Allergenicity Assessment of the Papaya Ringspot Virus Coat Protein Expressed in Transgenic Rainbow Papaya

Gustavo Fermín,[†,‡,||] Ronald C. Keith,[†] Jon Y. Suzuki,[†] Stephen A. Ferreira,[‡] Douglas A. Gaskill,[‡] Karen Y. Pitz,[‡] Richard M. Manshardt,[§] Dennis Gonsalves,[†] and Savarni Tripathi[*,†,‡]

[†]USDA-ARS-Pacific Basin Agricultural Research Center, Hilo, Hawaii 96720, United States

[‡]Department of Plant and Environmental Protection Sciences and [§]Department of Tropical Plant and Soil Sciences, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, Honolulu, Hawaii 96822, United States

ABSTRACT: The virus-resistant, transgenic commercial papaya Rainbow and SunUp (*Carica papaya* L.) have been consumed locally in Hawaii and elsewhere in the mainland United States and Canada since their release to planters in Hawaii in 1998. These papaya are derived from transgenic papaya line 55-1 and carry the coat protein (CP) gene of papaya ringspot virus (PRSV). The PRSV CP was evaluated for potential allergenicity, an important component in assessing the safety of food derived from transgenic plants. The transgene PRSV CP sequence of Rainbow papaya did not exhibit greater than 35% amino acid sequence homology to known allergens, nor did it have a stretch of eight amino acids found in known allergens which are known common bioinformatic methods used for assessing similarity to allergen proteins. PRSV CP was also tested for stability in simulated gastric fluid and simulated intestinal fluid and under various heat treatments. The results showed that PRSV CP was degraded under conditions for which allergenic proteins relative to nonallergens are purported to be stable. The potential human intake of transgene-derived PRSV CP was assessed by measuring CP levels in Rainbow and SunUp along with estimating the fruit consumption rates and was compared to potential intake estimates of PRSV CP from naturally infected nontransgenic papaya. Following accepted allergenicity assessment criteria, our results show that the transgene-derived PRSV CP does not pose a risk of food allergy.

KEYWORDS: papaya ringspot virus, transgenic papaya, Rainbow, allergenicity, coat protein

■ INTRODUCTION

Genetically engineered (GE) papaya, Rainbow and SunUp, were developed on the basis of the concept of pathogen-derived resistance[1] using the papaya ringspot virus (PRSV) coat protein (CP) gene to protect the host plants against PRSV infection. PRSV is an aphid-transmitted, single-stranded RNA virus that belongs with the family Potyviridae, and it is the most serious problem for papaya cultivation worldwide.[2] In Hawaii, PRSV severely damaged the papaya industry and reduced papaya production by 50%, from 24 million kg in 1992 to 12 million kg in 1998.[3] In 1998, PRSV-resistant transgenic papaya, Rainbow and SunUp, were deregulated for commercial cultivation in Hawaii, which subsequently saved the Hawaiian papaya industry. Today, Rainbow alone accounts for more than 70% of the total papaya acreage in the state.[4] The PRSV-resistant papaya Rainbow is an F1 hybrid of SunUp crossed with a nontransgenic cultivar, Kapoho, whereas SunUp is a cultivar homozygous for the PRSV CP transgene and is derived from transgenic line 55-1, a line which was created by transforming the nontransgenic inbred cultivar, Sunset.[5] Our previous report has shown that the nutritional composition of Rainbow papaya is similar to that of nontransgenic papaya,[6] a finding that addressed the biosafety issue that there should be "substantial equivalence" or in other words no significant differences between GE and non-GE counterparts.

Generally, food allergy is one of the main biosafety concerns for any food derived from GE organisms. To assess the allergenicity of the transgene PRSV CP, multiple criteria were evaluated in this study. The most widely used criteria to assess

their potential allergenicity, including comparison of transgene proteins to known allergens at the amino acid sequence level and digestibility analysis of the engineered proteins in controlled experiments using simulated gastric and intestinal fluids, were performed.[7−14] Although acidic conditions and proteolytic enzymes present in the mammalian gastrointestinal (GI) tract efficiently denature and degrade most ingested proteins into constituent amino acids and small peptides, causing loss of the protein structure and biological activity and serving as a nutrient source,[15] according to a January 2001 report by the joint FAO/WHO expert consultation on allergenicity of foods derived from biotechnology, there is a supposed correlation, albeit weak, between the indigestibility of a protein by enzymes present in the alimentary tract and potential allergenicity.

In addition to analysis of its potential allergenicity, PRSV CP was also evaluated for heat stability as a test for persistence during processing, and its potential exposure to humans was estimated. In this study, we present evidence that consumption of Rainbow and SunUp papaya transformed with the PRSV CP gene poses no increased risk of potential allergenicity.

■ MATERIALS AND METHODS

Bioinformatic Analysis of the PRSV CP Sequence. The PRSV CP transgene sequence (GenBank accession no. FJ467933) was used as

a query in the following databases: (1) the Structural Database of Allergenic Proteins (SDAP) of the University of Texas Medical Branch (http://fermi.utmb.edu/SDAP/);[16] (2) the Allergen Database for Food Safety (ADFS; http://allergen.nihs.go.jp/ADFS/index.jsp); (3) the AllergenOnline version 11 database (http://www.allergenonline.com/). SDAP was developed using the allergens list from the IUIS (International Union of Immunological Societies) Web site, http://www.allergen.org, supplemented with information from the literature and from major sequence (SwissProt, PIR, and NCBI) and structural (PDB) databases and contains 1425 allergen sequences. The ADFS Web site and AllergenOnline contain 1285 and 1491 peer-reviewed sequences, respectively. Our query sequence was subjected in all three databases to full FASTA searches (using <0.01 E score cutoff), >35% identity over an 80 amino acid window, and searches for eight contiguous identical amino acid matches to known allergenic proteins as recommended by Codex.[8]

Purification of Native PRSV Particles. Greenhouse-grown nontransgenic Sunrise papaya was inoculated with a 1:10 dilution of PRSV-infected papaya plant tissue in inoculation buffer (phosphate buffer, 0.01 M, pH 7.5, 0.1% sodium sulfate, 10 mM EDTA). Twenty-one days after inoculation, samples were taken from infected Sunrise papaya leaves and used in double antibody sandwich (DAS)-enzyme-linked immunosorbent assay (ELISA) experiments to test for the presence of PRSV CP. Infected leaves (10 g) were collected from samples that tested positive for PRSV CP and were used for purification of virus particles essentially by the method of Gonsalves and Ishii.[17] Infected papaya tissue was macerated in a blender with 10 mL of 0.01 M phosphate buffer, pH 7.5, followed by addition of 5 mL of carbon tetrachloride and 5 mL of chloroform. The resulting mixture was centrifuged for 10 min at 3697g. The supernatant was passed through glass wool, stirred for 1 h at 4 °C following the addition of PEG-8000 to 10% (w/v), and centrifuged at 1643g. The resulting pellet was resuspended in 2 mL of sterile distilled water, and NaCl was added to a concentration of 0.3 M, followed by one extraction with chloroform to eliminate the PEG. The aqueous phase was recovered and the virus protein concentration estimated using the Bradford method.[18]

Engineering of PRSV CP for Bacterial Expression. The CP gene harbored by transgenic papaya line 55-1 is a chimeric gene comprised of nucleotides corresponding to the first 16 amino acids of the cucumber mosaic virus CP gene and the coding region of the PRSV CP gene.[19] The coding region of the chimeric transgene was PCR amplified (from a clone of Rainbow papaya genomic DNA), cloned as a translational fusion with the intein sequence present in vector pTYB1 or pTYB11 (New England Biolabs, Inc., Ipswich, MA), and verified by sequencing following standard molecular biology methods.[20] Amplification of the chimeric gene was performed with the following primer pairs: Hilo06-02, GGTGGTCATATGGACAAATCTGAAT (with the ATG codon of the CP gene in bold and the NdeI restriction site underlined), and Hilo07-02, GGTGGTTGCTCTTCCGCAGTTGCGCATAC (with the SapI restriction site underlined) to engineer the CP gene into plasmid pTYB1 (for intein fusion at the C-terminus of the translated CP); Hilo08-02, GGTGGTTGCTCTTCCAACATGGACAAATCTG (with the ATG codon of the CP gene in bold and the SapI restriction site underlined) and Hilo09-02, GGTGGTCCCGGGTTAGTTGCGCATAC (with the SmaI restriction site underlined) to engineer the CP gene into plasmid pTYB11 (for intein fusion at the N-terminus of the translated CP).

Purification of the PRSV CP Expressed in *Escherichia coli*. A high expressing clone of CP, intein-tagged at the C-terminus in *E. coli* strain ER2566, was used for purification of PRSV CP. Isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration 0.5 mM) was added to a 1 L culture in Luria broth (LB) supplemented with ampicillin (50 μg/mL) at the late log phase, followed by further incubation at 16 °C for 24 h. Bacteria were pelleted by centrifugation at 5000g and 4 °C for

30 min and resuspended in 50 mL of cold lysis buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 500 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, and 20 μM phenylmethanesulfonyl fluoride (PMSF), pH 8.0]. Bacteria were sonicated and centrifuged at 20000g and 4 °C for 30 min, and the clarified supernatant was loaded onto a chitin bead column equilibrated with column buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, pH 8.0). The column was washed with 12 bed volumes of 1 M NaCl, and cleavage was induced by incubating the column in cleavage buffer [20 mM HEPES, 500 mM NaCl, 1 mM EDTA, pH 8.0, 50 mM dithiothreitol (DTT)] for 40 h. The PRSV CP was eluted from the column with 20 mM HEPES, 500 mM NaCl, 1 mM EDTA, pH 8.0. The protein concentration of 1 mL eluate fractions was measured using the Bradford reagent according to the manufacturer's instructions (BioRad, Hercules, CA). Fraction samples were also run in SDS—PAGE, and the CP identity was confirmed by Western blot analysis using antibodies raised against PRSV particles. Samples were dialyzed with at least 2000 volumes of 20 mM HEPES, pH 6.5. All purification steps were performed at 4 °C.

SDS—PAGE and Western Blot Analysis. Samples were boiled for 5 min, cooled on ice, and electrophoresed in 10% or 12% denaturing SDS—polyacrylamide gels using Tris—glycine—SDS running buffer prepared following standard methods[20] on a Mini Protean II gel system (Bio-Rad). Samples were visualized by Coomassie Brilliant Blue staining[21] and/or transferred to poly(vinylidene fluoride) (PVDF) or nitrocellulose membranes by submarine or semidry electroblotting according to the manufacturer's instructions (Bio-Rad). Membranes were equilibrated with 1× TBS (Tris-buffered saline: 20 mM Tris, 500 mM NaCl, pH 7.5) for 5 min and blocked with 1× TBS, 5% dry nonfat milk, or 5% casein for at least 1 h. Following three washes in 1× TBS containing 0.05% Tween-20 (1× TTBS), the blocked membrane was incubated for at least 2 h with primary antibody [serum raised against PRSV HA 5-1 (Gonsalves laboratory, Cornell University, Geneva, NY) or against the chitin binding domain (anti-CBD, New England Biolabs)]. After three washes in 1× TTBS, membranes were incubated in secondary antibody from the Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot Assay Kit (Bio-Rad), washed three more times in 1× TTBS, and incubated with the alkaline phosphatase—streptavidin complex for at least 1 h. After a final washing step, the blot was developed by either colorimetric (Bio-Rad) or chemiluminescent (Roche Applied Science, Indianapolis, IN) methods according to the manufacturers' instructions.

In Vitro Digestion of Coat Protein by Simulated Gastric Fluids. CP digestibility tests in simulated gastric fluid (SGF) were performed using two different conditions.[7,22] The first condition consisted of 50 μg of purified PRSV CP sample as the substrate plus the addition of 2 or 10 μg of the protease, pepsin (pepsin:PRSV CP (w/w) protein ratios of 1:25 and 1:5, respectively) in a total volume of 100 μL, 0.01 N HCl, pH 2.0. Samples were then incubated for 2 h at 37 °C. As a control, the protease inhibitor, pepstatin,[23] was added to a final concentration of 5 μM 10 min prior to addition of the substrate. For the second condition, 2 μg of purified PRSV CP was mixed with pepsin to obtain a final w/w pepsin:PRSV CP ratio of 13:1 or 6.5:1. SGF was prepared as a pepsin solution (3.2 mg/mL) in 30 mM NaCl, pH 1.2. For both experiments, samples were quenched after time course treatments with equal volumes of 2× Laemmli's buffer,[24] immediately boiled at 100 °C for 5 min, and cooled to room temperature, and an aliquot was analyzed by SDS—PAGE. Pepsin (P7000) was purchased from Sigma-Aldrich Inc., St. Louis, MO.

In Vitro Digestion of Coat Protein by Simulated Intestinal Fluids. CP digestibility tests in simulated intestinal fluid (SIF) were performed using two different conditions. For the first condition, CP was incubated with varying amounts of bovine trypsin or pancreatin (5, 10, or 50 μg) in 0.01 M Tris buffer, pH 8. As a control, the protease
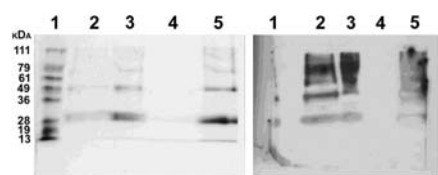
Figure 1. SDS—PAGE (left panel) and Western blot analysis (right panel) of CP-v in SGF containing pepsin A. Samples: lane 1, prestained protein markers; lane 2, CP-v only; lane 3, CP-v incubated with buffer (pH 2); lane 4, CP-v incubated with pepsin A (10 μg) for 2 h; lane 5, CP-v incubated with pepsin A (10 μg) and pepstatin for 2 h. The molecular masses (kDa) of the protein markers are shown at the left.

inhibitor, leupeptin,[25] was added to one of the samples at a final concentration of 10 μM 10 min prior to addition of the substrate. Following incubation at 37 °C for 1 or 2 h, each reaction was stopped by addition of an equal volume of Laemmli's sample buffer and boiled at 100 °C for 5 min. For the second condition, single-tube digestions were performed in SIF consisting of 10 mg/mL pancreatin in 0.05 M KH$_2$PO$_4$, pH 7.5. Reactions were stopped as described above. Bovine trypsin (TPCK-inhibited), pancreatin, pepstatin, and leupeptin were purchased from Sigma-Aldrich.

Heat Stability Assay. A heat stability assay was performed using native PRSV CP in host plant matrix which consisted of PRSV-infected Sunrise leaves. Leaf samples (100 mg) were ground in 600 μL of protein extraction buffer (50 mM Tris—HCl, pH 6.8, 4% SDS, 2% β-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and immediately heated at 100 °C for 2 or 4 h. PRSV-infected Sunrise papaya leaves (100 mg) were baked for the 206 °C heat treatment, reconstituted to 100 mg, and extracted in 600 μL of protein extraction buffer. Extracts for the different temperature treatments (15 μL each) were subjected to SDS—PAGE and Western blot analysis.

Estimation of PRSV Coat Protein Levels in Transgenic Papaya. Fully ripened (100% yellow skin color) papaya fruit of Rainbow, SunUp, and noninfected Sunset as well as PRSV-infected Kamiya cultivars was used for determining CP levels in the fruit. Leaf samples of Rainbow, SunUp, and noninfected Kapoho as well as PRSV-infected Kapoho were used for determining CP levels in the leaves. Coat protein levels were quantified using ELISA using a monoclonal antibody to PRSV CP. A standard volume of a dilution series (1.0×, 0.2×, 0.1×, 0.04×, 0.02×, and 0.01×) of purified PRSV CP particles ranging from 0.025 to 25 μg of CP was used to generate a standard curve for each ELISA assay, allowing the quantitation of CP in papaya samples from absorbance (A_{405}) values.

■ RESULTS

Sequence Analysis of PRSV CP for Predicting Potential Allergenicity. Potential CP allergenicity was assessed by searching for PRSV CP transgene amino acid sequence similarity to known allergens in the databases as described in the Materials and Methods. The similarity search for transgene PRSV CP amino acid sequences was performed by one or more of three standard methods: (1) full FASTA search for homologous proteins in the databases, (2) search for database proteins with >35% similarity to PRSV CP over an 80 amino acid window. No known allergen was found to be similar to PRSV CP by full FASTA search or by the criterion requiring >35% overall similarity over an 80 amino acid window. In addition, we also searched for short (eight amino acid) contiguous stretches of identical amino acids between proteins in the allergen database and PRSV CP. There were no eight contiguous amino acid matches between PRSV CP and known allergens in the searched databases.
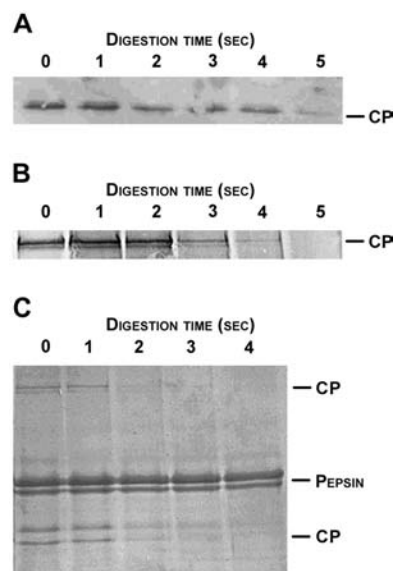

Figure 2. SGF digestion of partially purified native and purified recombinant PRSV CP (CP-v and CP-b, respectively) containing pepsin A. (A) Western blot analysis of 2 μg of CP-v digested with 13 μg of pepsin (w/w ratio of 6.5:1, pepsin:CP) for 0—5 s. (B) Western blot analysis of 2 μg of CP-v digested with 26 μg of pepsin (w/w ratio of 13:1, pepsin:CP) for 0—5 s. (C) SDS—PAGE analysis of 2 μg of purified CP-b digested with 26 μg of pepsin (w/w ratio of 13:1, pepsin:CP) for 0—4 s. Labels indicate the positions of CP and pepsin bands.

SGF Assay of PRSV CP. The digestibility of PRSV CP, tested by both simulated gastric fluid and simulated intestinal fluid, was performed on CP from isolated native virus or from bacterially expressed transgene sequence-derived CP. The protein purified from bacteria is referred to as CP-b, whereas CP partially purified from native PRSV particles is referred as CP-v.

Initial SGF experiments using a ratio of 1:25 or 1:5 of the protease, pepsin, to PRSV CP target protein resulted in the disappearance of PRSV CP when the reaction was incubated for 2 h (Figure 1). Minimal or no change in the CP-v level was observed in samples incubated for 2 h in medium similar to gastrointestinal fluids with respect to pH (pH 2.0), but lacking pepsin A. Similarly, a minimal change in the CP-v level was observed in samples containing the protease inhibitor pepstatin in addition to pepsin A, supporting the notion that pepsin A facilitated enzymatic degradation of PRSV CP in SGF. In addition to protein bands of molecular mass corresponding to that of a PRSV CP monomer, ~36 kDa, we observed higher order aggregates often observed in low ionic strength medium, as well as a ~25 kDa degradation product of PRSV CP that is commonly observed in PRSV CP purified from virus particles and other potyvirus preparations.[17,26]

In subsequent experiments we tested ratios of protease to target protein that have become the current standard and observed a more rapid time course for PRSV CP degradation. At a protease target protein ratio of 6.5:1 (pepsin:CP), a substantial amount of the CP-v was degraded within 5 s (Figure 2A), whereas nearly complete degradation was observed at 5 s using a ratio of 13:1 (Figure 2B). Similarly, degradation of CP-b was nearly complete after 5 s, when a 13:1 ratio (pepsin:CP-b) was used (Figure 2C). Similar to CP purified from the virus, higher order aggregates of bacterially expressed and purified PRSV CP were observed.
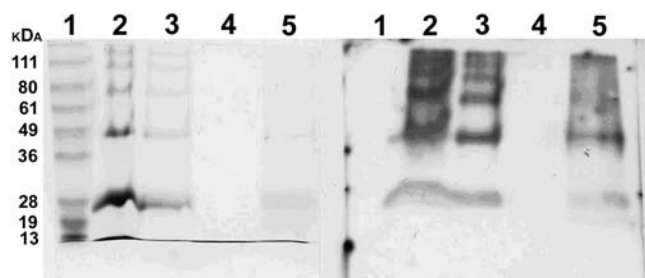
Figure 3. SDS−PAGE (left panel) and Western blot (right panel) analysis of CP-v in SIF containing trypsin. Samples: lane 1, prestained protein markers; lane 2, CP-v alone; lane 3, CP-v incubated with buffer (pH 8) only; lane 4, CP-v incubated with trypsin (50 μg) for 2 h; lane 5, CP-v incubated with trypsin (50 μg) and leupeptin. Trypsin ($\sim$24 kDa) comigrates with a $\sim$22 kDa fragment of PRSV CP. The molecular masses (kDa) of the protein markers are shown at the left.
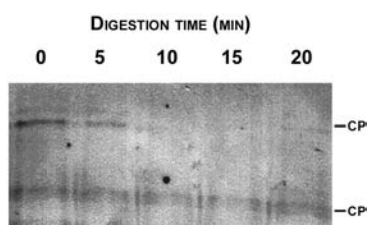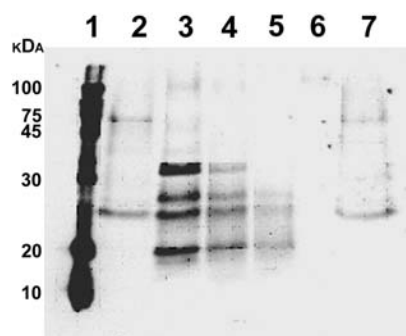

Figure 4. Western blot analysis of PRSV CP digestion in SIF containing pancreatin. The labels indicate the positions of the CP bands.

SIF Assay of PRSV CP. Initial SIF experiments with CP-v and trypsin, the serine protease found in the upper small intestine, with or without the serine and cysteine protease inhibitor leupeptin, indicated that trypsin could effectively degrade the CP in buffer at pH 8.0 within 2 h (Figure 3), whereas a minor proteolytic product of $\sim$20 kDa still remained when lower amounts of trypsin were used (data not shown).

Time course experiments using pancreatin, an enzyme mixture simulating pancreatic secretions of the GI tract and comprised not only of trypsin but also of lipase and amylase, showed that degradation was evident by 5 min and a significant amount of CP was digested by 10 min (Figure 4).

Heat Stability of Native PRSV Coat Protein in Plant Host Matrix. In experiments to test the effect of heat intensity and duration on PRSV CP stability, we observed a significant loss of signal in Western blots utilizing a PRSV CP specific antibody when crude leaf extracts of PRSV from infected papaya were treated at 100 °C for 2 h. Residual amounts of CP were observed in the sample treated at 100 °C for 4 h, whereas no bands were detected when the sample was treated at 206 °C for as short as 20 min. The multiple bands observed using the PRSV CP antibody, the highest of which correlated in size to the full-length PRSV CP ($\sim$36 kDa), are attributed to degradation products in the context of crude plant extracts as they were only detected in extracts from infected leaves, but not in extracts from the noninfected control. The higher molecular mass band corresponding to the full-length CP seemed to degrade faster than the proteins running as smaller molecular mass bands. This phenomenon was also observed using other treatments (Figure 5).

Human Consumption of PRSV CP. In vitro tests in this study suggest that PRSV CP is rapidly degraded by fluids simulating those found in the human gastrointestinal (digestive) tract.

Figure 5. Western blot analysis of heat-treated PRSV-infected papaya leaf samples using a polyclonal antibody to PRSV CP. Samples: lane 1, protein molecular mass marker; lane 2, noninfected papaya leaves with no treatment; lane 3, PRSV-infected sample with no treatment; lane 4, PRSV-infected papaya sample heated at 100 °C for 120 min; lane 5, PRSV-infected sample treated at 100 °C for 240 min; lane 6, PRSV-infected sample treated at 206 °C for 20 min; lane 7, noninfected Rainbow papaya sample used as a transgenic control. The molecular masses (kDa) of protein markers are shown at the left.

Table 1. PRSV Coat Protein Levels in Transgenic and Non-transgenic Papaya Fruit and Leaf Tissues[a]

type of tissue	papaya cultivar	no. of samples	coat protein level (μg/g of fresh mass) $\pm$ SD
fruit	Rainbow	5	6.3 $\pm$ 2.1
	SunUp	5	ND[b]
	Sunset	5	ND[b]
	Kamiya (infected)	5	48.5 $\pm$ 28.3
leaf	Rainbow	1	257.6
	SunUp	1	137.0
	Kapoho (infected)	1	3580.6
	Kapoho	1	ND

[a] Rainbow and SunUp are 55-1 line-derived transgenic papaya, while Kapoho, Kamiya, and Sunset are nontransgenic papaya cultivars. CP levels for the different cultivars and tissue types were quantified using ELISA. [b] ND = nondetectable, below the limit of detection of 0.25 μg of protein/g of fresh mass.

However, the potential amount of PRSV CP entering the human digestive tract from a transgene-derived source has not previously been thoroughly addressed. In an attempt to obtain an estimate of the levels of CP being consumed, the amount of PRSV CP in transgenic papaya fruit was measured by quantitative ELISA on a per gram of fresh mass basis, and total consumption levels were calculated taking into account average fruit mass as well as potential fruit consumption rates. The amount of virus-derived CP in PRSV-infected nontransgenic papaya fruit was also calculated to estimate for comparison the potential human exposure to CP, given a scenario where no GE, PRSV-resistant plants were available. The data indicate that the CP level in the fruit of Rainbow papaya, which is hemizygous for the transgene, was 7.7-fold lower than that of naturally infected nontransgenic papaya fruits (Table 1). CP levels in SunUp papaya, which is homozygous for the transgene, were below the level of detection by ELISA. It should be noted that, even for the Rainbow fruit sample, transgene-derived CP could not be detected unless a protease inhibitor (PMSF) was used in the assay, suggesting that the calculated transgene-derived CP amount likely overestimates

Table 2. Consumption Level Estimates (mg/year) of PRSV Coat Protein from Transgenic and Naturally PRSV-Infected Nontransgenic Papaya[a]

fruit consumption rate	Rainbow[b]	SunUp[c]	virus-infected fruit[d]
one fruit per day	1306.1	51.8	10055.0
one fruit per week	186.1	7.4	1432.5
one fruit per month	42.9	1.7	330.6

[a] Based on an estimated average fruit mass of 568 g. [b] Rainbow was measured to contain 6.3 μg of CP/g of fresh mass of fruit, or 3.6 mg of CP/fruit. [c] SunUp CP was not detected in the fruit. Assuming a CP level at the detection limit for CP, the level would equal 0.25 μg/g of fresh mass of fruit, or 0.14 mg of CP/fruit. [d] Nontransgenic infected fruit was measured to contain 48.5 μg of CP/g of fruit, or 27.5 mg of CP/fruit.

true consumption levels, since some level of CP degradation would be expected under normal food preparation conditions. By comparison to the levels measured in fruit, transgene-derived CP generally accumulates to higher levels in the leaves, at least in transgenic papaya line 55-1, which thus allowed for the detection of the CP in the leaves of SunUp. Similar to the relative differences in CP levels observed between the fruit, the amount of CP accumulating in the leaves of SunUp was measured to be 1.9 times lower than that found in the leaves of Rainbow. The amount of CP accumulating in the leaves of Rainbow, in turn, was 13.9-fold lower than that of the leaves of PRSV-infected nontransgenic papaya plants (Table 1).

On the basis of CP amounts measured in fruit, we calculated the potential annual cumulative CP consumption levels for Rainbow and naturally infected nontransgenic papaya fruit on the basis of different fruit consumption rates in the diet; in the case of SunUp, where CP was undetectable in fruit, an upper limit for CP consumption was estimated by using a theoretical number equal to the minimum CP amount detectable by our ELISA assay (0.25 μg/g of fresh mass). Using a theoretical maximum rate of one fruit per day, the cumulative annual CP consumption levels were estimated to be approximately 1306 and 52 mg for Rainbow and SunUp, respectively (Table 2). For naturally infected nontransgenic papaya, the annual CP consumption level is estimated to be at least 8-fold higher than that of Rainbow, the dominant and most widely consumed cultivar in Hawaii. In other words, the estimated annual human exposure level of transgene-derived CP from 365 Rainbow or SunUp fruits (based on a presumed consumption rate of one fruit a day) would be roughly equivalent to the amount of virus-derived CP obtained by consuming 47 or 2 naturally PRSV-infected papaya, respectively.

■ DISCUSSION

The PRSV CP is the major transgene protein expressed in Rainbow and SunUp papaya along with the plant transformation marker gene proteins NPTII and GUS. Our results of PRSV CP digestibility in gastrointestinal fluids as well as bioinformatic analysis have shown that the transgene CP expressed in Rainbow and SunUp does not pose a risk of food allergy. Furthermore, the amount of CP expressed in transgenic papaya was found to be 87−99% lower than the amount of CP found in naturally infected nontransgenic papaya fruits. Hence, the CP exposure from human consumption of transgenic papaya is much lower than that from consuming naturally infected nontransgenic papaya. Previous studies documented the biosafety aspect of NPTII and GUS.[27−29] Exposure to the transgene markers GUS

and NPTII in Rainbow and SunUp papaya was estimated to be lower than that of the CP in Rainbow (unpublished data; data submitted to the Japan Ministry of Health, Labor and Welfare and the Ministry of Agriculture, Forestry and Fisheries).

The predictions of allergenicity of novel proteins are based on bioinformatics and/or experimental approaches.[8,9] The bioinformatics analysis showed that the PRSV CP did not show significant similarity to any known allergenic proteins using the criterion of significant overall homology, greater than 35% similarity over a window of 80 amino acids and a contiguous eight amino acid identical match,[30] to any known allergen in the allergen database by full FASTA search.

Previously, Kleter and Peijnenburg[31] identified a peptide of six amino acids (EKQKEK) shared by PRSV CP and an alleged allergen, ABA-1, a protein of the human parasite *Ascaris lumbricoides* or the pig parasite *Ascaris suum*. However, numerous reports show that a high percentage of proteins identified by six amino acid matches are false positives.[32−37] Furthermore, we previously reported[38] that the PRSV CP six amino acid match to ABA-1 is not relevant with regard to allergenicity for several reasons: (1) the six amino acid sequence is not repeated in the coat protein sequence, and therefore, it will not trigger the IgE response associated with allergens, (2) the ABA-1 proposed allergenic peptide was not found to be inherently allergenic outside the context of other *Ascaris* proteins,[39] and (3) although ABA-1 was listed in the ADFS site, it is not among the officially recognized allergens found in the International Union of Immunological Societies (IUIS) allergen database (http://www.allergen.org).

Another important aspect of assessing potential allergenicity is experimental testing for properties common to allergenic proteins such as stability in simulated gastrointestinal fluids, which include SGF and SIF. SGF was developed to represent the conditions in the human stomach[22] and basically consists of the main gastric protease pepsin in low-pH medium. Some data suggest that proteins that are susceptible to gastrointestinal digestion are inherently safer than those that are stable, especially in terms of allergenicity.[7]

Our data show that the transgene sequence-derived PRSV CP is rapidly degraded in SGF. Many known allergens such as ovalbumin and phosvitin from egg, β-lactoglobulin from milk, β-conglycinin, lectin from soybean, sin a1 and bra j IE from mustard, and Ara h2 from peanut were stable up to 60 min in gastric fluid.[7] The PRSV CP was completely and rapidly digested in SGF within 5 s. Moreover, our results showed that the CP was also not stable in SIF and completely degraded within 10 min. The PRSV CP harbors from 34 to 50 pepsin and 46 trypsin cleavage sites according to the ExPASy peptide cutter bioinformatics tool;[40] therefore, it is not surprising that the coat protein is degraded rapidly in gastrointestinal fluids.

There is no general rule concerning the effect of heat on food allergens, and it is not considered a good predictor of allergenicity. Our heat treatment study of native PRSV CP in plant matrix indicated that the CP is unstable to heat treatments and it was completely degraded at 206 °C. At higher temperature loss of tertiary structure as well as various changes in intra- and intermolecular interactions, including the formation of aggregates, can occur. Heat can destroy the conformation and lead to loss of immunobinding sites that trigger the allergenic response as in the case of heat-labile allergens such as "Cor a" found in hazelnut.[41] In other cases, allergenicity can occur as of result of the linearization of epitopes during heating.[42] In the case of PRSV

CP, the CP pattern was altered after boiling or baking treatments perhaps due to the molecular interactions described above.

In Hawaii, the papaya industry was saved from the damage caused by the virus disease with the introduction of PRSV-resistant papaya Rainbow and SunUp, but the industry in Hawaii still faces the challenge of marketing GE papaya internationally. Japan and Canada have been and continue to be important export markets for Hawaii's papaya. In 2003, Canada approved the importation of SunUp and Rainbow transgenic papaya (http://www.hc-sc.gc.ca/fn-an/gmf-agm/appro/papaya-eng.php). On the other hand, application for the import and sale of line 55-1-derived transgenic papaya in Japan is in the final stages of approval by the Ministry of Health, Labor and Welfare, the Food Safety Commission, the Ministry of Agriculture, Forestry and Fisheries, and the Ministry of the Environment (http://www.fsc.go.jp/sonota/kikansi/21gou/21gou_1_8.pdf; http://www.fsc.go.jp/hyouka/hy/hy-tuuchi-papaya_55-1.pdf). One of the main food safety concerns of Japanese regulators was whether the PRSV CP gene in GE papaya had introduced any food allergenicity.

Finally, important evidence for supporting the safety of any food is a history of safe consumption. In Hawaii, transgenic papaya fruits have been consumed for more than a decade since their release in 1998 without any recorded adverse effect on human health. Furthermore, our detailed analysis of the Rainbow papaya and its nontransgenic counterpart show similar nutritional and mineral elements, including papain, carpain, and benzyl isothiocyanate (BITC).[6] Thus, we conclude that Rainbow and SunUp papaya consumption does not pose any threat to human and animal health.

■ AUTHOR INFORMATION

Corresponding Author
*Phone: (808) 959-4309. Fax: (808) 959-5470. E-mail: savarni.tripathi@ars.usda.gov.

Present Addresses
‖Centro Jardín Botánico, Universidad de los Andes, Mérida, Venezuela.

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