Identification and Detection Method for Genetically Modified Papaya Resistant to Papaya Ringspot Virus YK Strain

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Unauthorized genetically modified (GM) papaya (*Carica papaya* LINNAEUS) was detected in a commercially processed product, which included papaya as a major ingredient, in Japan. We identified the transgenic vector construct generated based on resistance to infection with the papaya ringspot virus (PRSV) YK strain. A specific detection method to qualitatively monitor papaya products for contamination with the GM papaya was developed using the real-time polymerase chain reaction.

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

Papaya (*Carica papaya* LINNAEUS) is an important fruit crop in tropical and subtropical areas.[1)] Infection with the papaya ringspot virus (PRSV) causes disastrous damage to papaya harvests.[2)] In response to this problem, genetically modified (GM) papayas have been developed in various places, such as Hawaii, Florida, China, Jamaica, Taiwan, Thailand, Australia, Malaysia, Philippines and Vietnam.[2)]

Japan has announced a mandatory safety assessment of GM foods and processed foods containing GM ingredients, and the importation of any unauthorized GM foods to Japan has been prohibited since April 1, 2001. Therefore, the establishment of qualitative detection methods for unauthorized GM foods was required for monitoring purposes. We previously developed and reported qualitative detection methods for various GM crops, such as potato,[3,4)] maize,[5—8)] rice,[9)] and flax,[10)] using polymerase chain reaction (PCR) methods. In the case of papaya, we established a qualitative detection method for GM papaya (Line 55-1), which was the first commercialized PRSV-resistant GM papaya developed in Hawaii, using a PCR test and a histochemical assay.[11—13)] A safety assessment for Line 55-1 by the Food Safety Commission of Japan was finished in 2009.[14)] Since Japan imports many papayas from Southeast Asia, we are required, in Japan, to monitor commercially processed products that include papaya as a major ingredient for contamination with other unauthorized GM papayas generated in the region.

GM papayas carry the transgenic vector construct generated based on resistance to PRSV infection by expressing the PRSV's coat protein (CP) gene. Since the other unauthorized GM papayas developed may differ in the transgenic vector construct of the authorized GM papaya (Line 55-1), we developed a method for detecting contamination with unauthorized GM papaya. In the present study, we found the unauthorized GM papaya, PRSV-YK, in processed products containing papaya as a major ingredient, papaya-leaf-tea, pickles and jam, and developed a method for the detection of PRSV-YK using the real-time PCR.

MATERIALS AND METHODS

Papaya Samples Papaya products were purchased through 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 leaves in papaya-leaf-tea, papayas in pickles and Sunset sarcocarp were ground using a mixing mill. Papaya jam was used for purification of DNA without grinding. 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) as follows: 30 ml Buffer G2 (QIAGEN), 20 μl 100 mg/ml RNase (QIAGEN) and 500 μl cellulase (Sigma-Aldrich, St. Louis, MO, U.S.A.) were added to the sample and vortexed thoroughly, then incubated at 50 °C for 1 h. The mixture was incubated at 50 °C for another 1 h after the addition of 200 μl Proteinase K (QIAGEN). During the incubation, the samples were mixed several times by inverting the tubes. The samples were then centrifuged at 3000×***g*** at 4 °C for 20 min. The supernatant was applied to a Genomic-tip 100/G column (QIAGEN), which was pre-equilibrated with 4 ml Buffer QBT (QIAGEN). The tip was washed three times with 7.5 ml Buffer QC (QIAGEN) and transferred to a fresh centrifuge tube, and 3 ml pre-warmed Buffer QF (QIAGEN) (50 °C) 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 12000×***g*** for 15 min. The pellet was rinsed with 1 ml 70% (v/v) ethanol and centrifuged at 12000×***g*** for 3 min. The supernatant was discarded and the precipitate was dried. The DNA was dissolved in 20 μl water for use in analyses. The DNA was quantified by measuring UV absorption at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.).

PCR Each PCR reaction mixture (25 μl) contained 2.5 μl 10×PCR buffer II (Life Technologies, Carlsbad, CA, U.S.A.), 0.16 mM of each deoxyribonucleotide triphosphate (dNTP) (Life Technologies), 1.5 mM $MgCl_2$, 1.2 μM forward and reverse primers, 0.8 U AmpliTaq Gold (Life Technologies) and 25 ng template DNA. The PCR conditions were as follows: 95 °C for 10 min, followed by 50 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s, extension at 72 °C for 60 s and terminal elongation at 72 °C for 7 min. PCRs were carried out using the GeneAmp PCR System 9700 (Life Technologies). To determine the nucleotide sequence of the transgenic vector construct harbored in GM

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papaya, DNA fragments were amplified by PCR using the following primer set. Forward primer: 5′-GACATCTCCA-CTGACGTAAGGG-3′ (p324). Reverse primer: 5′-CTATCR-CTCTCTCCAGTTTTTG-3′ (p323).

DNA Sequencing The PCR-amplified DNA fragments were extracted from the agarose-gel and purified using a QIAquick PCR purification kit (QIAGEN). The DNA fragments were directly sequenced from both strands using forward and reverse primers with an ABI PRISM 3700 DNA analyzer and Terminator v3.1 Cycle Sequencing Kit (Life Technologies), according to the manufacturer's instructions. Nucleotide sequences were analyzed using Lasergene version 7.2 software (DNASTAR Inc., Madison, WI, U.S.A.).

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 Universal Master Mix® (Life Technologies), 0.8 μM forward and reverse primers, and 0.1 μM probe. 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. GM papaya was detected using the following primers and probe. Forward primer: 5′-GATCCCCGGGTGGTCAGT-3′ (YK-1F). Reverse primer: 5′-CCGGTATCCACAGCTTCATTTT-3′ (YK-1R). Probe: 5′-FAM-AGACGCCATGGAAGG-MGB-3′ (YK-P).

For detecting the papaya endogenous internal control gene, *chymopapain* (*Chy*; GenBank accession No.: AY803756), we designed the following primers and probe referring to published report.[15] Forward primer: 5′-CCATGCGATCCTC-CCA-3′ (Q-Chy-1F2). Reverse primer: 5′-CATCGTAGCCA-TTGTAACACTAGCTAA-3′ (Q-Chy-2R). Probe: 5′-FAM-TTCCCTTCAT(BHQ1)CCATTCCCACTCTTGAGA-3′ (Q-Chy-P). Black-hole quencher 1 (BHQ1) was labeled for Q-Chy-P at the underlined thymidine in the nucleotide sequence.

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) for ABI PRISM™ 7900 Sequence Detection System.

Real-Time PCR Data Analysis Typically, the baseline was set to cycles 3 through 15. The ΔRn threshold for plotting the cycle threshold (Ct) values was set to 0.2 during exponential amplification. Reactions with Ct values of less than 48 and exponential amplification plots were scored as positive. If the Ct value could not be obtained, the reaction was

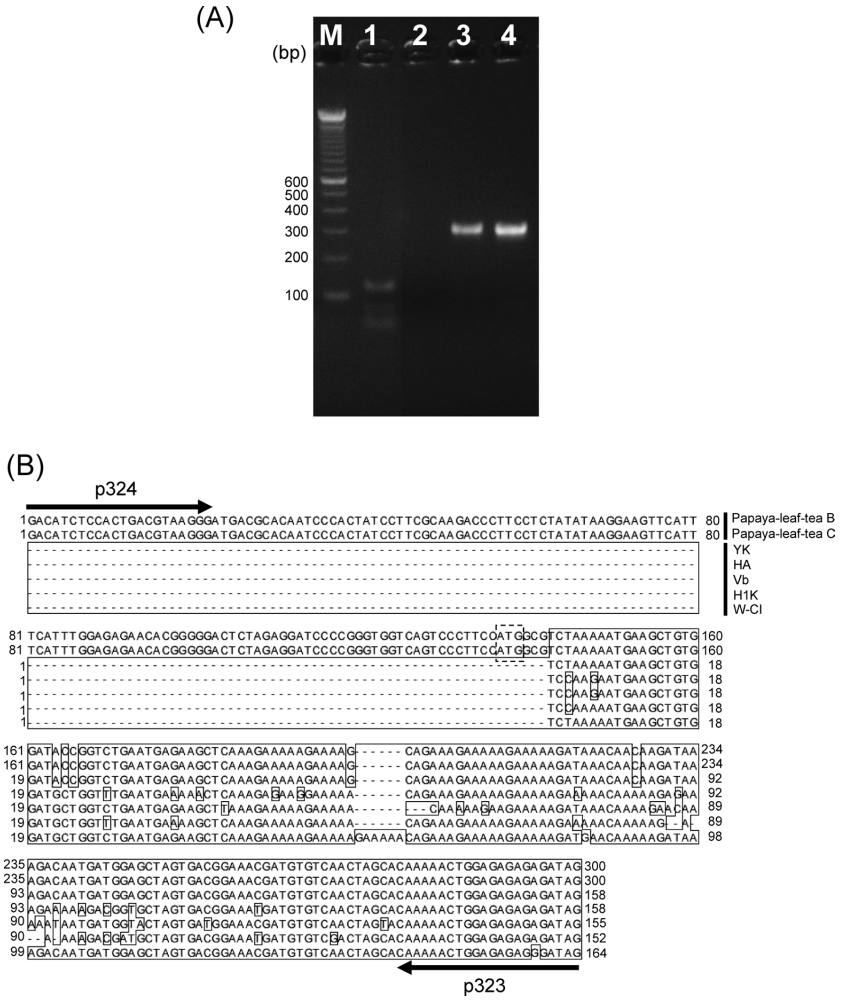

Fig. 1. PCR Targeting Construct Specific Sequence of PRSV-Resistant GM Papaya

(A) DNA templates (lane 1, non-GM papaya (Sunset); lane 2, papaya-leaf-tea A; lane 3, papaya-leaf-tea B; lane 4, papaya-leaf-tea C) were used for the PCR test using the p324 and p323 primer set. The PCR-amplified products were run on a 3% (w/v) agarose-gel. Lane M, 100-bp DNA ladder marker (B) Nucleotide sequence alignment of PRSV CP cloned from YK, HA, Vb, H1K, W-CI strains and the PCR products obtained using papaya-leaf-tea B and C. Homologous sequences were boxed. The initiation codon for the GM papaya was boxed with a dashed line. Numerals beside the sequence indicate the numbers of nucleotides from the 5′ terminus.

scored as negative. Reactions with Ct values of less than 48, but without exponential amplification as judged by visual inspection of the respective ΔRn plots and multi-component plots were scored as negative.

RESULTS AND DISCUSSION

Detection of Unauthorized GM Papaya To investigate the contamination with unauthorized GM papaya in commercially processed products, containing papaya as a major ingredient, in Japan, we used genomic DNA purified from the papaya-leaf-tea products as a template for the PCR test. The forward primer (p324) was designed to hybridize in the cauliflower mosaic virus (CaMV) 35S promoter sequence, which is the most common promoter used in the transformation of papaya for various GM papaya traits,[2] and the reverse primer (p323) was designed in the highly conserved sequence of the CP gene, which is cloned from various strains of PRSV (GenBank accession no.: YK, X97251; HA, S46722; Vb, AF243496.1; H1K, AF196839.1; W-CI, AY027810.2). Electrophoresis of the PCR products using p324 and p323 primers showed a single band of about 300 bp in length using DNA purified from two of the three papaya-leaf-tea products (papaya-leaf-tea B and C) (Fig. 1A). The DNA purified from non-GM papaya (Sunset) as a control and papaya-leaf-tea A generated no PCR products with the identical length. Direct sequence analysis of the PCR product and BLASTn analysis indicated that the 3′ end sequence was identical to the CP gene in a Taiwan isolate of PRSV (PRSV YK strain)[16] (Fig. 1B). Furthermore, the multiple cloning site (containing restriction sites for *Bam*HI and *Nco*I) and two amino acid mutations (methionine and alanine) between the CaMV 35S promoter and the N-terminus of CP gene were detected (Fig. 2A). According to the literature,[17] the design of this transgenic vector construct was identical to that of the GM papaya, which was generated to resist infection of the PRSV YK strain. These results suggest that the papaya-leaf-tea products were contaminated with the unauthorized GM papaya (PRSV-YK).

Development of a Construct-Specific Detection Method for PRSV-YK In order to qualitatively detect PRSV-YK in processed products, containing papaya as a major ingredient, with high specificity and sensitivity, we designed specific primers and a probe for a real-time PCR assay producing a short amplicon (57 bp), based on the detected transgenic construct sequence. The forward (YK-1F) and the reverse (YK-1R) primers were designed in the region between the transgenic vector backbone and the CP gene sequence. The probe

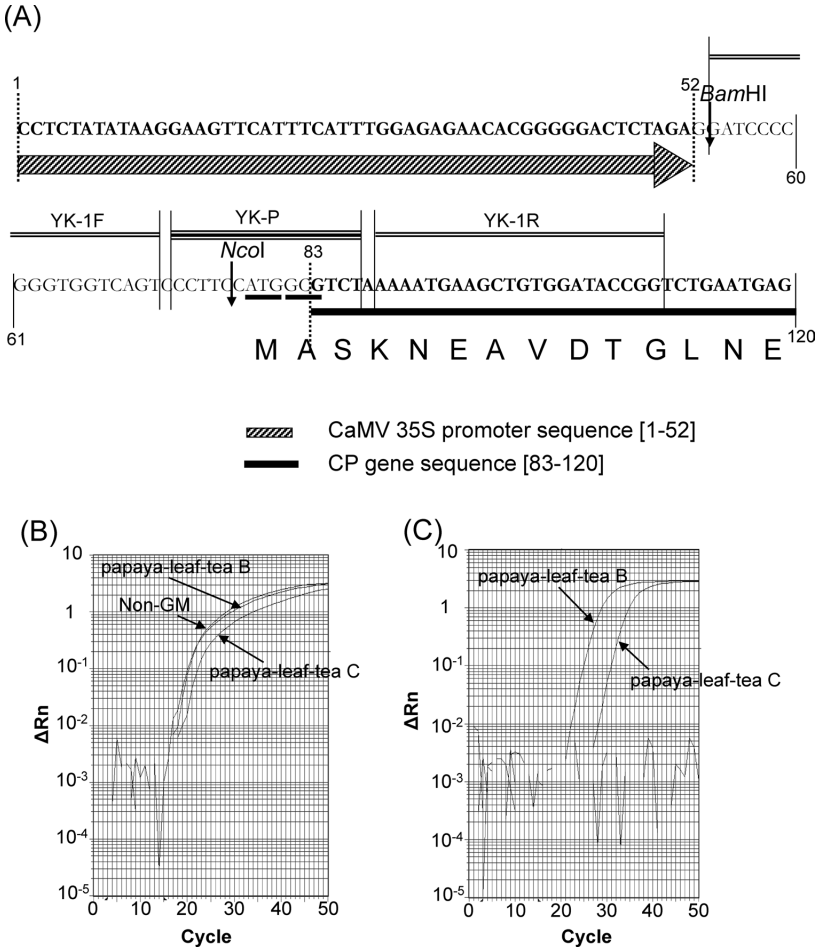

Fig. 2. Detection of PRSV-YK Using Real-Time PCR

(A) A fragment of the transgenic vector construct sequence was obtained and restriction sites were marked by vertical arrows. Design of the primers (YK-1F and YK-1R) and the probe (YK-P) for detecting construct-specific sequence of PRSV-YK is indicated by lines above the sequence. Numerals indicate the numbers of nucleotides from the 5′ terminus. (B) Endogenous *Chy* detection using a primer set (Q-Chy-1F2 and Q-Chy-2R) and probe (Q-Chy-P) (C) PRSV-YK detection using a primer set (YK-1F and YK-1R) and probe (YK-P). The threshold value was set at 0.2. Positive amplification curves are designated by arrows.

(YK-P) was designed on the site of the initiation codon of the CP gene (Fig. 2A).

Since the forward primer sequence for detecting the papaya endogenous internal control gene, *Chy*, had an unintentional error of a single nucleotide sequence in the previous report[15] (according to personal communication), we used the right sequence for the forward primer (Q-Chy-1F2), the reverse primer (Q-Chy-2R) and the probe (Q-Chy-P). The real-time PCR assay for PRSV-YK detection confirmed that the papaya-leaf-tea products B and C were positive for PRSV-YK, producing Ct values of 25.93 and 31.88 with a threshold value of 0.2, respectively. Endogenous *Chy* detection was positive for all samples, with the papaya leaf-tea product B, C and the non-GM papaya (Sunset) producing Ct values of 21.55, 23.82 and 21.45, respectively, with a threshold value of 0.2 (Figs. 2B, C). The copy numbers of PRSV-YK construct and *Chy* sequence were calculated from Ct values using standard curves which were generated using the positive control plasmid. Papaya-leaf-tea products B and C contained 1 copy of PRSV-YK construct sequence in 27 copies and 167 copies of *Chy* sequence, respectively (data not shown). Because the genetic background of PRSV-YK used in the processed papaya products was unknown, estimation of the content of PRSV-YK in a papaya product was not possible. The non-template control and the genomic DNA derived from other crops, such as maize, rice, soybean, flax and canola, gave no amplification signals in the PRSV-YK and the endogenous *Chy* detection systems (data not shown). These results indicated that the developed method is specific for detecting PRSV-YK.

In the present study, as a result of monitoring processed products, which included papaya as a major ingredient, for contamination with unauthorized GM papaya, we found a transgenic vector construct for expression of the CP gene, which was cloned from the YK strain, in papaya-leaf-tea products. The design of a part of the transgenic vector construct was identical to the one reported in 1996.[17] We also detected PRSV-YK contamination in 1 out of 7 products of papaya jam and 2 out of 3 products of papaya pickles in real-time PCR test for PRSV-YK detection (data not shown). The origin of the GM papaya contamination in the papaya products in Japan remains to be clarified. Furthermore, we successfully developed a construct-specific real-time PCR detection method for PRSV-YK. Further studies are required to determine the detection limits, and whether the method can be used for detection in other commercially processed products containing papaya as a major ingredient.

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