Identification and Detection of Genetically Modified Papaya Resistant to Papaya Ringspot Virus Strains in Thailand

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Many lines of genetically modified (GM) papaya (*Carica papaya* Linnaeus) have been developed worldwide to resist infection from various strains of papaya ringspot virus (PRSV). We found an unidentified and unauthorized GM papaya in imported processed papaya food. Transgenic vector construct that provides resistance to the PRSV strains isolated in Thailand was detected. An original and specific real-time polymerase chain reaction method was generated to qualitatively detect the PRSV-Thailand-resistant GM papaya.

Key words genetically modified organism; papaya; polymerase chain reaction; genomic DNA

Papaya (*Carica papaya* Linnaeus) is a widely grown fruit crop in tropical and subtropical areas.[1] A major constraint on papaya production has been infection by papaya ringspot virus (PRSV), which reduces papaya yields.[2] In response to this problem, genetically modified (GM) papaya that resists PRSV infection has been developed. Since the first successful generation of GM papaya in 1991,[3,4] many GM papaya lines, carrying different transgenic vector constructs, have been planted in various places, such as Hawaii, Florida, China, Jamaica, Taiwan, Thailand, Australia, Malaysia, the Philippines and Vietnam.[1]

Since Japan announced a mandatory safety assessment of GM foods, and processed foods containing GM ingredients, on April 1, 2001, foods on the Japanese market have been monitored for contamination with unauthorized GM products. We have developed qualitative detection methods for various GM crops, such as potato,[5,6] maize,[7–10] rice,[11,12] and flax,[13] using polymerase chain reaction (PCR) methods. For papaya, GM papaya line 55-1, a commercialized PRSV-resistant GM papaya developed in Hawaii, was the first authorized GM fruit allowed in Japan after a safety assessment by Food Safety Commission of Japan in 2009.[14] Subsequently, the use of any other GM papaya lines in foods has been prohibited. In 2011, an unauthorized GM papaya line, PRSV-YK, which was developed to resist the YK strain of PRSV in Taiwan, was found in some processed papaya products (papaya leaf tea, pickles and jam) on the Japanese market. The transgenic construct for PRSV-YK is similar to that of the GM papaya line 16-0-1/17-0-5 developed in Taiwan.[15] To monitor foods containing GM papaya on the Japanese market, we developed a qualitative detection method for GM papaya line 55-1 and for PRSV-YK using real-time PCR.[16–18] In addition, a histochemical assay[19] was developed to identify GM papaya line 55-1. Unauthorized GM papaya from Thailand has been found in Europe since 2012. Consequently, some papayas were rejected at the borders of some European countries.[20] Japan imports many papayas from overseas, thus it is required to monitor commercially processed products that include papaya as a major ingredient for contamination with the unauthorized GM papaya lines. In the present study, the presence of a new unauthorized GM papaya, PRSV-SC, was found in a processed papaya commodity (dried papaya) in Japan, and a new detection method for detecting PRSV-SC was developed using real-time PCR.

MATERIALS AND METHODS

Papaya Samples Processed papaya product (dried papaya) was purchased over the Internet in Japan. Hawaiian non-GM papaya (Sunset) fruit was purchased from a Japanese trade agency *via* the Hawaii Papaya Industry Association through the Consumer Affairs Agency, Government of Japan.

Purification of DNA Dried papaya was ground using a mixing mill. DNA was extracted and purified from 2 g of the samples using an ion-exchange resin-type DNA extraction and purification kit (Genomic-tip; Qiagen, Hilden, Germany) according to a previous report.[16] The purified DNA was quantified by measuring UV absorption at 260 nm using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.).

PCR Each PCR reaction mixture (50 μL) contained 5 μL 10× cloned *Pfu* reaction buffer (Agilent Technologies, Santa Clara, CA, U.S.A.), 0.18 mM deoxyribonucleotide triphosphate (dNTP) (Agilent Technologies), 0.3 μM forward and reverse primers, 2.5 U *Pfu Turbo* DNA polymerase (Agilent Technologies) and 50 ng template DNA. The PCR conditions were 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. A final terminal elongation occurred at 72°C for 10 min. PCR was performed using the GeneAmp PCR System 9700 (Life Technologies, Carlsbad, CA, U.S.A.). To determine the nucleotide sequence of the transgenic vector construct harbored in the GM papaya, DNA fragments were amplified by PCR using the following primer set:

Forward primer: 5′-GAC ATC TCC ACT GAC GTA AGG G-3′ (p324)

Reverse primer: 5′-CTATCRCTC TCT CCA GTT TTT G-3′ (p323)

DNA Sequencing PCR-amplified DNA fragments were extracted from the agarose gels and purified using a QIAquick PCR purification kit (Qiagen). The fragments were directly sequenced from both strands using forward and reverse primers with an ABI PRISM 3700 DNA analyzer and BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies), according to the manufacturer's instructions. Nucleotide se-

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quences were analyzed using GENETYX software ver. 10.0.3 (GENETYX Corp., Tokyo, Japan).

Real-Time PCR Real-time PCR assays were performed using the ABI PRISM 7900 Sequence Detection System (Life Technologies). The 25 μL reaction mixture consisted of 2.5 μL sample DNA solution (25 ng), 12.5 μL Gene Expression Master Mix (Life Technologies), 0.8 μM forward and reverse primers, and 0.1 μM probe. The PCR conditions were 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. GM papaya was detected using the following primers and probe:

Forward primer: 5′-CAT TTC ATT TGG AGA GAA CAC G-3′ (SC-F)

Reverse primer: 5′-ACC AGC ATC CAC AGC TTC-3′ (SC-R)

Probe: 5′-FAM-ACT CTA GAG GAT CCA TGT CCA A-TAMRA-3′ (SC-P)

To detect the papaya endogenous internal control gene, *chymopapain* (*Chy*; GenBank accession no. AY803756), the fol-

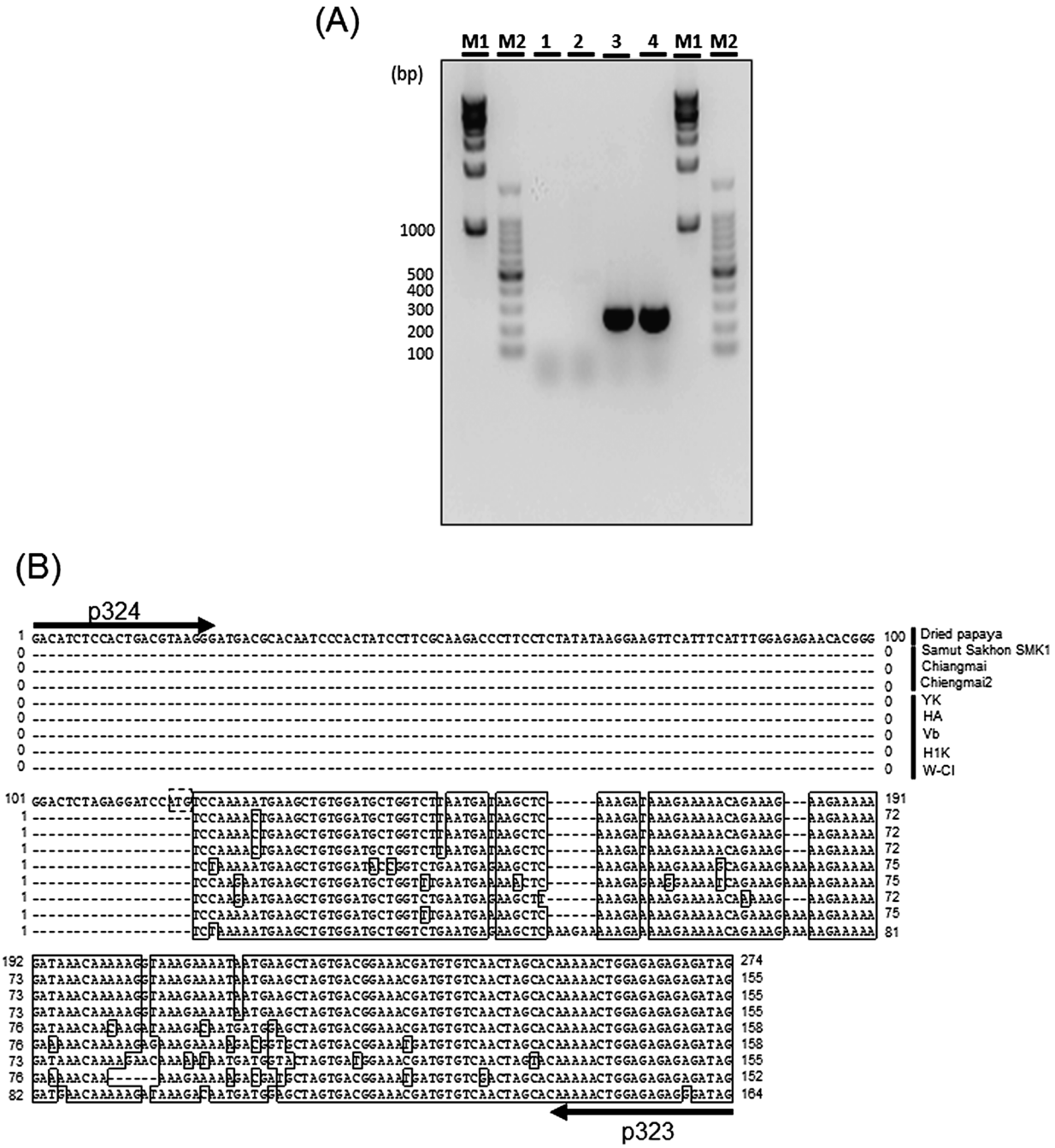

Fig. 1. PCR Targeting Construct-Specific Sequence of Papaya Ringspot Virus (PRSV)-Resistant Genetically Modified (GM) Papaya (*Carica papaya* Linnaeus)

(A) DNA templates (lane 1, no DNA was added; lane 2, non-GM papaya (Sunset); lanes 3 & 4, dried papaya product) were used for the PCR test using the primer set, p324 and p323, that hybridize to the cauliflower mosaic virus (CaMV) 35S promoter and the PRSV's coat protein (CP) gene, respectively. The PCR-amplified products were run on a 2% (w/v) agarose gel. Lane M1, 1-kbp DNA ladder marker, lane M2, 100-bp DNA ladder marker (B) Nucleotide sequence alignment of PRSV CP cloned from various isolates and strains (Samut Sakhon SMK1 [Thailand]; Chiangmai [Thailand]; Chiengmai2 [Thailand]; YK [Taiwan]; HA [Hawaii]; Vb [China]; H1K [Florida]; W-CI [Taiwan]) and the PCR products obtained using dried papaya product. Homologous sequences are boxed. The start codon for the GM papaya is boxed with a dashed line. Numerals beside the sequences indicate the number of nucleotides from the 5′ terminus. Arrows indicate the position of the primers p324 and p323.

lowing primers and probe were used according to a previous report[17]:

Forward primer: 5′-CCA TGC GAT CCT CCC A-3′ (Q-Chy-1F2)

Reverse primer: 5′-CAT CGT AGC CAT TGT AAC ACT AGC TAA-3′ (Q-Chy-2R)

Probe: 5′-FAM-TTCCCTTCAT(BHQ1)CCA TTC CCA CTC TTG AGA-3′ (Q-Chy-P)

BHQ1 (black-hole quencher 1) was labeled in Q-Chy-P at the thymidine underlined in the nucleotide sequence.

All primers and probes were diluted with an appropriate volume of distilled water and stored at −20°C until used. Each real-time PCR reaction was tested in duplicate. Results were analyzed using SDS 2.1 sequence detection software (Life Technologies) for the ABI PRISM 7900 Sequence Detection System.

RESULTS AND DISCUSSION

Identification of Unauthorized GM Papaya To investigate the potential contamination of processed papaya products with a new unauthorized GM papaya, we used genomic DNA purified from the products as the PCR template. The primer set p323 and p324 was used as previously reported.[16] The forward primer (p324) was designed to hybridize to the sequence of the most common promoter, the cauliflower mosaic virus (CaMV) 35S promoter, which is used in the transformation of papaya to alter various papaya traits.[1] The reverse primer (p323) was designed to hybridize to the highly conserved sequence of the coat protein (CP) gene, which has been cloned from various isolates and strains of PRSV (GenBank accession nos. Samut Sakhon SMK1, DQ085864; Chiangmai, DQ085856; Chiengmai2, AY010720; YK, X97251; HA, S46722; Vb, AF243496.1; H1K, AF196839.1; W-CI, AY027810.2). When using DNA purified from a dried papaya product as the template, electrophoresis of the PCR products showed a single band of 200–300 bp (Fig. 1A). The DNA purified from the non-GM papaya (Sunset) as a control generated no PCR products of this length. A direct sequence analysis of the PCR product and a BLASTn analysis indicated that the 3′ end sequence was homologous to the CP gene in some Thai PRSV isolates (Samut Sakhon SMK1, Chiangmai, Chiengmai2), except that the fourth codon after the start codon had a single nucleotide polymorphism (“aat” in the dried papaya and “act” in Samut Sakhon SMK1, Chiangmai, and Chiengmai2) (Fig. 1B). The predicted amino acid se-

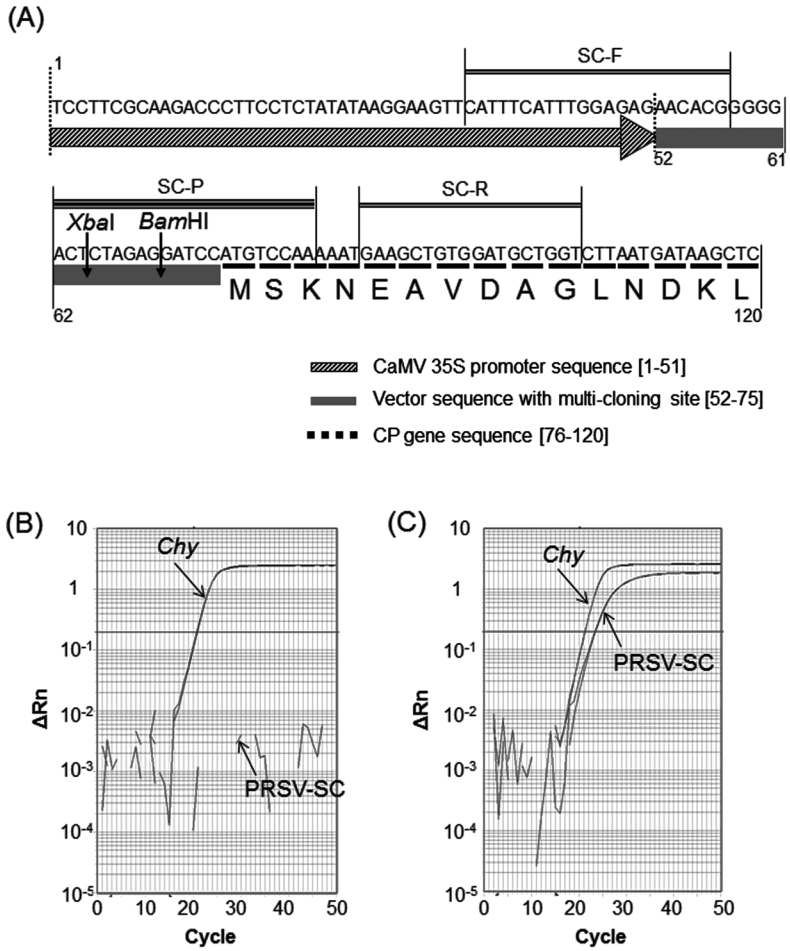

Fig. 2. Detection of a Papaya Ringspot Virus (PRSV)-Resistant Genetically Modified (GM) Papaya (*Carica papaya* Linnaeus) Line, PRSV-SC, Using Real-Time PCR

(A) A fragment of the transgenic vector construct sequence from the line PRSV-SC was obtained; restriction sites are marked by vertical arrows. The primers (SC-F and SC-R) and probe (SC-P) designed for detecting the construct-specific sequence of PRSV-SC are indicated by lines above the sequence. Numerals indicate the number of nucleotides from the 5′ terminus. DNA purified from non-GM papaya (Sunset) (B) and the PRSV-SC contaminated dried papaya product (C) were tested using real-time PCR for endogenous *Chy* detection with primer set (Q-Chy-1F2 and Q-Chy-2R) and probe (Q-Chy-P) and for PRSV-SC detection with SC-F, SC-R and SC-P. The threshold value was set at 0.2. Positive amplification curves are designated by arrows.

quences of the CP gene were identical to that of Thailand's PRSV isolates.[21] Furthermore, a multiple cloning site (containing restriction sites for *Xba*I and *Bam*HI) and the insertion of a start codon between the CaMV 35S promoter and the N-terminus of the CP gene were detected (Fig. 2A). The transgenic vector construct sequences of the CaMV 35S promoter and the CP gene in the GM papaya developed in Hawaii (line 55-1, GenBank accession no. FJ467933.1) and Taiwan (lines 16-0-1, 17-0-5)[15] showed no similarity (data not shown) to the sequence from the dried papaya product. In addition, our preliminary study using real-time PCR showed that the dried papaya product also contained high concentrations of two transgenic sequences, the *nopaline synthase* terminator, which is used in transgenic cassettes, and *neomycin phosphotransferase II*, which is used as a selectable marker (data not shown). These results suggest that the dried papaya product was contaminated with a new unauthorized GM papaya line, which we named PRSV-SC.

Designing a Novel Construct-Specific Detection Method for PRSV-SC DNA fragmentation occurs during the manufacturing of processed foods.[22,23] To qualitatively detect PRSV-SC in processed foods, specific primers and a probe for a real-time PCR assay were designed to produce a short amplicon (70 bp) based on the detected transgenic construct sequence. Also, to prevent false-negative results using real-time PCR, the PRSV-SC detection method was designed to generate a target amplicon shorter than the endogenous papaya *Chy* detection method (amplicon size 72 bp). The primers were designed to amplify the region between the transgenic vector backbone and the CP gene sequence. The probe (SC-P) was designed to target the site of the CP gene's start codon (Fig. 2A).

The real-time PCR assay for PRSV-SC detection confirmed that the dried papaya was positive for PRSV-SC, producing Ct values of 23.48 and 23.34 with a threshold value of 0.2 in a duplicate test (Fig. 2C). Endogenous *Chy* detection was positive for all samples, with Ct values of 21.27 and 21.28 for the dried papaya and 20.77 and 20.87 for the non-GM papaya (Sunset), each with a threshold value of 0.2 in a duplicate test (Figs. 2B, C). No amplification signals were obtained from the non-template control, from genomic DNA derived from 14 other crops (maize, rice, soybean, flax, canola, chickpea, wheat, sugarbeet, cottonseed, potato, papaya, tomato, eggplant, and green pepper), or from other GM papaya lines, such as 55–1 (Hawaii) and PRSV-YK (Taiwan), using the developed PRSV-SC detection method (data not shown). These results indicated that the method is specific for detecting PRSV-SC.

In the present study, as a result of monitoring processed foods for contamination with unauthorized GM papaya, we found a dried papaya product containing a transgenic vector construct for the expression of PRSV's CP gene, which was cloned from isolates in Thailand. A novel construct-specific real-time PCR detection method was developed for detecting PRSV-SC. Because the genetic background of PRSV-SC was unknown, it was not possible to estimate the content level of PRSV-SC in the papaya product. Further studies are underway to determine the PRSV-SC detection limits of the real-time PCR, and whether the qualitative PCR method using the p323/p324 primer set can function as an initial screening for the presence of GM papaya in foods.

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