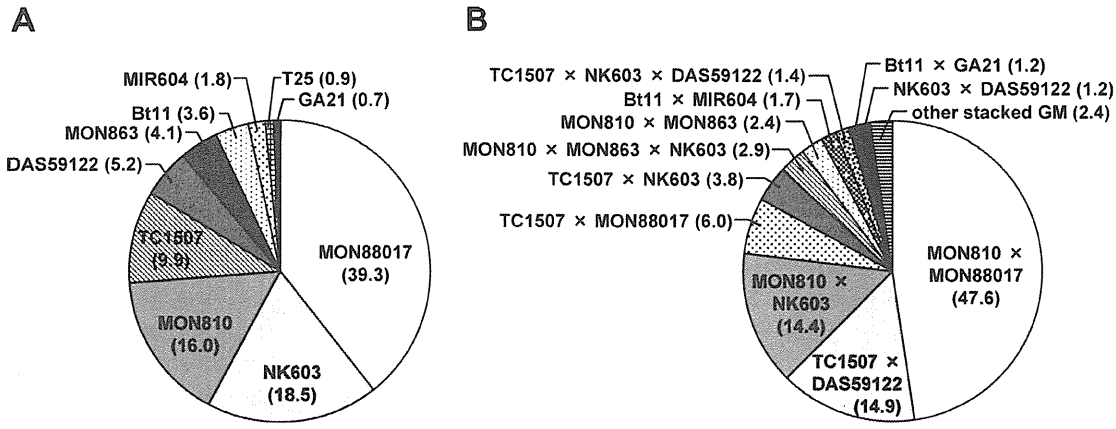

Fig. 3. Pie chart of the average populations of single GM event grains (A) and stacked GM event grains (B) in the non-IP maize kernels analyzed. The values in parentheses show the percentage of each population in the single and stacked GM event grains.

1188 kernels of non-IP samples tested in 2009 are shown in Table 2. The average percentage of GM grains in the samples was 81.9%, and, in the GM grains, the average percentage of the single GM event grains was 46.9% and that of the stacked GM event grains was 35.0%. These results show that the average ratio (35.0%) of the stacked GM event grain population in non-IP maize samples in 2009 was higher than in 2005[5] (12.0%).

The populations of single GM event grains and stacked GM event grains are indicated in the pie chart of Fig. 3. For single GM events, MON88017 (39.3%) and the NK603 grains (18.5%) were mainly detected, followed by MON810 (16.0%), TC1507 (9.9%) and DAS 59122 grains (5.2%). Most of the detected stacked GM event grains were MON810×MON88017 (47.6%), followed by the TC1507×DAS59122 (14.9%), MON810×NK603 (14.4%), TC1507×MON88017 (6.0%), TC1507×NK603 (3.8%) and MON810×MON863×NK603 (2.9%).

Discussion

To date, many GM maize events have been authorized for import into Japan. The allowed single GM events include resistance to feeding damage by the European corn borer (ECB) (Event 176 and Bt11 from Syngenta Seeds AG (formerly Novartis Seeds), MON810 from Monsanto Company), resistance to corn rootworm (*e.g.*, MON863 from Monsanto Company), tolerance to the herbicide phosphinothricin (PPT) (*e.g.*, T25 from Bayer Crop Science), resistance to the ECB and tolerance to the herbicide PPT (TC1507 from Pioneer Hi-Bred International, Inc., Mycogen Seeds/Dow AgroSciences LLC), tolerance to the herbicide glyphosate (GA21 and NK603, Monsanto Company), resistance to corn rootworm and tolerance to the herbicide glyphosate (MON88017, Monsanto Company), resistance to corn rootworm and tolerance to the herbicide PPT (DAS59122 from Dow AgroSciences LLC/Pioneer Hi-Bred International, Inc.). Furthermore, many stacked event maize varieties (*e.g.*, MON863×NK603, MON810×NK603, MON810×GA21, MON810×T25, MON88017×MON810, TC1507×NK603, MON863×MON810 and MON863×MON810×NK603) have already been authorized in Japan.

In this study, we found that GM maize grains of non-IP maize samples produced in 2009 were present at a high level, in the range of 79.7% to 85.5%. In addition, we found that the ratio of the stacked GM event grains in the non-IP maize samples in 2009 was higher than in 2005[5]. The evidence implies that the cultured area of GM maize in the USA has increased, and in particular, the ratio of the stacked GM maize grains in non-IP maize samples has increased from 2005 to 2009.

The National Agricultural Statistics Service (NASS) reported that the percentage of the cultivation area planted with any GM maize events in 2005, 2008 and 2009 in the USA was 52%, 80% and 85%, respectively, and the percentage for stacked GM events in 2005, 2008 and 2009 was 9%, 40% and 46%, respectively[*9, *10]. Thus, the percentage of the cultivation area planted with GM maize events has increased substantially from 2005 to 2009. In particular, the area planted with GM maize events in Illinois, Indiana and Ohio has increased more than that in other states. In addition, the percentage of the cultivation area planted with stacked GM maize events has significantly increased from 2005 to 2009. We estimated that our multiplex qualitative PCR analyses can cover almost all the GM maize events cultivated in 2009, since it has been reported that other authorized GM maize events such as DBT41 and DDL25 are not cultivated anymore. Thus, it appears that our results are reasonable and probably reflect the average GM grain mixing in 2009.

For the analyses of GM events, we clarified that MON88017 grains (39.3%) and NK603 grains (18.5%) were the major single GM events and that MON88017×MON810 grains were the major stacked GM event in non-IP samples in 2009. We could not follow the

[*9] Acreage, No. 06.30.2009, 2009, National Agricultural Statistics Service, Agricultural Statistics Board, USA. Department of Agriculture.

[*10] http://www.ers.usda.gov/Data/BiotechCrops/ExtentofAdoptionTable1.htm

MON89034 event because a detection method for the MON89034 event had not yet been developed. However, we considered that MON89034 had not yet been widely cultivated in 2009 because the event was authorized worldwide from 2008 to 2010.

This study showed that the major cultured single GM maize event changed from MON810 to MON88017 and the major stacked GM event changed from MON810×NK603 to MON88017×MON810 between 2005[5] and 2009. The MON88017 event maize has both resistance to corn rootworm and tolerance to the herbicide glyphosate, and MON810 has resistance to the ECB. Therefore, MON88017×MON810 event maize has three advantageous features: resistance to corn rootworm, tolerance to the herbicide glyphosate and resistance to the ECB. Therefore, we presumed that the MON88017×MON810 event maize may be more productive than other conventional events and so the area planted with MON88017×MON810 may have been increased. So far, there is little information on the ratio of areas planted with each GM maize event, or which GM maize event is the main planted maize in the USA. NASS reported that the percentage of the cultivation area planted with insect-resistant (Bt) traits in 2005, 2008 and 2009 in the USA was 26%, 17% and 17%, respectively, and the percentage of herbicide-tolerant traits in 2005, 2008 and 2009 was 17%, 23% and 22%, respectively[*9, *10]. The data do not indicate which events for insect-resistant (Bt) traits or herbicide-tolerant traits were planted. However, the data suggests that herbicide-tolerant traits are becoming more popular than insect-resistant (Bt) traits over the period from 2005 to 2009.

In addition, Marra *et al.* reported a survey of the average shares of total corn acres planted by the survey respondents to each GM maize event by agronomic zone in the USA in 2009. The report indicated that MON88017×MON810 (Monsanto's YieldGard VT Triple hybrids) made up the largest share of total planted acres in the survey in 2009[8]. The stacked GM maize event with the second largest share by agronomic zone varied, with MON810×NK603 (TieldGard Con Borer Roundup Ready hybrids) in the western corn belt agronomic zone (13.8%), and TC1507×DAS59122 (Herculex Xtra) in the central corn belt agronomic zone (8.2%) and the east corn belt agronomic zone (13.1%). Among single GM maize events, NK603 made up 15% of planted corn acres in the western corn belt agronomic zone, 12.2% in the central corn belt agronomic zone, and 13.2% in the east corn belt agronomic zone[8]. According to this information, we considered that MON88017×MON810 is the largest share event and TC1507×DAS59122 or MON810×NK603 is the second largest share event among stacked GM maize events, and NK603 is the largest share event as a single GM maize event, in GM maize planted in the USA in 2009. Considering the survey[8] and Fig. 3, we presumed that MON88017 grains or MON810 grains mainly detected as single GM events in the present study could be derived from the cultivation of MON88017×MON810 maize seeds. Consequently, it appears that the results of the present analysis of GM maize events are reasonably consistent with the data on actual planting in the USA in 2009 (Fig. 3).

It should be noted that there are limitations to the present study in terms of estimation of the ratio of GM maize content and GM maize events in a non-IP maize sample. There are many factors that would influence the estimation, such as the time and place of the sampling for non-IP maize. The present study is only based on analyses of limited numbers of samples obtained in 2009 in Japan. However, to our knowledge, this is the first report of analysis of GM maize content and GM maize events in recent maize samples on a kernel basis, except for our study published in 2008[5]. Although, the samples used in the present study may not be a representative group, they provide important information about the GM maize content and the main GM maize events in non-IP maize samples in 2009.

In conclusion, we successfully determined the GM maize grain content on a kernel basis in non-IP maize samples imported from the USA in 2009 using the individual kernel detection system[2, 3]. In addition, we analyzed the GM events in GM maize grains of non-IP maize samples in 2009 using two multiplex qualitative PCR detection methods[4, 6] coupled to microchip electrophoresis and partially real-time PCR array analyses[7]. MON88017 and NK603 were the major single GM events and MON88017×MON810 was the major stacked GM event in the non-IP samples. This type of study should provide useful information on GM maize mixing in imported maize samples on a kernel basis and the method permits precise quantification of the GM maize content in GM maize kernels for labeling regulation. It will be necessary to obtain the latest information on the GM maize ratio and GM maize events in non-IP maize grains to investigate the level of GM mixing and the probability of stacked GM maize mixing.

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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.
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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 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 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 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

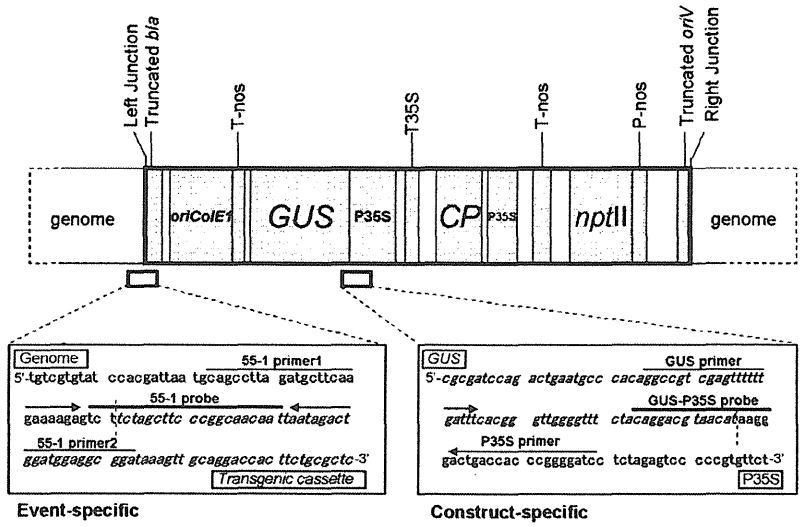

Fig. 1. Transgenic construct in 55-1. Positions of real-time PCR primers and probe for event- and construct-specific detection are depicted as two arrows and a bar, respectively. Oligonucleotide sequences of the primers and probe are shown underneath. The transgenic construct contained oriColE1 (origin 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/μl) 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 (ΔRn) 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 ΔRn 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 ΔRn 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 (ΔΔCt) between GM papaya cultivars were obtained, and the relative copy number of the transgenic construct was calculated using the following formula:

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

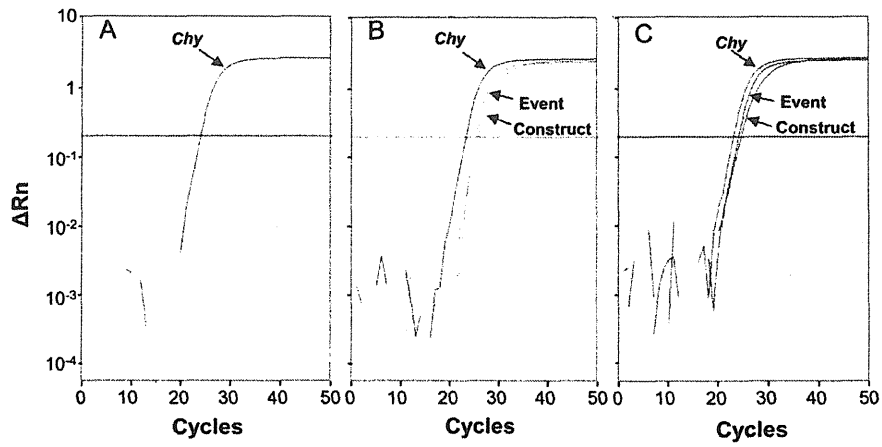

Fig. 2. Amplification plots of real-time PCR. DNA templates purified from Sunset (A), Rainbow (B), or SunUp (C) were used for real-time PCR using a primer and probe set for an endogenous *Chy* and 55-1 detection method.

Table 1
Oligonucleotide primers and probes used in this study.

Target	Primer name	Sequence (5′–3′)	Amplicon (bp)	Reference
Endogenous gene (*Chy*)	Q-Chy-1 F2	CCATGCGATCCTCCCA	72	This study
	Q-Chy-2R	CATCGTAGCCATTGTAACACTAGCTAA		
	Q-Chy-P′	FAM-TTCCCTTCAT(BHQ1)CCATTCCCACTCTTGAGA-Ⓟ		
Event-specific sequence (55-1)	55-1 primer1	CAGCCTTAGATGCTTCAAGAAAAGA	71	This study
	55-1 primer2	TCCGCCTCCATCCAGTCTATT		
	55-1 probe	FAM-TCTTCTAGCTTCCCGGCAACAAT-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	Waiblinger, 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, Waiblinger, and Grohmann (2007)
	180-R	TTGTTTTCTATCGCGTATTAAATGT		
	TM-180	FAM-ATGGGTTTTTATGATTAGAGTCCCGCAA-TAMRA		

Ⓟ, 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 (ΔCt at a threshold value of 0.2) obtained using event- /construct-specific methods and the *Chy* detection method were calculated. The ΔCt 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 ΔCt values (ΔΔCt) 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	*Chy*	Event	Construct	ΔCt_{Event}		$\Delta\Delta Ct_{Event}$		Relative copy No.	$\Delta Ct_{Construct}$		$\Delta\Delta Ct_{Construct}$		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.

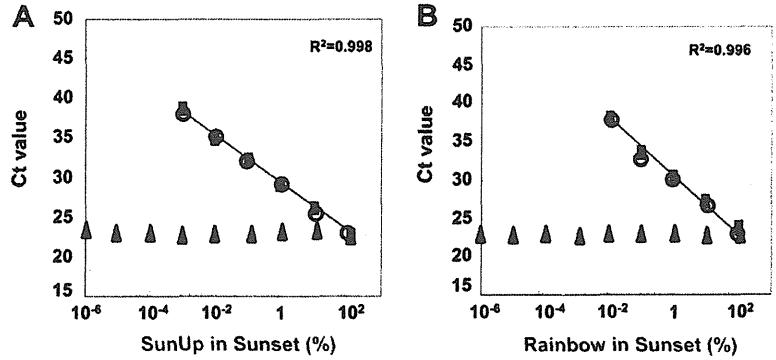

Fig. 3. Detection limits of 55-1. DNA template (25 ng) containing 0.000001%, 0.00001%, 0.0001%, 0.001%, 0.01%, 0.1%, 1%, and 10% (w/w) of DNA purified from SunUp (A) or Rainbow (B) was diluted with Sunset DNA and used for real-time PCR. Event-specific (filled square), construct-specific (open circle) 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, Diolez, 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 ⩽5% and a RSD of reproducibility of ⩽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 ⩽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 (%)		
	Chy	Event	Construct	*Chy*	Event	Construct	*Chy*	Event	Construct	*Chy*	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 (Unsulfured)	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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