JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Toxicity Assessment of Transgenic Papaya Ringspot Virus of 823-2210 Line Papaya Fruits

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ABSTRACT: The transgenic papaya is a valuable strategy for creating plants resistant to papaya ringspot virus (PRSV) infection and increasing production. This study was further performed to evaluate the comparative toxicity effects of the newly developed transgenic line of the fruits of two backcross transgenic papaya lines (2210 and 823) and one hybrid line (823-2210) and compare to their parent non-transgenic (TN-2) counterparts. The stability analysis of coat protein (CP) of PRSV was investigated using the digestion stability assays in simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and bile salts to detect the CP fragments. Results revealed that the CP fragments were rapidly hydrolyzed in SGF and were undetectable in organs and gastrointestinal contents in rats. For the genotoxicity, three *in vitro* assays were conducted and exhibited that non-transgenic and backcross transgenic papaya fruits were negative. Moreover, a repeated animal feeding study was conducted by feeding 2 g/kg of body weight (bw) of non-transgenic and backcross transgenic papaya fruits for 28 days in rats. There were no biological or toxicological significances between non-transgenic and backcross transgenic papaya fruits in rats. The results demonstrated that the backcross transgenic papaya fruit can be recognized as an equivalent substitution for traditional papaya in food safety.

KEYWORDS: *Transgenic papaya, stability analysis, genotoxicity, food safety*

INTRODUCTION

Transgenic crops were first commercially introduced in 1996. The main transgenic crops being produced are soybeans, corn, cotton, and canola. The majority of transgenic crops is herbicide-resistant, followed by insect-resistant (Bt) and multiresistant (herbicide-resistant and insect-resistant).[1] Transgenic plants are also considered to be an alternative system for the useful production of recombinant proteins, such as antibodies, antigens, and therapeutics. For example, transgenic tomatoes expressing human β-amyloid have been created for use as a vaccine against Alzheimer's disease.[2]

Papaya is an important fruit that provides a variety of vitamins with nutritional value. However, papaya ringspot virus (PRSV) infection is a destructive disease that affects the production of papaya. Recently, a transgenic papaya has been developed, which can be used as a valuable strategy to fight infection and to increase papaya production. Molecular characterization of the transgenic papaya demonstrated that the coat protein (CP) gene of Taiwan strain PRSV YK was inserted into the genome by liquid-phase wound infection of Tainung No. 2 (TN-2). The transgenic papaya lines 16-0-1, 17-0-5, and 18-2-4 provide broad-spectrum resistance against various PRSV strains under greenhouse conditions and have a potential benefit for controlling PRSV infection.[3−5]

For the food safety, the concept of "substantial equivalence" is used by regulatory agencies to compare transgenic crops to their conventional counterparts, including nutritional equivalency, levels of natural toxicants, and the potential for allergenicity, in addition to a number of agronomic and environmental factors.[6] Up to date, reviews on the assessment of the health impact of transgenic plant diets in long-term and multigenerational animal feeding trials from 2000 to 2011, included 12 long-term studies (of more than 90 days and up to 2 years in duration) and 12 multigenerational studies (from 2 to 5 generations), presented evidence to show that transgenic plants are nutritionally equivalent to their non-transgenic counterparts and can be safely used in food and feed.[7−9] Food allergies can be caused by a wide variety of foods, including some transgenic crops. For the allergic potency, the Balb/c mouse has been widely used to evaluate the sensitizing potential of novel proteins in transgenic crops.[10] Allergenicity assessment of the PRSV CP expressed in transgenic Rainbow papaya was conducted, and results showed that the transgene-derived PRSV CP did not pose a risk of food allergy.[11] Also, previous assessments of the effects of transgenic papaya (TPY10-4) fruit supplementation on immune responses have revealed that it does not increase the allergenic potential of

Received: November 26, 2012
Revised: January 19, 2013
Accepted: January 25, 2013
Published: January 25, 2013

dx.doi.org/10.1021/jf305036x | *J. Agric. Food Chem.* 2013, 61, 1585−1596

ovalbumin-sensitized mice but may induce protective immunity via increasing the total serum immunoglobulin M (IgM) level.[12]

Because lines 823 and 2210 used in this study were obtained from T0 transgenic line 18-2-4 backcrossing with breeding parental lines "Sunrise" or "Thailand" for several generations, T0 line 18-2-4 is heterozygous and lines 823 and 2210 are homozygous for transgenes. They showed divergences in transgene dosage and horticultural traits. The aim of this study was conducted to compare the possible toxicity of the newly developed backcross transgenic papaya fruits, including the fruits of two backcross transgenic papaya lines (2210 and 823) and one hybrid line (823-2210) using molecular identification on its CP fragments, genotoxicity *in vitro* assays, and repeated animal feeding test for *in vivo* toxicological study to compare these transgenic crops to their conventional counterparts.

MATERIALS AND METHODS

Test and Control Substances. The fruits of two backcross transgenic papaya lines (2210 and 823) and one hybrid line (823-2210) were used in this study. Line 2210 was obtained by backcrossing the monoresistant transgenic papaya line 18-2-4 with non-transgenic variety Sunrise. When 18-2-4 backcrossed with non-transgenic variety Thailand, line 823 was obtained. The hybrid line 823-2210 was generated by crossing 2210 with 823. The non-transgenic control is TN-2. They were cultivated in the isolated greenhouses at the Department of Plant Pathology, Taiwan Agricultural Research Institute, Wufeng, Taiwan, Republic of China. Fresh papaya fruit pulp was harvested and lyophilized. The ratio of fresh/lyophilized papaya fruit was approximately 8:1. The lyophilized papaya fruits were ground into powder and stored at −20 °C before use. The molecular markers for the characterization of these two transgenic papaya lines, which were resistant to the PRSV and non-transgenic TN-2, were confirmed in our previous report.[13]

Construct-Specific Detection. Papaya genomic DNA was isolated from a 30 mg cryostat sample of the non-transgenic papaya line TN-2. The backcross transgenic papaya lines 2210, 823, and 823-2210 were extracted with a plant genomic DNA purification kit. DNA quality was analyzed using 1% agarose gel, and DNA concentrations were measured by absorbance at 260 nm (U-3000 spectrophotometer, Hitachi Instruments, San Jose, CA). Primers papa32/papa59 were previously used to amplify a conserved portion of papaya endogenous DNA fragment of 345 base pairs (bp), and primers S5/S6 were used to amplify the papain gene of 69 bp. On the other hand, PRSV-F/PRSV-R and S9-2/S10-2 were amplified as part of the PRSV CP gene of 840 and 151 bp, respectively (Table 1).[13] All polymerase chain reaction (PCR) amplifications were performed on a Gene Amp PCR System 2400 (Applied Biosystems, Foster City, CA). The PCR amplification reaction mixture (final volume of 25 μL) contained 1 μL of DNA template, 5 μL of 5× PCR buffer (GeneMark Technology Co., Ltd., Dali, Taiwan, Republic of China), 17 μL of sterilized distilled water, 1 μL of forward primer, and 1 μL of reverse primer. According to the study by Fan et al., the PCR was performed for 1 min at 94 °C for the melting phase, 1 min at 55 °C, and 2 min at 72 °C for synthesis, for 30 cycles. The PCR products were analyzed by electrophoresis on 2% agarose gel in TAE buffer.[13]

Table 1. Sequences of Primers and Probes Used in the Molecular Biological Assay of PRSV Transgenic Papaya Fruits[a]

primer/probe	sequence (5′ → 3′)	target gene
Papa32	AAT ATC AAA TGG ACG TGT TAG TG	RB flanking
Papa59	TGG TTA TCA ATA TAG CAA TTA TGT AG	LB flanking
PRSV-F	TCC AAG AAT GAA GCT GTG GA	PRSV
PRSV-R	GTG CAT GTC TCT GTT GAC AT	PRSV
S9-2	AGT AAC GCG GCA GAG GCA TA	PRSV
S10-2	GAG CCC TAT CAG GTG TTT TCG A	PRSV
S5	TGG GTT TGT CAT TTG GTG ATT TT	papain
S6	GTC TTT CAG TGG ATG TCA AGT CAT TT	papain

[a]Cited from ref 13.

Simulated Gastric Fluid (SGF) Digestion Stability Assay. The SGF that was prepared consisted of 3.2 mg/mL pepsin in 0.03 M NaCl at pH 1.2. Aliquots (200 μL) of SGF were placed in 1.5 mL microcentrifuge tubes and incubated in a water bath at 37 °C. The ratio of test gene fragment solution, including PRSV CP gene fragment of 840 or 151 bp, to SGF was about 1:1 (w/w). At intervals of 0, 5, 15, 30, 60, and 120 min, 75 μL of 1 N NaOH or 0.2 M $NaCO_3$ was added to each vial to stop the reaction. The samples were then analyzed using 2% agarose gel to detect the CP gene.[14,15]

Simulated Intestinal Fluid (SIF) Digestion Stability Assay. The SIF components were prepared with 10 mg/mL pancreatin in 0.05 M KH_2PO_4 at pH 7.5. Aliquots (64 μL) of SIF were placed in 1.5 mL microcentrifuge tubes and incubated at 37 °C for 10 min in a water bath. The ratio of test gene fragment solution, including PRSV CP gene fragment of 840 or 151 bp, to SIF was about 1:1 (w/w). At intervals of 0, 5, 15, 30, 60, and 120 min, 15 μL of 6× Laemmli buffer was added to each tube and the reaction was immediately stopped by placing the tube in a boiling water bath for 10 min. The samples were then analyzed using the 2% agarose gel to detect the CP gene.[14,15]

Bile Salt Digestion Stability Assay. The bile salt was diluted to 15% bile salts (Sigma, St. Louis, MO) with saline. The ratio of test gene fragment solution, including PRSV CP gene fragment of 840 or 151 bp, to bile salt was about 1:1 (w/w). The samples were incubated at intervals of 0, 5, 15, 30, 60, and 120 min and then analyzed using the 2% agarose gel to detect the CP gene.[14,15]

Semi-quantitative Analysis of CP Protein Stability. The products of PRSV CP gene fragment of 840 or 151 bp were quantified using a charge-coupled device (CCD) video image analysis software system (Image-Pro 6.0, Santa Barbara, CA). Areas of each band were calculated in time courses of 5, 15, 30, 60, and 120 min incubations. The band area of each point was compared to that of the 0 min incubation. Data were present as the ratio (%) = (each point band area/0 min band area) × 100%.

CP Fragment Detection in Rats after the 7 Day Feeding Experiment. Four rats, 4-week-old male [Sprague-Dawley (SD) strain], obtained from Biolasco Taiwan Co., Ltd. (I-Lan, Taiwan, Republic of China), were housed individually in stainless-steel, wire-mesh cages. The animal housing room was maintained at 20−22 °C, and an approximately 12 h light/12 h dark cycle was provided by automated illumination. Rats were daily oral-gavaged with 2 g/kg body weight (bw) of non-transgenic TN-2 and backcross transgenic papaya strains of 2210, 823, and 823-2210 for 7 days. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung-Hsing University (IACUC: 98-94). At the end of treatment, each animal was anesthetized with 2% isoflurane, and organs from rats (brain, heart, liver, spleen, lung, kidney, and testes) were collected and homogenized with Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) at a ratio of 1:4. The contents (stomach contents, cecal contents, colon contents, urine, whole blood, and serum) of rats were taken in 25 μL samples (approximately 25 mg). DNA extraction was performed using the DNA extraction kit QIAmp DNA Mini kit (Qiagen, Hilden, Germany). The primers and PCR analyses were performed as previously described in Table 1.

***In Vitro* Bacterial Reverse Mutation Test (Ames Test).** The Ames test was performed as described previously.[16,17] Five test histidine-dependent *Salmonella typhimurium* strains (TA98, TA100, TA102, TA1535, and TA1537) were used. The TA98 and TA100 strains (histidine needed a mutant) were purchased from the Bioresources Collection and Research Center (BCRC, Hsinchu, Taiwan, Republic of China). The TA102, TA1535, and TA1537 strains were obtained from Discovery Partners International (DPI, San

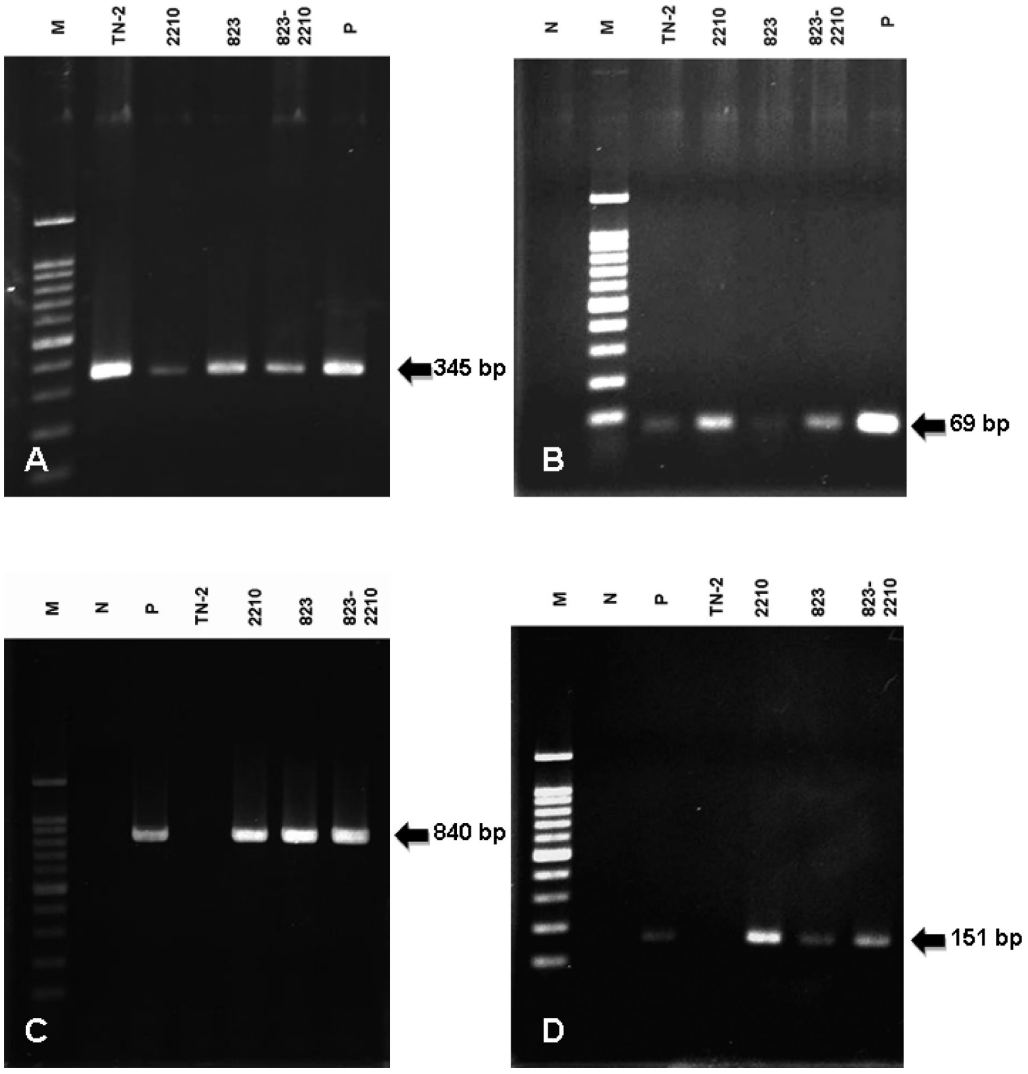

Figure 1. PCR amplification of non-transgenic and backcross transgenic papaya fruits. Detection of papaya endogenous genes included the (A) 345 bp with papa32/papaS9 primers and (B) 69 bp with S5/S6 primers. The PRSV CP genes included the (C) 840 bp with PRSV-F/PRSV-R primers and (D) 151 bp with S9-2/S10-2 primers. M, 100 bp DNA ladder; N, negative control (DW); and P, positive control (18-2-4 line of backcross transgenic papaya fruits with transgenic gene). TN-2, Tainung No. 2 with non-transgenic papaya fruit; 2210, 823, and 823-2210, transgenic papaya fruits.

Diego, CA). The positive control (PC) mutagens were 4-nitroquinoline-*N*-oxide (4-NQO), sodium azide, 9-aminoacridine (9-AA), 2-aminoanthracene (2-AA), mitomycin C ethyl methanesulfonate, cyclophosphamide, acridine orange, and colchicine. They were purchased from Sigma Co. (St. Louis, MO). Histidine was obtained from Merck (Germany). Other chemicals used were of analytical grade. The non-transgenic TN-2 and backcross transgenic strains of 2210, 823, and 823-2210 papaya fruit powders were dissolved in sterilized distilled water (DW). The Ames test (plate incorporation method) was administered at no cytotoxic doses of 0.3125, 0.625, 1.25, 2.5, and 5 mg/plate of TN-2, 2210, 823, and 823-2210. The test was performed with or without the S9 fraction from Aroclor 1254-induced rat livers (36.5 mg/mL, Lot 1452, Moltox, Boone, NC). The S9 fraction was prepared freshly by adjusting the S9 fraction to a final concentration of 10% with a dilution buffer containing 4 μM nicotinamide adenine dinucleotide phosphate (sodium salt), 5 μM glucose-6-phosphate (monosodium salt), 8 μM $MgCl_2$, 33 μM KCl, and 100 μM sodium phosphate buffer (pH 7.4). Briefly, 0.1 mL of DW-soluble sample was first added to culture tubes containing 2 mL of top agar (containing 0.5% NaCl), 0.2 mL of 0.5 mM histidine/biotin, and 0.1 mL of tester strain suspension. The sample-loaded cultures were then plated. Metabolic activation experiments were conducted using the rat liver S9 fraction at a volume of 0.5 mL in activation mixtures. Plates were incubated at 37 °C for 48 h. Control (DW) and PC experiments were also conducted. The PC mutagen incubations without S9-fraction treatment were 4-NQO (2.5 μg/plate), sodium azide (5 μg/plate), 9-AA (50 μg/plate), and mitomycin C (0.5 μg/plate). Otherwise, all tester bacterial strain incubations with S9 fraction in the PC groups used 2-AA (5 μg/plate). Test samples were assayed in triplicate at five concentrations, and two independent experiments were performed for each bacterial strain.

***In Vitro* Chromosomal Aberration Assay.** To assess the ability of non-transgenic TN-2 and the backcross transgenic varieties of 2210, 823, and 823-2210 papaya fruit to induce structural and numerical chromosome aberrations, an *in vitro* chromosome assay was applied to Chinese hamster ovary cell clone K1 (CHO-K1) according to methods described previously.[18] The CHO-K1 cells were obtained from the BCRC. The cells (5 mL of 1.5×10^5 in a 25 cm^2 flask) were seeded overnight before the treatment day. For cytotoxicity, 5 mg/mL TN-2, 2210, 823, and 823-2210 were dissolved in culture media and incubated with cells for 24 h. Cultures treated with no cytotoxic doses of 1.25, 2.5, and 5 mg/mL TN-2, 2210, 823, and 823-2210, with or without the metabolic activation system, were incubated with the S9 fraction for 3 h before treatment. The PC reagents, ethyl

dx.doi.org/10.1021/jf305036x | *J. Agric. Food Chem.* 2013, 61, 1585−1596

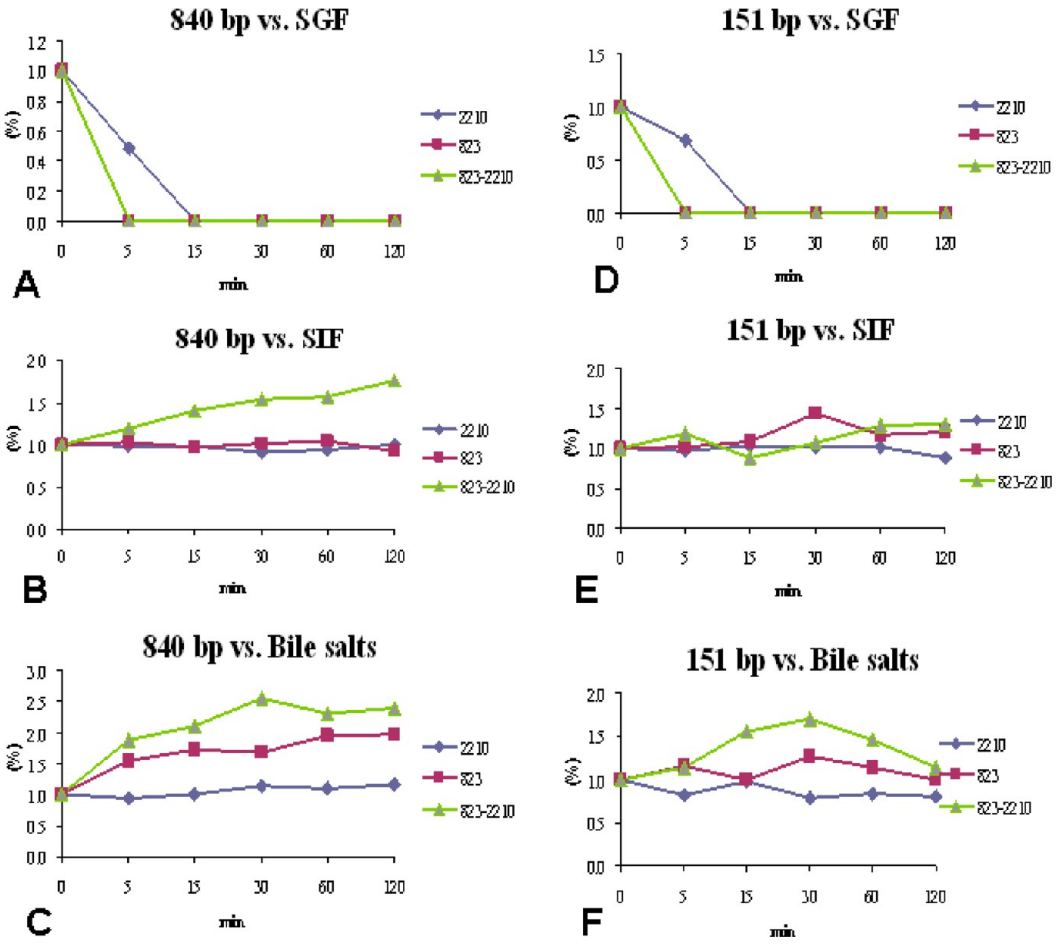

Figure 2. Digestion stability of CP gene fragments of PRSV of the backcross transgenic papaya fruits was incubated with SGF, SIF, and bile salts. Stability of PRSV gene 840 bp was incubated with (A) SGF, (B) SIF, and (C) bile salts; PRSV gene 151 bp was incubated with (D) SGF, (E) SIF, and (F) bile salts for 120 min. Transgenic papaya fruits = 2210, 823, and 823-2210.

methanesulfonate (2.5×10^{-3} mg/mL) and cyclophosphamide (25×10^{-3} mg/mL), were used without or with S9-fraction incubation, respectively. After treatment for 21 h, the cells were harvested and then added to colchicine (0.1 μg/mL) for 3 h before harvesting. After trypsinization, the cells were treated with hypotonic solution (0.5% KCl) for 5−7 min at 37 °C pre-incubation, fixed with an acetic acid and methanol (1: 3) solution, dropped onto slides, air-dried, and stained with 10% Giemsa. For the analysis of chromosomal aberrations, at least 100 metaphases were scored for each group. The number of cells with damaged chromosomes was calculated as aberration rate (%) = (number of cells with damaged chromosomes/total number of cells examined) × 100%. Two independent treatments were conducted.

***In Vivo* Mammalian Erythrocyte Micronucleus Test.** Healthy 6-week-old male mice (ICR strain, 25−35 g bw), obtained from Biolasco Taiwan Co., Ltd. (I-Lan, Taiwan, Republic of China), were subjected to a general physical examination upon receipt and acclimatized for 1 week. The animals were housed in cages (five per cage) and provided with food (Lab Diet 5001 Rodent diet, Purina Mills LLC, St. Louis, MO) and water *ad libitum*. The stainless-steel cages were kept at 21 ± 2 °C with 50−70% relative humidity under a 12 h light/12 h dark cycle. This study was approved by the IACUC of National Chung-Hsing University (IACUC: 98-94). The micronucleus assay was conducted as previously described.[19,20] Mice were orally administered non-transgenic TN-2 and backcross transgenic strains of 2210, 823, and 823-2210 papaya fruit powder at a limited dose of 5 g/kg of bw. The administered volume was 10 mL/kg of bw. The PC group was intraperitoneously injected with 0.05 μg/kg of bw of cyclophosphamide and DW as a control. Five mice were allocated randomly to each group. After dosing, the animals were examined for mortality and clinical signs. The animals were anesthetized using 2% isoflurane (Halocarbon Laboratories, River Edge, NJ), and 100 μL of orbital peripheral blood was withdrawn at 48 and 72 h. Slides were prepared for staining with 0.1% acridine orange. Reticulocytes (RETs) stained orange and micronuclei (MN) in RETs stained yellow−green on each slide were counted under a florescence microscope (BX50, Olympus, Münster, Germany). In total, 1000 RETs per animal were analyzed for the existence of MN. The ratio of RETs/normochromatic erythrocytes (NCEs) was determined on the basis of 1000 NCEs. The MN/NCE ratio was recorded while counting 1000 RETs per animal, and the MN-RETs/1000 RETs (‰) was calculated.

The 28-Day Repeated Feeding Study. The 28-day repeated feeding study for the non-transgenic and backcross transgenic papaya fruits was performed on the basis of the experimental guidelines reported by the Organization for Economic Co-operation and Development (OECD).[21] The 5-week-old male and female SD albino rats were obtained from Biolasco Taiwan Co., Ltd. (I-Lan, Taiwan, Republic of China). The housing facility was maintained under appropriate environmental conditions, as mentioned above. For the 28-day repeated oral feeding study, 10 male and 10 female rats were orally administered the non-transgenic TN-2 and backcross transgenic strains of 2210, 823, and 823-2210 papaya fruit powder at a daily dose of 2 g/kg of bw. The control animals were only given DW. The weekly bw and daily feed consumption were recorded. At the end of day 28, all animals were fasted overnight and then anesthetized with 2% isoflurane in an inhalation chamber. Blood was withdrawn from the abdominal aorta into tubes (K3 EDTA syringes, Vacutainer, NJ). Routine hematology, coagulation, clinical chemistry, and urinary examinations were conducted on all animals. A thorough necropsy was performed on all animals, and organs were weighed after dissection

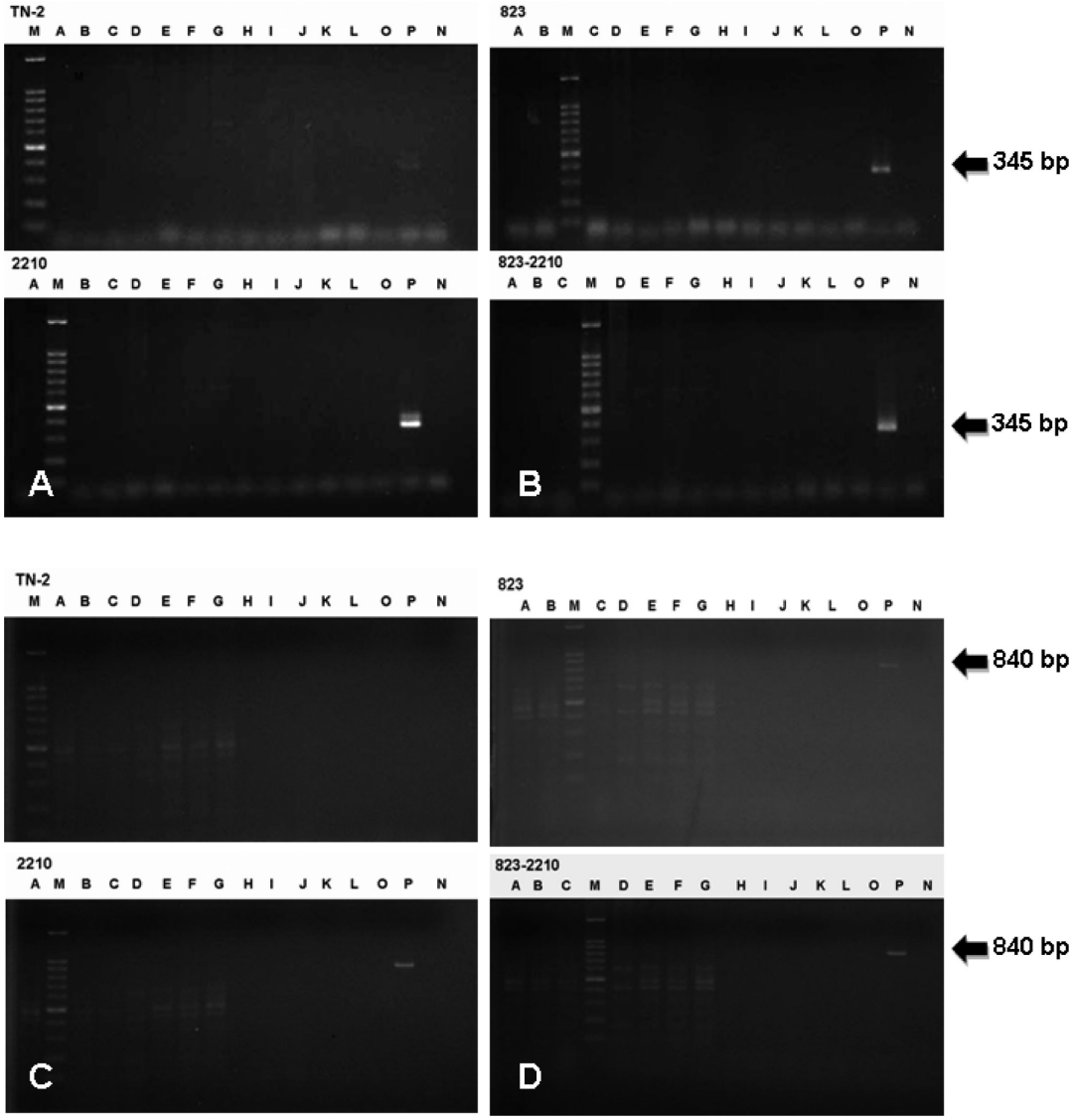

Figure 3. PCR amplification of CP gene fragments of PRSV in the various organ tissues of rats fed with non-transgenic and backcross transgenic papaya fruits for 7 days. (A and B) Papaya endogenous gene 345 bp with papa32/papa59 primers and (C and D) PRSV gene 840 bp with PRSV-F/PRSV-R primers were not detectable in all examined organs. TN-2, Tainung No. 2 with non-transgenic papaya fruit; 2210, 823, and 823-2210, transgenic papaya fruits. M, 100 bp DNA ladder marker; A, brain; B, heart; C, liver; D, spleen; E, lung; F, kidney; G, testes; H, stomach content; I, cecum content; J, colon content; K, urine; L, whole blood; O, serum; N, negative control, (DW); and P, positive control (18-2-4 line of backcross transgenic papaya fruit sample).

and then examined grossly, removed, and fixed in 10% buffered formalin. The organs, including the brain, heart, thymus, liver, spleen, kidneys, adrenal glands, testes (males), and ovaries (females) were trimmed for histopathological examination. For semi-quantitative grading, lesion severity was graded using the criteria developed by Shackelford et al.[22] Lesion severity was graded as follows: 1, minimal (<10%); 2, slight (11−25%); 3, moderate (26−50%); 4, moderate/severe (51−75%); and 5, severe/high (76−100%).

Statistical Analysis. Data are expressed as the mean ± standard deviation (SD). For the genotoxicity evaluation of *in vitro* and *in vivo* assays, statistical difference was performed between the control and treated groups. For the 28-day repeated animal feeding study, comparisons were designed to determine whether differences were attributable to the non-transgenic TN-2 and backcross transgenic strains of 2210, 823, and 823-2210 treated groups using Student's *t* test. Differences are regarded as significant at $p < 0.05$.

RESULTS

Construct-Specific Detection. The primer pair papa32/papa59 is designed for detecting the papaya endogenous protein of papain, a 345 bp product from the non-transgenic papaya TN-2 and the backcross transgenic strains of 2210, 823, and 823-2210 papaya fruits. The primer pair S5/S6 is also designed for a smaller papain fragment, a 69 bp product from all of the transgenic and non-transgenic papaya fruits. Both of them were positive, indicating the test substances are papaya fruits (panels A and B of Figure 1). Furthermore, the primer pair PRSV-F/PRSV-R is designed for transgene-specific detection of an 840 bp product from the backcross transgenic papaya fruits but not from the non-transgenic TN-2 sample. Also, for the sequence of transgene-specific detection, the primer pair S9-2/S10-2 amplified a 151 bp product from the three transgenic papaya but was not shown in the non-transgenic TN-2 (panels C and D of Figure 1).

Table 2. Revertant Colonies of Backcross Transgenic Ppapaya Fruits in the Absence of the S9 Fraction in *Salmonella* Mutagenicity

		number of revertants (colonies/plate)[a]				
		TA98	TA100	TA102	TA1535	TA1537
test article	concentration (mg/plate)	−S9	−S9	−S9	−S9	−S9
C[b]		33.3 ± 2.4[a]	306.3 ± 5.6	306.3 ± 5.6	9.0 ± 1.4	8.3 ± 0.5
PC[c]		506.7 ± 38.8[d]	1204.0 ± 172.4[d]	1304.0 ± 172.0[d]	488.0 ± 37.0[d]	519.0 ± 47.4[d]
2210	0.3125	26.0 ± 2.4	357.7 ± 9.0	290.7 ± 9.0	10.3 ± 0.9	8.0 ± 0.8
	0.625	31.0 ± 5.4	338.3 ± 8.7	313.3 ± 14.8	7.3 ± 0.9	8.3 ± 1.2
	1.25	28.3 ± 2.5	336.3 ± 16.8	311.3 ± 17.6	13.0 ± 1.6	7.7 ± 0.5
	2.5	26.3 ± 2.5	337.3 ± 15.5	313.3 ± 22.5	10.3 ± 3.8	8.0 ± 0.8
	5	29.3 ± 0.5	353.7 ± 17.9	307.3 ± 8.7	13.7 ± 4.8	7.7 ± 0.9
823	0.3125	35.7 ± 0.9	374.0 ± 8.5	312.3 ± 6.3	11.0 ± 4.5	7.3 ± 0.5
	0.625	36.3 ± 2.4	365.7 ± 27.0	302.0 ± 7.5	9.0 ± 2.4	7.7 ± 0.5
	1.25	32.0 ± 5.4	362.0 ± 15.5	317.0 ± 19.6	13.0 ± 1.6	9.0 ± 1.4
	2.5	30.3 ± 1.9	334.3 ± 15.0	313.3 ± 22.5	12.0 ± 4.3	7.3 ± 0.5
	5	29.3 ± 3.7	350.0 ± 16.3	313.7 ± 3.1	13.7 ± 4.9	7.7 ± 0.5
823-2210	0.3125	33.7 ± 0.9	376.7 ± 12.7	317.0 ± 5.9	8.7 ± 2.1	8.3 ± 1.2
	0.625	31.3 ± 2.1	385.0 ± 17.7	319.3 ± 10.8	11.3 ± 2.6	8.7 ± 0.9
	1.25	31.0 ± 4.3	379.3 ± 5.7	320.3 ± 8.5	9.0 ± 3.6	7.7 ± 0.9
	2.5	28.0 ± 2.8	371.7 ± 12.7	312.7 ± 12.3	11.0 ± 4.2	9.7 ± 1.2
	5	32.7 ± 3.8	379.3 ± 9.6	310.7 ± 12.4	14.0 ± 5.7	9.3 ± 1.2

[a]Data are presented as the mean ± SD (n = 3). [b]C, control (DW); 2210, 823, and 823-2210, backcross transgenic papaya fruits. [c]Positive reagents without S9-fraction reactions were 1 μg/plate 4-NQO for TA98, 5 μg/plate sodium azide for TA100 and TA1535, 0.5 μg/plate mitomycin C for TA102, and 50 μg/plate 9-aminoacridine for TA1537. [d]Significant difference in the number of colonies was more than twice that of the control and treated groups at $p < 0.05$.

Table 3. Revertant Colonies of Backcross Transgenic Papaya Fruits in the Presence of the S9 Fraction in *Salmonella* Mutagenicity

		number of revertants (colonies/plate)[a]				
		TA98	TA100	TA102	TA1535	TA1537
test article	concentration (mg/plate)	+S9	+S9	+S9	+S9	+S9
C[b]		36.0 ± 3.7[a]	345.7 ± 12.0	395.3 ± 9.5	10.7 ± 4.5	8.0 ± 0.8
PC[c]		2759.3 ± 167.9[d]	2921.0 ± 233.1[d]	2096.0 ± 132.0[d]	370 ± 36[d]	117 ± 2[d]
2210	0.3125	35.3 ± 2.6	317.0 ± 12.0	402.3 ± 7.6	16.7 ± 1.9	7.3 ± 0.5
	0.625	32.7 ± 1.2	325.7 ± 11.7	398.3 ± 10.7	14.0 ± 0.8	8.3 ± 2.6
	1.25	34.7 ± 3.1	353.7 ± 1.9	402.7 ± 12.0	15.7 ± 1.2	8.3 ± 1.2
	2.5	32.0 ± 2.8	366.0 ± 2.2	393.3 ± 6.3	13.3 ± 5.3	7.7 ± 1.7
	5	31.7 ± 1.7	365.7 ± 8.2	397.7 ± 10.7	15.3 ± 5.3	8.3 ± 1.2
823	0.3125	29.7 ± 3.9	303.7 ± 16.8	391.3 ± 8.2	17.0 ± 2.8	7.7 ± 1.7
	0.625	29.7 ± 3.3	344.0 ± 9.6	383.7 ± 11.1	19.7 ± 2.1	7.3 ± 1.2
	1.25	31.7 ± 2.4	355.7 ± 15.4	403.7 ± 12.7	15.0 ± 3.3	6.7 ± 0.5
	2.5	33.0 ± 2.4	348.7 ± 11.5	386.3 ± 15.6	7.7 ± 1.7	7.7 ± 0.9
	5	37.3 ± 1.7	322.3 ± 24.5	387.0 ± 3.6	10.7 ± 2.4	6.3 ± 0.5
823-2210	0.3125	37.7 ± 1.9	289.3 ± 21.1	398.7 ± 13.0	16.7 ± 4.1	6.7 ± 0.5
	0.625	37.3 ± 0.9	324.7 ± 11.5	382.3 ± 10.2	17.7 ± 1.7	7.7 ± 1.7
	1.25	35.3 ± 2.1	292.0 ± 9.4	393.3 ± 9.6	10.7 ± 3.3	8.0 ± 1.6
	2.5	36.0 ± 3.7	323.0 ± 8.0	392.0 ± 12.3	15.3 ± 3.4	7.7 ± 0.0
	5	36.0 ± 3.7	311.7 ± 4.6	396.0 ± 13.1	13.3 ± 2.4	7.7 ± 0.5

[a]Data are presented as the mean ± SD (n = 3). [b]C, control (DW); 2210, 823, and 823-2210, backcross transgenic papaya fruits. [c]Positive reagent with the S9 fraction was 50 μg/plate 2-aminoanthracene for all *Salmonella* strains. [d]Significant difference in the number of colonies was more than twice that of the control and treated groups at $p < 0.05$.

Stability Analysis of CP Fragments *in Vitro*. *In vitro* resistance of the CP fragments, 840 and 151 bp products of the backcross transgenic papaya fruits, was assessed by incubation with digestive enzymes, SGF, SIF, and bile salts. The 840 and 151 bp products of 823 and 2210 were rapidly (less than 5 min) hydrolyzed in SGF containing pepsin, and the product of 2210 was hydrolyzed in less than 15 min. Otherwise, the 840 and 151 bp products of CP fragments in three transgenic samples were not hydrolyzed more than 120 min in SIF (containing pancreatin) and bile salts (Figure 2).

CP Fragment Detection in Rats. Rats were orally administered the backcross transgenic papaya fruit of 2210, 823, and 823-2210 at a dose of 2 g kg^{-1} day^{-1} for 7 consecutive days. Neither the conserved portion of the papaya endogenous DNA fragment of 345 bp nor the CP fragments of 840 bp were found in any organs, including the brain, heart, liver, spleen,

lung, kidneys, and testes, or in the stomach contents, cecal contents, colon contents, urine, whole blood, and serum (Figure 3).

Ames Test. Before testing, the bactericide of non-transgenic TN-2 and backcross transgenic strains of 2210, 823, and 823-2210 papaya fruit powder was conducted and the results showed that there was no toxicity to the *S. typhimurium* TA98, 100, 102, 1535, and 1537 tester strains in a high dose (5 mg/plate) exposure (data not shown). For the Ames test, concentrations of 0.3125, 0.625, 1.25, 2.5, and 5 mg/plate samples were co-cultured with or without S9 metabolic activation with tester strains for 48 h. The results revealed that, except for the positive control mutagens, all test samples of backcross transgenic papaya fruits were negative when treated with or without S9 metabolic activation (Tables 2 and 3).

Chromosomal Aberration Test. No cytotoxicity of the backcross transgenic papaya fruits to the CHO-K1 cells at a concentration of 5 mg/mL after 24 h of incubation was found (data not shown). For the chromosomal aberration assay, the tested concentrations of the transgenic papaya fruits at concentrations of 1.25, 2.5, and 5 mg/mL were tested and the results presented no significant difference when compared to the control group in the frequency and specific site of chromosomal aberration in either the absence or presence of S9 metabolic activation (Table 4).

Table 4. Frequency of Chromosomal Aberration of Backcross Transgenic Papaya Fruits Treated with or without the S9 Fraction in Cultured CHO-K1 Cells

		frequency of chromosomal aberration (%)	
group[a]	concentration (mg/mL)	−S9	+S9
C	0	4.7 ± 3.8[b]	4.3 ± 2.1
EMS	0.0025	31.7 ± 9.0[c]	ND
CP	0.025	ND	27.7 ± 1.5[c]
2210	1.25	6.3 ± 0.6	7.0 ± 1.7
	2.5	4.7 ± 1.5	6.3 ± 4.0
	5	5.7 ± 1.5	6.0 ± 1.0
823	1.25	4.7 ± 2.5	3.7 ± 1.5
	2.5	5.7 ± 1.5	4.7 ± 0.6
	5	6.0 ± 1.0	4.0 ± 1.0
823-2210	1.25	3.3 ± 1.5	3.0 ± 1.7
	2.5	5.3 ± 4.7	1.7 ± 1.2
	5	6.7 ± 1.5	1.7 ± 0.6

[a]C, control (DW); EMS, ethyl methanesulfonate; CP, cyclophosphamide; 2210, 823, and 823-2210, backcross transgenic papaya fruits; and ND, not done. [b]A total number of 100 metaphases of chromosomes of CHO cells were counted in each treatment. The number of cells with damaged chromosomes was calculated as the aberration rate (%) = (number of cells with damaged chromosomes/100) × 100%. [c]Significant difference between the control and treated groups at $p < 0.05$.

Micronucleus Test. Three groups of mice were singly orally administered with backcross transgenic strains of 2210, 823, and 823-2210 papaya fruit powder at a dose of 5 g/kg of bw. The results of the micronucleus test in all groups at 48 and 72 h points are shown in Table 5. The percentage of reticulocytes in the positive control (PC) group was significantly inhibited by CP treatment ($p < 0.05$) and increased the frequency of micronuclei in the erythrocytes ($p < 0.05$) when compared to the control group. Both the percentage of reticulocytes and frequency of micronuclei in the peripheral blood erythrocytes showed no significant difference between non-transgenic TN-2 and backcross transgenic papaya fruit treatment groups.

Table 5. Micronuclei Assay of Backcross Transgenic Papaya Fruits in the Peripheral Red Blood Cells of Mice

sampling intervals/group[a]	dose (g/kg)	RETs/1000 RBCs (‰)	MN-RETs/1000 RETs (‰)
		48 h	
C[a]	0	18.2 ± 1.8[b]	0.8 ± 0.8
CP	0.05	9.6 ± 0.9[c]	11.8 ± 3.1[c]
2210	5	22.2 ± 2.7[c]	0.0 ± 0.0
823	5	19.8 ± 1.1	0.0 ± 0.0
823-2210	5	22.6 ± 4.8	1.2 ± 1.3
		72 h	
C	0	21.2 ± 3.9	0.6 ± 0.9
CP	0.05	12.4 ± 2.7[c]	4.2 ± 1.9[c]
2210	5	21.4 ± 2.1	1.0 ± 0.7
823	5	18.9 ± 2.4	0.2 ± 0.4
823-2210	5	25.0 ± 4.7	0.2 ± 0.4

[a]C, control (DW); TN-2, Tainung No. 2; 2210, 823, and 823-2210, backcross transgenic papaya fruits; RETs, reticulocytes ; RBCs, red blood cells; MN-RETs, micronucleated reticulocytes; and CP, cyclophosphamide (intraperitoneal injection). [b]Data are expressed as the mean ± SD ($n = 5$). [c]Significant difference between the control and treated groups at $p < 0.05$.

The 28-Day Repeated Feeding Study. No death or abnormality of rats occurred in the non-transgenic TN-2 and backcross transgenic papaya fruit treatment groups. An increase in food consumption (g/day) of control male rats that caused a decrease in the backcross transgenic papaya fruit-treated groups at the interval of 21 days ($p < 0.05$), but it became normal at the end of the experiment. Finally, no significant difference of feed efficacy was observed in all treated groups (Table 6). No significant difference in bw was observed between the groups that were administered the non-transgenic TN-2 and three backcross transgenic papaya fruits (Figure 4). Hematological examination revealed a significant elevation of white blood cells (WBCs) and lymphocytes (%) of male rats for the three backcross transgenic papaya fruits when compared to the non-transgenic papaya TN-2 group (Table 7). Furthermore, there was a decrease of segmented neutrophils (%) in males that were administered the 823 and 823-2210 fruit and a decrease of banded neutrophils in the females rats that were administered the 2210 and 823-2210 fruit when compared to the TN-2 group (Table 8). These statistical differences were not considered biologically significant. Both responses of WBCs and lymphocytes (%) were within age-matched (8−16-week-old) historical control values.[23] In clinical biochemistry, there were significant differences of albumin, creatine kinase (CK), total cholesterol (TC), triglyceride (TG), total protein (TP), and some ion concentrations in 823- and 823-2210-treated male and female rats ($p < 0.05$). However, these parameters were within age-matched (8−16-week-old) historical control values,[23] and statistical differences were not considered biologically significant in male rats (Table 9) and female rats (Table 10). No adverse effects were observed in the clinical hematology, biochemistry, and blood coagulation parameter response variables of treatment groups. Furthermore, no biological significances of organ weight (Table 11) and urinary parameters (data not shown) were observed in the backcross

Table 6. Weekly Feed Consumption and Feed Efficiency of Rats Fed with Non-transgenic and Backcross Transgenic Papaya Fruits for 28 Days

	non-transgenic line	transgenic lines		
group[a]/day	TN-2	2210	823	823-2210
		Male		
7	23.2 ± 5.1[b,c]	23.7 ± 2.6	25.0 ± 3.1	26.0 ± 1.4
14	30.9 ± 8.4	25.8 ± 3.2	26.7 ± 2.6	28.4 ± 2.0
21	35.8 ± 4.3	26.6 ± 2.1[d]	27.8 ± 3.8[d]	29.0 ± 3.4[d]
28	29.0 ± 2.7	26.0 ± 2.9	26.9 ± 2.7	30.1 ± 3.9
feed efficiency (%)[e]	18.8 ± 2.7	23.3 ± 2.8	22.6 ± 3.3	23.4 ± 3.2
		Female		
7	21.4 ± 1.7	19.9 ± 2.2	21.4 ± 3.4	21.3 ± 2.0
14	21.0 ± 4.3	21.0 ± 2.8	20.3 ± 3.3	21.2 ± 2.4
21	23.0 ± 2.4	22.3 ± 2.0	20.8 ± 2.9	22.1 ± 2.2
28	28.4 ± 1.5	28.7 ± 2.5	27.7 ± 3.4	29.0 ± 2.9
feed efficiency (%)	9.7 ± 1.8	11.4 ± 2.5	9.5 ± 2.2	11.1 ± 2.5

[a]TN-2, Tainung No. 2; 2210, 823, and 823-2210, backcross transgenic papaya fruits. [b]Feed consumption (g/day) = [total feed intake (g)/test period (28 days)]. [c]Data are expressed as the mean ± SD (n = 10). [d]Significant difference between the non-GM (TN-2) and GM papaya-treated groups at $p < 0.05$. [e]Feed efficiency (%) = [daily bw gain (g)/daily feed intake (g)] × 100%.

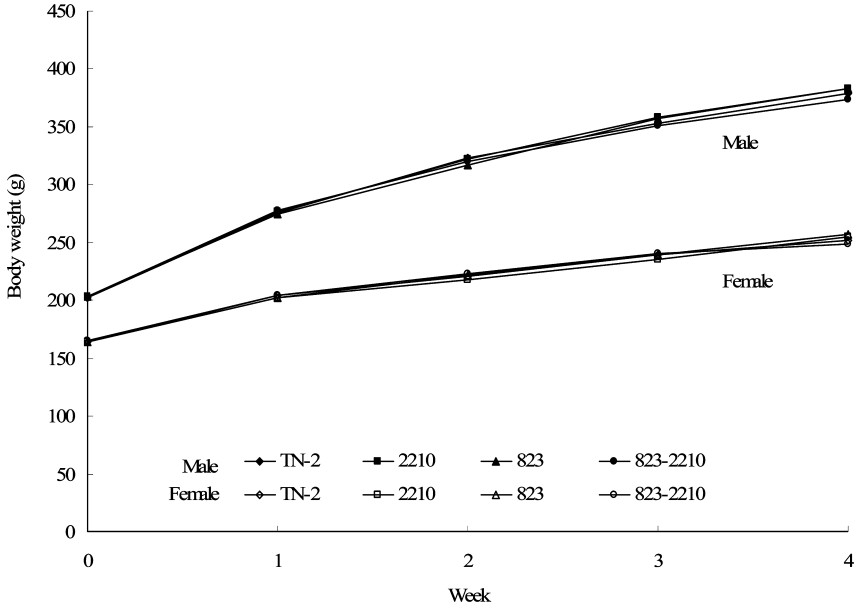

Figure 4. Weekly bw changes of rats fed with non-transgenic and backcross transgenic papaya fruits in the 28 day feeding toxicity trial. TN-2, non-transgenic papaya of Tainung No. 2; 2210, 823, and 823-2210, backcross transgenic papaya fruits.

transgenic papaya fruit-treated rats. Some of the non-specific lesions observed during pathological examination included congenital abnormality of the brain, mononuclear cell infiltration of the heart, renal cysts and tubular regeneration of the kidneys, liver necrosis, and a foreign-body-induced granuloma of the lung. These lesions were randomly found in the non-transgenic TN-2- and backcross transgenic papaya fruit-treated rats (Table 12). A foreign-body-induced granuloma in the lung was noted in a TN-2-treated male rat and was considered the result of an error operation during oral administration. There were no significant gross or microscopic lesions in male or female rats attributable to the non-transgenic and backcross transgenic papaya fruit treatments.

DISCUSSION

As stated by the Codex, the inserted DNA and flanking DNA at the insertion site and the expressed substance should be described to evaluate the safety risk of transgenic plants.[24] The goal of the safety assessment is not to demonstrate that foods obtained from transgenic crops are absolutely safe but rather to demonstrate they are as safe as those obtained from non-transgenic comparators.[25] For this, the newly developed backcross transgenic papaya of 823, 2210, and 823-2210 lines were evaluated for the presence of plant fragments, genotoxicity to bacterial and mammal cells, and tested for the levels of certain health parameters in animals fed on backcross transgenic papaya fruits.

One of main food safety issues that raises some concerns when considering the use of transgenic crops is whether exogenous genes from transgenic crops can affect tissues or organs.[26] In the present study, the non-transgenic papaya TN2 was also used as the control group to compare to the backcross transgenic papaya fruits and to distinguish differences that are related to transgenic or papaya itself effects too. From our bioinformatic results, the transgenic-specific gene fragment of backcross transgenic papaya fruits was degraded in acid or

Table 7. Hematological and Coagulate Parameters of Rats Fed with Non-transgenic and Backcross Transgenic Papaya Fruits for 28 Days

	non-transgenic line	transgenic lines		
group[a]/items	TN-2	2210	823	823-2210
		Male		
RBC ($10^6/\mu L$)	7.8 ± 0.4[b]	8.0 ± 0.5	7.9 ± 0.5	8.1 ± 0.4
HGB (g/dL)	15.5 ± 0.5	15.6 ± 0.9	15.4 ± 0.7	15.7 ± 0.7
HCT (%)	47.2 ± 1.8	47.7 ± 3.0	47.2 ± 2.8	48.4 ± 2.1
MCV (fL)	60.4 ± 2.4	60.0 ± 2.1	59.9 ± 1.5	59.8 ± 1.3
MCH (pg)	19.8 ± 0.7	19.7 ± 0.8	19.6 ± 0.9	19.4 ± 0.6
MCHC (g/dL)	32.8 ± 0.6	32.8 ± 0.8	32.7 ± 1.0	32.5 ± 0.5
PLT ($10^3/\mu L$)	1203.3 ± 154.2	1206.1 ± 117.9	1058.3 ± 549.6	1241.7 ± 143.4
PT (s)	11.6 ± 0.2	11.8 ± 0.3	11.7 ± 0.2	11.5 ± 1.0
APTT (s)	20.0 ± 0.7	20.4 ± 0.4	21.8 ± 0.6	21.9 ± 0.4
Fbg (mg/dL)	180.0 ± 6.5	181.1 ± 4.0	188.9 ± 3.1	188.7 ± 2.9
		Female		
RBC ($10^6/\mu L$)	7.8 ± 0.4	7.3 ± 0.3[c]	7.8 ± 0.4	7.6 ± 0.3
HGB (g/dL)	15.0 ± 0.5	14.8 ± 0.4	15.3 ± 0.6	15.1 ± 0.4
HCT (%)	46.0 ± 1.7	43.5 ± 1.5[c]	45.7 ± 2.1	44.6 ± 1.2
MCV (fL)	58.9 ± 1.9	59.1 ± 1.5	58.7 ± 1.7	59.0 ± 1.5
MCH (pg)	19.2 ± 0.7	20.2 ± 0.5	19.6 ± 0.5	20.0 ± 0.7
MCHC (g/dL)	32.6 ± 0.7	31.2 ± 9.5	33.5 ± 0.7	33.9 ± 0.8
PLT ($10^3/\mu L$)	1194.7 ± 116.8	1183.2 ± 418.3	1141.6 ± 139.7	1140.9 ± 331.1
PT (s)	10.2 ± 0.1	10.1 ± 0.1	10.5 ± 0.2	9.8 ± 0.1
APTT (s)	20.4 ± 1.1	22.3 ± 1.5	20.4 ± 1.2	20.5 ± 0.8
Fbg (mg/dL)	177.0 ± 8.7	179.9 ± 5.2	178.8 ± 4.8	176.3 ± 4.4

[a]TN-2, Tainung No. 2; 2210, 823, and 823-2210, backcross transgenic papaya fruits; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; and PLT, platelets. [b]Data are expressed as the mean ± SD ($n = 10$). [c]Significant difference between the non-transgenic (TN-2) and transgenic papaya-treated groups at $p < 0.05$.

Table 8. WBC Differentiation of Rats Fed with Non-transgenic and Backcross Transgenic Papaya Fruits for 28 Days

	non-transgenic line	transgenic lines		
group[a]/items	TN-2	2210	823	823-2210
		Male		
WBC ($10^3/\mu L$)	4.4 ± 0.8[b]	6.4 ± 1.8[c]	6.1 ± 2.2[c]	6.8 ± 1.6[c]
lymphocyte (%)	82.4 ± 4.2	87.2 ± 4.5[c]	88.2 ± 4.4[c]	90.0 ± 4.6[c]
neutrophil (%)				
band	1.1 ± 1.3	0.6 ± 0.8	0.9 ± 0.9	1.0 ± 1.2
segment	15.0 ± 3.4	12.1 ± 4.7	10.1 ± 4.4[c]	8.1 ± 4.2[c]
monocyte (%)	1.5 ± 0.8	0.1 ± 0.3[c]	0.8 ± 0.5	0.7 ± 1.3
eosinophil (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
basophil (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		Female		
WBC ($10^3/\mu L$)	5.2 ± 1.6	5.3 ± 1.7	6.5 ± 2.2	6.1 ± 2.1
lymphocyte (%)	85.4 ± 5.7	86.7 ± 4.9	85.6 ± 8.0	89.9 ± 3.9
neutrophil (%)				
band	1.0 ± 0.7	0.3 ± 0.5[c]	0.9 ± 1.2	0.1 ± 0.3[c]
segment	12.5 ± 5.5	12.1 ± 4.9	13.3 ± 7.7	9.6 ± 4.1
monocyte (%)	1.1 ± 1.7	0.9 ± 0.6	0.2 ± 0.4	0.4 ± 0.5
eosinophil (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
basophil (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a]TN-2, Tainung No. 2; 2210, 823, and 823-2210, backcross transgenic papaya fruits. [b]Data are expressed as the mean ± SD ($n = 10$). [c]Significant difference between the non-transgenic (TN-2) and transgenic papaya-treated groups at $p < 0.05$.

pepsin but not in alkali (such as bile salt) or pancreatin. In the 7 day feeding test, the transgenic-specific gene fragment was not detectable in any organs or gastrointestinal contents, indicating the backcross transgenic papaya fruits could be completely digested by the gastrointestinal tract of rats. This is unlike the foreign DNA fragments of transgenic soybean diets that can be detected in different tissues and organs in tilapias, suggesting that exogenous transgenic genes were absorbed systemically and not completely degraded by the tilapia's alimentary canal. However, these studies were unable to confirm whether these fragments were integrated into cellular DNA.[26]

Table 9. Serum Biochemistry of Male Rats Fed with Non-transgenic and Backcross Transgenic Papaya Fruits for 28 Days

	non-transgenic line	transgenic lines		
group[a]/items	TN-2	2210	823	823-2210
albumin (g/dL)	2.8 ± 0.5[b]	2.7 ± 0.4	3.3 ± 0.4[c]	3.1 ± 0.2
ALP (units/L)	126.2 ± 40.1	119.1 ± 30.5	149.7 ± 27.8	126.8 ± 25.9
amylase (units/L)	635.2 ± 133.1	587.9 ± 149.0	729.5 ± 86.2	675.6 ± 93.3
ALT (units/L)	16.4 ± 3.2	19.6 ± 7.9	24.3 ± 6.2[c]	18.3 ± 3.3
AST (units/L)	53.7 ± 10.3	57.6 ± 10.7	69.8 ± 11.1	58.5 ± 7.5
BUN (mg/dL)	12.3 ± 2.2	11.2 ± 2.3	13.8 ± 2.0	11.7 ± 2.3
creatinine (mg/dL)	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
CK (units/L)	73.1 ± 22.5	145.7 ± 190.4	141.1 ± 217.8	102.9 ± 29.3[c]
glucose (mg/dL)	153.7 ± 34.1	141.4 ± 38.4	156.6 ± 29.3	145.8 ± 14.6
LDH (units/L)	93.8 ± 32.7	145.7 ± 108.3	158.2 ± 204.7	123.2 ± 82.0
TC (mg/dL)	42.8 ± 8.0	40.8 ± 10.8	55.9 ± 10.9[c]	52.0 ± 9.4[c]
TG (mg/dL)	26.7 ± 6.0	27.7 ± 8.5	33.8 ± 8.0[c]	28.9 ± 8.4
TP (g/dL)	4.9 ± 0.8	4.9 ± 1.0	6.1 ± 0.6[c]	5.4 ± 0.4
K^+ (mequiv/dL)	5.3 ± 0.7	4.9 ± 1.0	5.6 ± 0.7	5.3 ± 0.5
P^{-3} (mg/dL)	7.5 ± 1.3	7.5 ± 1.5	9.6 ± 1.3[c]	9.0 ± 0.5[c]

[a]TN-2, Tainung No. 2; 2210, 823, and 823-2210, backcross transgenic papaya fruits; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase; LDH, lactate dehydrogenase; TC, total cholesterol; TG, triglyceride; and TP, total protein. [b]Data are expressed as the mean ± SD (n = 10). [c]Significant difference between the non-transgenic (TN-2) and transgenic papaya-treated groups at $p < 0.05$.

Table 10. Clinical Biochemistry of Female Rats Fed with Non-transgenic and Backcross Transgenic Papaya Fruits for 28 Days

	non-transgenic line	transgenic lines		
group[a]/items	TN-2	2210	823	823-2210
albumin (g/dL)	3.1 ± 0.4[b]	3.0 ± 0.4	3.3 ± 0.2	3.5 ± 0.2[c]
ALP (units/L)	80.9 ± 26.7	72.9 ± 17.8	72.8 ± 16.6	75.9 ± 13.6
amylase (units/L)	411.7 ± 75.5	447.6 ± 108.5	390.9 ± 63.8	415.5 ± 67.0
ALT (units/L)	17.2 ± 4.6	14.1 ± 2.0	16.3 ± 5.9	17.0 ± 7.7
AST (units/L)	61.9 ± 10.7	51.1 ± 5.8[c]	66.0 ± 13.5	58.0 ± 11.1
BUN (mg/dL)	11.9 ± 2.0	12.3 ± 2.9	25.7 ± 40.2	13.3 ± 2.1
creatinine (mg/dL)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.6 ± 0.1[c]
CK (units/L)	70.1 ± 24.3	60.0 ± 6.8	140.3 ± 227.1	64.2 ± 9.5
glucose (mg/dL)	124.6 ± 21.9	116.1 ± 23.7	133.0 ± 19.1	132.7 ± 9.9
LDH (units/L)	122.0 ± 110.1	73.7 ± 17.4	110.8 ± 95.1	77.0 ± 27.6
TC (mg/dL)	44.0 ± 6.3	44.1 ± 9.3	52.8 ± 11.7	54.0 ± 14.6
TG (mg/dL)	27.2 ± 4.4	27.6 ± 5.4	26.3 ± 9.9	30.5 ± 5.9
TP (g/dL)	5.4 ± 0.6	5.2 ± 0.8	5.8 ± 0.2	6.0 ± 0.3[c]
K^+ (mequiv/dL)	5.3 ± 0.7	4.6 ± 0.5[c]	5.3 ± 0.4	5.5 ± 0.4
P^{-3} (mg/dL)	7.7 ± 1.5	7.4 ± 1.4	9.0 ± 0.9[c]	8.2 ± 0.9

[a]TN-2, Tainung No. 2; 2210, 823, and 823-2210, transgenic papaya fruits; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase; LDH, lactate dehydrogenase; TC, total cholesterol; TG, triglyceride; and TP, total protein. [b]Data are expressed as the mean ± SD (n = 10). [c]Significant difference between the non-transgenic (TN-2) and transgenic papaya-treated groups at $p < 0.05$.

In vitro and *in vivo* genotoxicity tests are used worldwide as a test system for detecting the degree of mutagenicity and predicting the carcinogenicity of certain chemicals.[16] Otherwise, various *in silico* and *in vitro* methods may contribute to the safety assessment of genetically modified (GM) plant-derived food and feed and components thereof, such as *in vitro* genotoxicity test methods that screen for point mutations, chromosomal aberrations, and DNA damage/repair, which are also recommended by European Food Safety Authority (EFSA).[27] For example, genotoxicity and animal toxicity tests were used as clinical risk assessments in biresistant anti-pesticide crylAc/sck transgenic rice and anti-cucumber leaf mosaic virus transgenic tomatoes and sweet peppers.[28] Regardless of the presence of S9 enzyme, the results of the Ames test revealed that both non-transgenic TN-2 and backcross transgenic papaya fruits had no mutagenicity toward *S. typhimurium* mutation strains TA 98, TA 100, TA 102, TA 1535 and TA 1537, including frame shifts, base-pair substitution, and transitions. Consequently, our data suggested that the backcross transgenic papaya fruits exhibited no toxicity and mutagenicity in CHO-K1 cell lines, either with or without S9 treatment. The number of chromosomal aberrations and the site of aberration, including gap, ring, break, interchange, and intrachange, were under the normal range after incubation with non-transgenic TN-2 and backcross transgenic papaya fruits. Furthermore, no significant increase in the number of reticulocytes and frequency of micronuclei occurred when mice were orally administered non-transgenic TN-2 and backcross transgenic papaya fruits. From these results, we

Table 11. Absolute Organ Weight (g) of Rats Fed with Non-transgenic and Backcross Transgenic Papaya Fruits for 28 Days

	non-transgenic line	transgenic lines		
group[a]/items	TN-2	2210	823	823-2210
		Male		
brain	2.0 ± 0.1[b]	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
heart	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.2	1.2 ± 0.2
thymus	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.2	0.5 ± 0.1
liver	9.4 ± 1.8	9.1 ± 1.2	9.5 ± 1.6	9.4 ± 1.3
kidney	2.6 ± 0.5	2.6 ± 0.2	2.6 ± 0.3	2.7 ± 0.4
adrenal	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
spleen	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.4	0.7 ± 0.1
testis	3.0 ± 0.2	2.9 ± 0.3	3.1 ± 0.1	3.1 ± 0.3
		Female		
brain	2.0 ± 0.1	1.9 ± 0.1	2.0 ± 0.0	1.9 ± 0.0
heart	0.9 ± 0.1	0.9 ± 0.0	0.8 ± 0.1	0.9 ± 0.1
thymus	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
liver	6.2 ± 0.5	6.6 ± 0.4	6.3 ± 0.1	6.3 ± 0.5
kidney	1.8 ± 0.2	1.7 ± 0.4	1.7 ± 0.1	1.7 ± 0.2
adrenal	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
spleen	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.1
ovary	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0

[a]TN-2, Tainung No. 2; 2210, 823, and 823-2210, backcross transgenic papaya fruits. [b]Data are expressed as the mean ± SD (n = 10).

concluded that backcross transgenic papaya fruit did not elicit genotoxicity in both *in vitro* and *in vivo* assays.

The second tier of food safety evaluation is animal feeding toxicity assessment. In experiments that involve the administering of transgenic foods to rats and mice over specific time periods, parameters, such as bw, feed consumption, blood chemistry, organ weight, and histopathology, should be examined.[27] No evidence of adverse health effects were observed in male or female SD rats following 28 consecutive days of oral exposure to backcross transgenic papaya fruits. During the period of this study, although some differences were observed, no biologically significant differences were noticed in bw, bw gain, feed consumption, and relative organ weight between backcross transgenic papaya fruits and non-transgenic papaya groups.

Finally, the transgenic-specific gene fragment of backcross transgenic papaya fruit is able to be completely digested by gastric acid (with pepsin) and has no genotoxicity in the Ames test, micronucleus test, and chromosomal aberration assays. This repeated feeding toxicity study of backcross transgenic papaya fruit demonstrated that there are no biologically adverse effects in rats. These results presented here support and strengthen the claim that backcross transgenic papaya fruits can be recognized as an equivalent and safe substitute for conventional non-transgenic papaya fruit.

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Funding

The authors thank the Food and Drugs Administration, Department of Health, Taiwan, Republic of China, for financially supporting this research under Contract FDA99-FS034 and the Ministry of Education, Taiwan, Republic of China, under the "Aiming for the Top University Plan (ATU)".

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Ian Cochrane-Lusk for his review of the manuscript.

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Table 12. Summary of Histopathology of Rats Fed with Non-transgenic and Backcross Transgenic Papaya Fruits for 28 Days

	non-transgenic line		transgenic lines					
	TN-2		2210		823		823-2210	
histopathological findings	M	F	M	F	M	F	M	F
adrenal	–[a]	–	–	–	–	–	–	–
brain								
congenital hydroencephalopathy, massive, severe[b]	–	1/10	–	–	–	–	–	–
heart								
infiltration, mononuclear cell, focal, minimal	–	–	–	–	1/10	–	–	–
kidney								
cyst, tubule, focal, minimal	1/10[c]	–	–	–	2/10	–	–	1/10
regeneration, tubule, focal, minimal	–	–	1/10	–	–	–	1/10	–
liver								
necrosis, focal, minimal	–	–	1/10	–	–	–	–	–
lung								
granulation, foreign body reaction, multifocal, moderate	1/10	–	–	–	–	–	–	–
spleen	–	–	–	–	–	–	–	–
ovary	–	–	–	–	–	–	–	–
testes	–	–	–	–	–	–	–	–
thymus	–	–	–	–	–	–	–	–

[a]"–", no significant lesion; TN-2, Tainung No. 2; 2210, 823, and 823-2210, backcross transgenic papaya fruits; M, male rats; and F, female rats. [b]Degree of lesions was graded from 1 to 5 depending upon severity: 1, minimal (<1%); 2, slight (1–25%); 3, moderate (26–50%); 4, moderate/severe (51–75%); and 5, severe/high (76–100%). [c]Incidence = number of rats affected/total number of rats examined (n = 10).

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