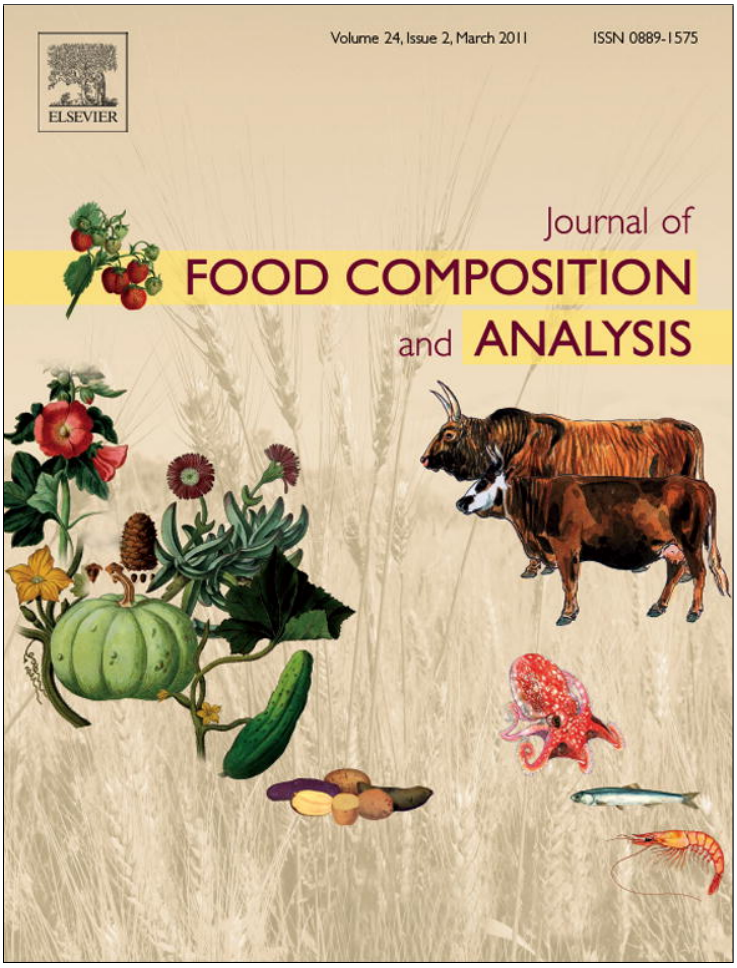
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Original Article

Nutritional composition of Rainbow papaya, the first commercialized transgenic fruit crop

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ABSTRACT

Rainbow papaya (*Carica papaya* L.) is a genetically engineered (GE) cultivar with resistance to *papaya ringspot virus* (PRSV). This cultivar currently accounts for about 70% of Hawaii's papaya acreage. The nutritional composition of Rainbow papaya and a non-transgenic control were analyzed to address GE food safety concerns regarding the potential for altered nutritional composition and altered expression of inherent allergens and toxic proteins. Rainbow papaya fruit were analyzed at three ripening stages and the data compared to that of a non-transgenic papaya which shares a similar pedigree. No differences were observed between GE and non-GE papaya for 36 nutrients at any of the tested fruit ripeness stages. However, vitamin A was higher and calcium levels were lower in the GE fruit. The GE fruit showed higher levels of protein and papain at the earliest stage of ripening (color break), but in ripened fruit these differences were insignificant. Benzyl isothiocyanate (BITC) levels were very low and similar for both Rainbow and the non-transgenic control fruit at all ripeness stages. Our data show that the contents of nutrients, BITC and papain of GE Rainbow papaya are within the range of those of non-GE papaya and that the Rainbow cultivar is substantially similar to the non-GE cultivar.

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1. Introduction

Papaya (*Carica papaya* L.) is produced commercially in many tropical and subtropical areas of the world for domestic consumption and for export. Global papaya production increased about 40% in a single decade (1998–2008), with an estimated 9.1 million tonnes produced in 2008. The top papaya producing countries are India, Brazil, Nigeria, Indonesia and Mexico (FAO, 2010). Papaya is a fruit that is well known for its nutritional and medicinal values. In fact, the Center for Science in the Public Interest, Washington, DC ranked papaya in the top 5 (with guava, watermelon, grapefruit and kiwifruit) among 38 common fruits based on nutritional scores and the percentage Recommended Daily Allowance (RDA) for vitamin A, vitamin C, potassium, folate, niacin, thiamine, riboflavin, iron, and calcium plus fiber (CSPI, 1998).

The most limiting factor for papaya production worldwide is *papaya ringspot virus* (PRSV), an aphid transmitted potyvirus (Tripathi et al., 2008). In Hawaii, the papaya industry was severely damaged by PRSV and production declined from 25.3 million kg in the early 1990s to 16.1 million kg in the latter part of the decade due to virus infection (Gonsalves, 1998; Fuchs and Gonsalves, 2007). In 1998, two transgenic cultivars with resistance to PRSV (Rainbow and SunUp) were released for commercial cultivation in Hawaii. The original transformant (R_0) named 55-1 was obtained via particle bombardment of the red-fleshed cultivar Sunset (Ss) with the transformation vector pGA482GG/cpPRV4 containing the PRSV coat protein (CP) transgene (Ling et al., 1991; Fitch et al., 1992). SunUp (Su) is a red-fleshed cultivar that is essentially a transgenic Ss, obtained by selecting progeny of transformant line 55-1 that were homozygous for the PRSV CP transgene. Rainbow (Rb) is an F_1 hybrid resulting from a cross between the transgenic Su and a yellow-fleshed non-transgenic cultivar, Kapoho (Kp) (Manshardt, 1998; Manshardt, 1999). The development and commercialization of transgenic papaya expressing the PRSV CP gene controlled PRSV and saved the papaya industry in Hawaii (Tripathi et al., 2008). Since then, transgenic papaya cultivars have

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been widely grown in Hawaii with Rb representing the dominant cultivar, accounting for more than 70% of the papaya acreage (NASS, 2008).

Although a major constraint to papaya production in Hawaii was eliminated with the introduction of PRSV-resistant plants, the papaya industry still faces some challenges in bringing these transgenic papaya to market outside the US. Japan and Canada are large export markets for Hawaii's papaya. Canada approved the importation of Su and Rb in 2003 (Health-Canada, 2003), and transgenic papaya shipments to Canada are continuing. On the other hand, the application for deregulation of transgenic papaya in Japan is ongoing, and reached a milestone when Japan's Ministry of Health, Labor and Welfare (MHLW) approved the food safety of the transgenic papaya in May 2009 (http://www.fsc.go.jp/sonota/kikansi/21gou/21gou_1_8.pdf).

One area of concern for regulatory authorities in Japan was the possibility of the introduction or alteration of allergenic proteins in transgenic papaya, and changes in the nutritional composition of the fruit. Papain and benzyl isothiocyanate (BITC) are the main components in papaya with potential allergenic or toxic properties. The present investigation compares the levels of major nutritional components including papain and BITC in Rb papaya with those of a closely related, non-transgenic commercial papaya variety at different stages of fruit ripening.

2. Materials and methods

2.1. Source of papaya fruit

Rb is a F_1 hybrid obtained from a cross between transgenic Su (the transgenic version of Ss), and non-transgenic Kp. Since a non-transgenic hybrid consisting of a cross between non-transgenic Ss and non-transgenic Kp is not commercially grown in Hawaii, a selection (F_8 to F_{10}) derived from a hybrid between the cultivar Sunrise (Sr) and Kp was chosen as a comparable control. Sr is an inbred sibling selection of Ss and is therefore closely related to Ss. The non-transgenic hybrid selection line (Hyb) produced fruit that had thick, deep orange flesh, a crown at the blossom end of the fruit, and skin freckling that closely resembled the fruits of Rb.

The transgenic Rb and the Hyb papaya fruit were collected from the same commercial plantation located near Hilo, Hawaii. The grower managed the papaya field under the guidelines of Hawaii Department of Agriculture's (HDOA) Identity Preservation Protocol (IPP) that insures that the transgenic or non-transgenic identity of each tree in a field has been checked with a biochemical assay (Camp III, 2003). The selected farm had (a) good agronomic practices for commercial production, (b) papaya trees in the field that were in good health with no virus symptoms, (c) both transgenic and non-transgenic trees of similar age in adjacent fields, and (d) plantings of a selection of non-transgenic papaya that shares a similar pedigree with Rb.

2.2. Fruit sampling

Papayas are typically harvested at the color break to 1/8 ripe stages for commercial markets. For nutritional comparison, fruit of Rb and Hyb were harvested or treated to obtain three stages of fruit maturity or ripeness: for stage 1, fruits were harvested at color break (mature green fruit) and processed immediately; for stage 2, fruits were harvested at color break and allowed to fully ripen at 20 °C to full yellow color (ripened off of the tree) before processing; and stage 3, tree-ripened fruit were harvested at full color (ripened on the tree) and processed immediately (Fig. 1). Ripening stage was assessed visually according to commercial practices followed by the Hawaiian Papaya Industry (HPIA, 2010). The ripening stages correspond in general to the following color index and firmness data determined for Rb fruit. Stage 1 (color break) fruit are characterized by a dark yellowish-green peel color averaging 48.35 ± 1.16 for lightness (L*), 32.65 ± 0.83 for chroma (C*), and 121.45 ± 0.88 for hue angle (H°). The peel firmness of color break fruit averages 90.8 N ± 2.9. Stage 2 fruit (ripened off of the tree) have peel colors that are a bright, vivid yellow (L* = 68.52 ± 0.40, C* = 62.95 ± 0.56, and H° = 88.08 ± 0.55), and peel firmness averaging 17.5 ± 0.71 N. Stage 3 fruit (ripened on the tree) also have bright yellow peels (L* = 70.03 ± 0.38, C* = 65.66 ± 0.62, and H° = 84.48 ± 0.44), but the peel firmness (13.61 ± 0.61 N) is lower than stage 2 fruit.

2.3. Sample preparation

A total of 32 fruit were collected for each ripening stage of Rb and another 32 fruit were collected from each ripening stage of the non-transgenic papaya, Hyb. Individual fruit weight and size were measured. From the 32 fruit of each stage/cultivar type, four composite samples were created, each comprised of eight fruits (Fig. 1). Therefore, compositional analyses were conducted on a total of 24 composite samples [12 composite samples of Rb (3 stages × 4 composites/stage) and 12 composite samples of non-transgenic Hyb (3 stages × 4 composites/stage)].

To prepare composite samples, individual fruit were cut longitudinally and the seed, placenta tissue and peel (5 mm, measured from the surface of the fruit) were removed. Eight slices of papaya flesh of equal amounts (i.e. 100 g), one from each of the eight fruit were homogenized in a blender. About 200 g of each homogenized, composite sample was placed in a brown plastic

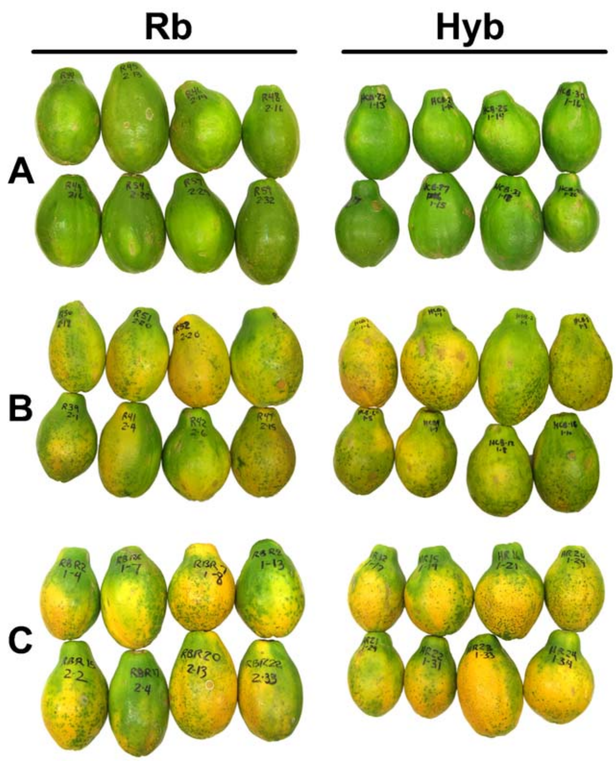

Fig. 1. Different ripeness stages of fruit from transgenic Rainbow (Rb) and non-transgenic (Hyb) varieties. A total of 32 fruit samples were collected for each stage per cultivar. The three different stages are designated as: (**A**) mature green/color break stage (stage 1), fully developed fruits with dark green skin color (1/8 ripe), which contain the fully developed black seed and ripen when stored at 20 °C for 9 days, (**B**) ripen at 20 °C (stage 2), the fruit harvested at mature green stage and allowed to fully ripen at 20 °C for 9 days, which developed the yellow color and ready-to-eat pulp, and (**C**) tree ripened (stage 3), a fully ripe papaya fruit with yellow skin color and ready-to-eat pulp. Stage (B) is the normal stage for commercial markets. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

bottle and frozen at −80 °C. The set of 24 homogenized composite samples was done in triplicate. One set was used for nutritional analyses, the second was used for papain analysis, and the third set was used for BITC analysis.

2.4. Papain analysis

The papain content of papaya fruit at the various ripening stages was determined by an indirect ELISA technique. A 0.25 g composite sample was homogenized in 7.5 mL of 0.1 M carbonate/bicarbonate buffer (pH 9.6). The ELISA plate was coated with 200 μL of the homogenized extract and incubated overnight at 4 °C. The analysis was performed by the standard ELISA protocol using a polyclonal antibody raised against papain (Abcam Inc., Cambridge, MA). Detection was performed by monitoring the color reaction resulting from the use of alkaline phosphatase conjugated anti IgG antibody (Abcam Inc., Cambridge, MA), as secondary antibody, and the substrate p-nitrophenyl phosphate (Sigma, St. Louis, MO). ELISA readings were taken at 405 nm wavelength with an MRX plate reader (Dynatech Laboratories, Chantilly, VA). The amount of papain in fruit samples was calculated by a regression (sigmoidal) equation, formulated based on standard curves generated in this experiment.

A standard curve was created with known amounts of papain (from 0.5 ng/μL to 3 ng/μL) standard (Sigma, St. Louis, MO) against ELISA 405 nm reading values. Using the papain standard, the lower limit of detection was 1 ng/μL. The precision of the assay was determined with the standards having a mean ± SD of 2.04 ng/μL ± 0.08 and a relative standard deviation (RDS) = 4.0% (n = 3). The formula to calculate the level of papain in fruit samples was:

$$\text{Amount of papain in mg/g FW of papaya} = \frac{[-b\ln(a/E-1)+x_0]}{[150/1000]};$$

where, b = 0.3091; a = 0.9488; E = ELISA 405 nm values; x_0 = 1.6716; FW = fresh weight.

2.5. Benzyl isothiocyanate (BITC) analysis

BITC levels were measured in papaya fruit based on a method described by Tang (1971), with modification. For each composite sample, 10 g of tissue was homogenized with 10 mL water, and 200 μL of crude papaya thioglucosidase enzyme extract. Thioglucosidase was added to ensure that BITC generation was not limited by insufficient enzyme in the reaction mixture. The crude enzyme extract was obtained by rupturing the sarcotestae of 2 g of fresh papaya seed and collecting the fluid contents in 1 mL water without breaking the actual seeds. Homogenates were incubated at 4 °C for 1 h, extracted with 10 mL of chloroform, and centrifuged at 2000 rpm for 40 min. A 5 mL aliquot of the supernatant was removed, placed into glass vials, and the chloroform (Fisher Scientific, Pittsburgh, PA) was evaporated under nitrogen gas. Samples were re-suspended in 100 μL chloroform for BITC analysis, and 100 μL of internal standard [(60 ppm octadecane in methylene chloride) (Fisher Scientific, Pittsburgh, PA)] was added to account for GC loading variation. Samples (1 μL) were injected into a gas chromatograph (GC-14B, Shimadzu, Columbia, MD) equipped with a flame ionization detector and a capillary column (Agilent J & W Scientific DB-5, 0.25 μm × 30 m, Agilent Technologies, Santa Clara, CA). The GC conditions were: injector temperature at 250 °C, detector temperature at 280 °C, and oven temperature at 140 °C for 1 min then programmed to increase at a rate of 12 °C/min to 280 °C. Helium was the carrier gas at a flow rate of 1 mL/min. Hydrogen and air for the flame ionization detector were set at 30 mL/min flow rates. Calibration curves were developed using authentic standards of BITC (Sigma, St. Louis, MO). The quantitation limit for BITC in papaya samples was 0.08 ppm. The precision of the assay was determined with internal standards having a mean ± SD of 2.04 ppm ± 0.13, and relative standard deviation (RSD) = 6.4% (n = 10).

2.6. Nutritional analyses

All analyses in this section were conducted by NP Analytical Labs (St. Louis, MO.) using standard methodologies. Frozen homogenized papaya samples were sent by air-freight to NP Analytical Labs and remained frozen during shipment.

2.6.1. Moisture

Fruit moisture content was determined by gravimetric measurement of weight loss after drying the samples in a vacuum oven at 70 °C until constant weight was obtained.

2.6.2. Protein

Total nitrogen content was determined by the Kjeldahl method (AOAC, 1995) using an autoanalyzer (Alpken RFA-300, OI Analytical, College Station, TX). Protein was calculated as Kjeldahl nitrogen × 6.25. The protein assay had a high level of reproducibility with a mean ± SD of 21.72 ± 0.16% and RSD = 0.74% (n = 80 internal controls).

2.6.3. Fat analysis

For total fat analysis, samples were hydrolyzed with hydrochloric acid (GFS Chemical, Powell, OH), and the digest was extracted repeatedly with ethyl ether and petroleum ether (AOAC, 1995). The solvents were volatilized, and the extracted fat was dried, weighed and quantified as percent fat.

2.6.4. Fiber

Fiber was determined by grinding the dried samples to pass a 1.0 mm screen, and extracting with ether to remove excess fat. Samples were then digested in dilute sulfuric acid, filtered, digested in dilute sodium hydroxide (Fisher Scientific, Pittsburg, PA), and filtered again. The residue was washed, dried, weighed, ignited, and reweighed. Fiber was calculated from the loss on ignition of the residue (AOAC, 1995). The reproducibility of the fiber analysis was determined with internal controls (n = 80) having a mean ± SD of 3.19% ± 0.22, RSD = 7%.

2.6.5. Ash

Ash content was determined by gravimetric measurement of the sample residue after ignition in a muffle furnace at 500 °C. The residue was calculated as percent ash.

2.6.6. Carbohydrates

Carbohydrates were calculated by subtracting the sum of protein, fat, moisture, and ash from 100%.

2.6.7. Vitamin A (β-carotene and α-carotene)

Papaya samples were extracted and analyzed for β-carotene and α-carotene concentrations using the methods of Bushway et al. (1985) and Quakenbush and Smallidge (1986). Samples were saponified by refluxing in alcoholic potassium hydroxide solution (NP Analytical Lab, St. Louis, MO). Carotenes were extracted with hexane (Fisher Scientific, Pittsburgh, PA), evaporated to dryness, and re-suspended in chloroform/methanol (Fisher scientific, Pittsburgh, PA). Samples were injected into a Beckman System Gold high pressure liquid chromatograph (HPLC, Beckman Coulter, Brea, CA) for separation through a C18 reverse phase column (Vydac TP20154, 25 cm × 4.6 mm ID; Alltech Assoc., Deerfield, IL), with methanol:chloroform (90:10, v/v) as the mobile phase at a flow rate of 1 mL/min. Individual carotenes were detected with a visible wavelength detector set at 475 nm (Bushway et al., 1985;

Quakenbush and Smallidge, 1986). β-Carotene and α-carotene were quantified from standards (Fluka, St. Louis, MO) of known concentration. The analysis was repeated 32 times with internal controls to determine reproducibility, and the mean ± SD for carotenes was 7919 ± 1124 UI A/100 g, RSD = 14%.

2.6.8. Vitamin C

Total vitamin C was measured using fluorometric determination (AOAC, 2000; Egberg et al., 1975). Ascorbic acid and dehydroascorbic acid were extracted from 10 g samples with a solution of metaphosphoric acid/methanol/water. Norit (Fisher Scientific, Pittsburgh, PA) was added to remove colored interferences from the sample extracts and to oxidize ascorbic acid to dehydroascorbic acid in the samples and standards (Fluka, St. Louis, MO). The extracts were filtered, and vitamin C was quantified using a rapid flow system (Alpkem RFA-300 system, OI Analytical, College Station, TX). Aliquots of the sample solutions and standards were buffered and reacted with o-phenylenediamine (Sigma, St. Louis, MO), forming quinoxaline-1-one, a fluorescent condensation product of dehydroascorbic acid and o-phenylenediamine. Background or interfering fluorescence was measured by reacting aliquots of the sample and standard solutions with boric acid (Fisher Scientific, Pittsburgh, PA), which complexes dehydroascorbic acid, preventing the condensation with o-phenylenediamine. Sample fluorescence values were corrected for background interference and the samples were quantified from standards (Fluka, St. Louis, MO) of known concentration. Repeated analyses of samples with internal controls (n = 80) showed a mean value of 110.5 mg/100 g ± 8.8, RSD = 8%.

2.6.9. Vitamin E

Samples were extracted and saponified with ethanolic 60% potassium hydroxide (NP Analytical Lab, St. Louis, MO) in the presence of 6% pyrogallol (Sigma, St Louis, MO) in a 70 °C water bath according to the methods of McMurray et al. (1980). Extracts were partitioned 3 times into hexane, evaporated to dryness in rotary evaporator, resuspended in methanol (Fisher Scientific, Pittsburgh, PA), and analyzed by HPLC (Beckman System Gold, Beckman Coulter, Brea, CA) using fluorescence detection (290 nm excitation and 320 nm emission). Samples (25 μL) were injected onto a Zorbax ODS column (25 cm × 4.6 mm ID; Agilent Technologies, Santa Clara, CA), with a mobile phase of methanol:water (95:5, v/v) at a flow rate of 3 mL/min. Vitamin E results were reported as mg of α-tocopherol acetate. The detection limit for α-tocopherol was 0.4 mg/100 g. The reproducibility of the analysis was determined with internal controls (n = 80) having a mean of 16.8 mg/100 g ± 1.15, RSD = 6.8%.

2.6.10. Minerals

Mineral analysis was performed using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Varian Vista, Varian, Palo Alto, CA). Prior to ICP-AES analysis, fruit samples were ashed in a muffle furnace at 500 °C and acid-extracted (AOAC, 2000). Mineral concentrations were determined by comparing the emission of the samples to the emissions of standard solutions. The reproducibility (%RSD; n = 80) of ICP analysis ranged from 5.4% for calcium to 12.8% for potassium, with the majority of minerals having RSD values of 5–7%.

2.7. Statistical analysis

Data from each fruit physical measurement, nutrient analysis, papain analysis and BITC analysis were subjected to analysis of variance (ANOVA) using the best fit linear models procedure of JMP statistical analysis software package (JMP, 2007). Each analysis was conducted using a two-way full factorial design ANOVA (2 cultivars × 3 ripeness stages) with four replications for each treatment. A replication consisted of a composite sample of eight papaya fruit. The Tukey HSD Test, at α = 0.05, was used to test for differences in groups means (JMP, 2007).

3. Results and discussion

3.1. Fruit sampling

The fruit samples of both Rb and Hyb cultivars ranged from 493 to 583 g in weight, 13.6–14.3 cm in length, and 29–30 cm in girth among the different stages of fruit ripening (Table 1). Although the physical measurements were variable taking all fruits into account, more uniformity was observed among samples of Rb and Hyb at comparable stages of ripenness. Fruit weight, length and girth of Rb were not statistically different from that of non-transgenic control papaya at comparable stages. The results of PCR analysis confirmed that all trees that were sources of the Rb fruit were positive for the PRSV CP transgene, whereas trees that were sources of the non-transgenic Hyb fruit were negative for the PRSV CP transgene (data not shown). For commercial markets, fruit are normally harvested at color break and allowed to fully ripen (Fig. 1). In both papaya varieties, fruits that were harvested at color break ripened fully after about nine days at 20 °C.

3.2. Nutritional composition analysis

3.2.1. Macronutrients

The macronutrient concentrations for transgenic Rb fruit in comparison with the non-transgenic Hyb control are shown at the three stages of ripening in Table 2. As expected, moisture content decreased and carbohydrates increased significantly in ripe fruit. Rb fruit lost 2.7% moisture and increased 2% in carbohydrate content from the color break to fully ripened stages. A similar trend was observed for the Hyb fruit, and differences in macronutrients between ripened fruits of Rb and Hyb were insignificant ($P > 0.05$). A decrease in moisture and an increase in carbohydrate level as papaya fruit ripen were also reported by Roberts et al. (2008).

The protein analysis was important in this study because the ratio of new protein (due possibly to protein expressed from the transgene) to total papaya protein content was not known. The protein content in Rb was not elevated when compared to

Table 1
Physical measurements for transgenic Rainbow (Rb) and non-transgenic hybrid line (Hyb) papaya fruit used for various nutritional analyses[z].

	Papaya cultivars and fruit maturity stages tested					
	Mature green fruit (stage 1)		Fruit ripened off the tree (stage 2)		Fruit ripened on the tree (stage 3)	
	Transgenic (Rb)	Non-transgenic (Hyb)	Transgenic (Rb)	Non-transgenic (Hyb)	Transgenic (Rb)	Non-transgenic (Hyb)
Fresh weight (g)	493.2 ± 66.3[c]	544.6 ± 80.9[abc]	516.8 ± 72.6[bc]	533.8 ± 111.0[abc]	578.2 ± 67.4[ab]	583.3 ± 126.2 [a]
Length (cm)	13.6 ± 0.9[ab]	14.0 ± 0.9[ab]	14.3 ± 0.7[b]	14.3 ± 1.4[ab]	13.6 ± 0.7[a]	13.9 ± 1.1[a]
Girth (cm)	28.6 ± 1.6[b]	29.9 ± 1.6[ab]	30.2 ± 1.7[ab]	30.2 ± 2.3[ab]	29.2 ± 1.5[a]	29.6 ± 2.0[a]

[z] Values shown in the table are the mean of 32 papaya fruits, mean ± SD.
Means within the same row followed by the same letters are not significantly different at the α = 0.05 level, based on a Tukey HSD test for mean separation.

Table 2
Macronutrient, vitamin A and vitamin C composition of transgenic Rainbow (Rb) and non-transgenic hybrid line (Hyb) papaya fruits at different stages of ripening[z].

	Papaya cultivars and fruit maturity stages tested					
	Mature green fruit (stage 1)		Fruit ripened off the tree (stage 2)		Fruit ripened on the tree (stage 3)	
	Transgenic (Rb)	Non-transgenic (Hyb)	Transgenic (Rb)	Non-transgenic (Hyb)	Transgenic (Rb)	Non-transgenic (Hyb)
Moisture (g/100 g)	87.1 ± 0.3[ab]	87.7 ± 0.7[a]	86.1 ± 0.1[cd]	86.6 ± 0.2[bc]	85.0 ± 0.1[e]	85.5 ± 0.2[de]
Protein (g/100 g)	0.743 ± 0.05[b]	0.831 ± 0.03[a]	0.843 ± 0.01[a]	0.829 ± 0.04[a]	0.779 ± 0.06[ab]	0.702 ± 0.01[b]
Fat (g/100 g)[*]	<0.100	0.157 ± 0.05	0.171 ± 0.02	0.169 ± 0.003	0.158 ± 0.07	0.141 ± 0.00
Fiber (g/100 g)	0.632 ± 0.06[a]	0.631 ± 0.06[a]	0.560 ± 0.05[ab]	0.576 ± 0.04[ab]	0.490 ± 0.09[b]	0.535 ± 0.04[ab]
Ash (g/100 g)	0.437 ± 0.02[ab]	0.460 ± 0.03[a]	0.388 ± 0.04[ab]	0.364 ± 0.06[b]	0.445 ± 0.03[a]	0.410 ± 0.01[ab]
Energy (kJ/100 g)	209.3 ± 4.6[de]	200.5 ± 11.7[e]	229.0 ± 5.0[bc]	219.8 ± 4.6[cd]	245.3 ± 2.5[a]	236.6 ± 4.6[ab]
Vitamin A (IU A/100 g)	105 ± 9.6[cd]	50.3 ± 5.6[e]	156 ± 6.8[b]	87.6 ± 29.6[de]	262 ± 18.2[a]	138 ± 18.6[bc]
Vitamin C (mg/100 g)	57.4 ± 1.6[cd]	46.3 ± 7.9[d]	68.3 ± 13.0[bc]	65.8 ± 2.6[bc]	84.9 ± 2.7[a]	75.9 ± 3.5[ab]

[z] Values shown in the table are the mean of four composite samples consisting of 32 papaya fruits, mean ± SD. All values were calculated based on fresh weight basis.
[*] Statistical analysis was not performed because of insufficient replicates.
Means within the same row followed by the same letters are not significantly different at the $\alpha = 0.05$ level, based on a Tukey HSD test for mean separation.

non-transgenic Hyb papaya at different fruit ripening stages (Table 2). Rather, a lower protein content was observed in Rb when compared to Hyb at the color break stage, and this difference subsequently became insignificant at the fully ripened stage. All other macronutrients were not significantly different between Rb and Hyb ($P > 0.05$) at their various comparable stages of fruit ripening. In general, the macronutrient levels (moisture, protein, fat, carbohydrate, fiber, ash) in Rb papaya were within the range of the non-transgenic control and were similar to earlier published reports (Roberts et al., 2008; USDA, 2008; Wall, 2006).

3.2.2. Vitamins

All tested vitamin contents in papaya fruits of transgenic Rb and non-transgenic Hyb are summarized in Table 2. Vitamin E (α-tocopherol acetate) content was below the detection limit (<0.4 mg/100 g) for all of the samples. Vitamin A and vitamin C increased significantly as fruits ripened, with the highest contents measured in tree-ripened fruit. The highest vitamin A concentrations (based on β-carotene) for Rb and Hyb fruit ripened on the tree were 262 IU/100 g fresh weight (FW) and 138 IU/100 g FW, respectively. Vitamin C content was highest for Rb (84.9 mg/100 g FW), compared to the non-transgenic Hyb (75.9 mg/100 g FW) for tree-ripened papayas. The changes in vitamin contents for Rb followed a similar trend for Hyb among the different ripening treatments. However, vitamin A was significantly higher in Rb fruit at all stages of ripening ($P > 0.05$), whereas the differences in vitamin C content between Rb and Hyb were insignificant.

The attractive color of papaya is due to the presence of carotenoids which make papaya a rich source of vitamin A (Cano et al., 1996). According to Wall (2006), Rb tends to have higher vitamin A concentrations than either Kp or Su cultivars; our results confirmed this observation. A similar trend was also reported between transgenic and non-transgenic control fruit by Roberts et al. (2008). Differences between Rb and Hyb reported here could also be attributed to varietal differences, or possibly, to subtle differences in the age of harvested fruit. Although papaya were harvested at stages designated as color break or fully ripened, even slight variability in maturity and ripening at these stages could potentially contribute to a wide range of differences in vitamin A levels. Indeed, while the vitamin A contents of Rb reported in this study agree in general with the vitamin content of papaya in other published reports (Kimura et al., 1991; Philip and Chen, 1988; Roberts et al., 2008; USDA, 2008; Wall, 2006) other studies have reported that vitamin A (or carotenoids) levels in papaya fruit are highly variable between and within cultivars. Our data indicated differences in vitamin A level between Rb and the non-transgenic Hyb of up to 1.9-fold. This observation is not surprising considering the even greater variability reported in other published data. For example, variations in vitamin A levels of up to 5.7-fold between papaya cultivars have been reported (Chandrika et al., 2003; Gouado et al., 2007; Wall, 2006; Yano et al., 2005). Variations in vitamin A levels of up to 3.9-fold also were reported within a single papaya variety (Sr), and a 1.6-fold variation was reported among Rb fruit (Wall, 2006). Considering the high variability in vitamin A levels reported for papaya, the greater level of vitamin A in Rb as compared to Hyb does not appear to be related to an effect of the transgene. This observation is also supported by the results published by Mutsuga et al. (2001) where differences in the total carotenoid content between Su and Ss papaya were statistically insignificant.

Table 3
Mineral contents in transgenic Rainbow (Rb) and non-transgenic hybrid line (Hyb) papaya fruits at different stages of ripening[z].

Minerals (mg/ 100 g fresh weight)	Papaya cultivars and fruit maturity stages tested					
	Mature green fruit (stage 1)		Fruit ripened off the tree (stage 2)		Fruit ripened on the tree (stage 3)	
	Transgenic (Rb)	Non-transgenic (Hyb)	Transgenic (Rb)	Non-transgenic (Hyb)	Transgenic (Rb)	Non-transgenic (Hyb)
Macroelements						
Calcium	14.7 ± 1.84[bc]	23.9 ± 1.59[a]	11.9 ± 1.92[c]	21.3 ± 4.53[a]	9.51 ± 0.86[c]	19.6 ± 2.41[ab]
Magnesium	20.8 ± 1.49[a]	18.7 ± 0.92[ab]	19.1 ± 2.85[ab]	19.8 ± 2.85[ab]	15.9 ± 1.74[b]	17.4 ± 2.03[ab]
Phosphorus	6.58 ± 0.48[ab]	6.88 ± 0.83[a]	5.07 ± 0.20[c]	5.57 ± 0.17[bc]	6.20 ± 0.46[ab]	6.30 ± 0.49[ab]
Potassium	166 ± 14.7[a]	133 ± 22.2[ab]	138 ± 6.61[ab]	122 ± 4.1[b]	162 ± 18.8[ab]	135 ± 25.4[ab]
Sodium	2.86 ± 0.19[ab]	3.17 ± 0.31[a]	2.34 ± 0.29[b]	2.81 ± 0.24[ab]	2.51 ± 0.43[ab]	2.69 ± 0.47[ab]
Microelements						
Copper	0.06 ± 0.004[a]	0.05 ± 0.02[ab]	0.02 ± 0.003[c]	0.03 ± 0.02[bc]	0.04 ± 0.004[abc]	0.04 ± 0.005[abc]
Iron	0.07 ± 0.03[a]	0.08 ± 0.07[a]	0.05 ± 0.04[a]	0.07 ± 0.03[a]	0.07 ± 0.004[a]	0.08 ± 0.02[a]
Manganese	0.02 ± 0.002[a]	0.01 ± 0.004[ab]	0.01 ± 0.002[bc]	0.01 ± 0.002[c]	0.01 ± 0.002[ab]	0.01 ± 0.002[bc]
Zinc	0.04 ± 0.006[a]	0.06 ± 0.02[a]	0.03 ± 0.01[a]	0.04 ± 0.008[a]	0.05 ± 0.005[a]	0.07 ± 0.03[a]

[z] Values shown in the table are the mean of four composite samples consisting of 32 papaya fruits, mean ± SD.
Means within the same row followed by the same letters are not significantly different at the $\alpha = 0.05$ level, based on a Tukey HSD test for mean separation.

Table 4
Contents of papain and benzyl isothiocyanate (BITC) in transgenic Rainbow (Rb) and non-transgenic hybrid line (Hyb) papaya[z].

Contents (mg/100 g of fresh weight)	Papaya cultivars and fruit maturity stages tested					
	Mature green fruit (stage 1)		Fruit ripened off the tree (stage 2)		Fruit ripened on the tree (stage 3)	
	Transgenic (Rb)	Non-transgenic (Hyb)	Transgenic (Rb)	Non-transgenic (Hyb)	Transgenic (Rb)	Non-transgenic (Hyb)
Papain	8.60 ± 1.06[a]	6.41 ± 0.24[b]	5.86 ± 0.18[b]	5.55 ± 0.34[b]	5.81 ± 0.09[b]	5.43 ± 0.22[b]
BITC	0.040 ± 0.03[a]	0.056 ± 0.04[a]	0.041 ± 0.02[a]	0.042 ± 0.02[a]	0.061 ± 0.02[a]	0.057 ± 0.06[a]

[z] Values shown in the table are the mean of four composite samples consisting of 32 papaya fruits, mean ± SD.
Means within the same row followed by the same letters are not significantly different at the $\alpha = 0.05$ level, based on a Tukey HSD test for mean separation.

The average vitamin C content in ripe papaya fruits in this study was slightly greater than the vitamin C level reported by Wall (2006) and in the USDA Nutrient Database (2008), and generally agrees with other published vitamin C data (Franke et al., 2004; Nisperos-Carriedo et al., 1992). The vitamin C concentrations increased as the fruit approached ripeness, with the highest contents recorded when fruit were harvested at full color from the tree (ripened on the tree). Vitamin C levels are affected by the amount and intensity of sunlight exposure to individual fruit. Longer day lengths and higher light intensities in the summer months, for example, can increase the concentrations of ascorbic acid and glucose in fruits (Lee and Kader, 2000; Mozafar, 1994).

3.2.3. Minerals

Mineral analysis of Rb and Hyb papaya showed a slight decreasing trend for Ca, P, Na, and Cu as fruit reached full color from the color break stage (Table 3). Other minerals showed no obvious pattern of change as fruit approached full color. The levels of Ca, Mg, Cu and Zn are slightly greater and the K is lower in Rb papaya than those reported in the USDA Nutrient Database (2008) for Solo papaya. However, the mineral levels in Rb papaya were within the range reported in other studies (Bari et al., 2006; Roberts et al., 2008; Wall, 2006; Wenkam, 1990).

Among all the minerals analyzed for Rb, only calcium was noted to be significantly different from Hyb, but was within the range of reported values for papaya (Bari et al., 2006; Roberts et al., 2008; Wall, 2006; Wenkam, 1990). A similar observation was reported by Roberts et al. (2008) where the calcium contents of the control were significantly different from the transgenic line. Mineral composition is dependent on several environmental factors and tends to reflect the mineral content of a location and region. The conditions of the root system of the host plant are known to affect the levels of minerals (Baldwin, 1975). The transgenic Rb and non-transgenic Hyb were grown in the same orchard under identical fertility practices, and the results indicate that mineral content of the two cultivars were not statistically different.

3.3. Papain

Papain is one of the cysteine endopeptidases found in the latex of a wide range of plants including papaya, where it composes up to 80% of the latex (El Moussaoui et al., 2001). Latex is a complex mixture of chemical compounds with important proteolytic activity and is believed to be involved in defending the host plant from various insect pests. Papain is considered to be a possible cause of allergenic reactions in humans, because allergenicity is known to be associated with cysteine proteases (Chambers et al., 1998). Therefore, the papain level in Rb fruit was compared with the non-transgenic Hyb fruit to determine whether the papain levels were altered by the process of genetic modification.

Mean papain values of Rb and Hyb fruit ranged from 5.81 to 8.60 mg/100 g FW and 5.43–6.41 mg/100 g FW, respectively (Table 4). Papain was highest (8.60 mg/100 g FW) in mature green fruit of Rb and gradually declined by up to 32% as the fruit ripened. A similar downward trend was observed in the ripening fruit of Hyb. This decrease in papain levels was reported earlier for papaya (Azarkan et al., 2003; Mezhlumyan et al., 2003). It was reported that papain levels were lowest at the initial stages of fruit development (i.e. immature green fruits) and gradually increased when fruit approached maturity (i.e. mature green) (Madrigal et al., 1980; Skelton, 1969). Papain levels were reported to be highest when fruits were fully developed (i.e. mature green) and later gradually decreased again as the fruit approached ripeness (Skelton, 1969). The papain level in mature green fruit of Rb was significantly higher than the Hyb fruit, however, these differences became insignificant as the fruit ripened (Table 4). Papain levels were highly variable within papaya cultivars, locations, and sampling methods in reports by others (Balamohan et al., 2008; Harjadi et al., 1995; Kunkalikar et al., 2007).

In some countries such as Thailand, green papaya which contains higher level of papain as compared to ripe papaya is consumed. However, a dietary value for papain has not been established. NIH Dietary Supplements Labels Database listed 81 dietary supplements available in the market which contain papain at levels as high as 130 mg/serving (NIH, 2009). Green mature fruit of transgenic Rb papaya on the other hand contain only 8.60 mg of papain per 100 g FW of papaya. Therefore, a person consuming a 100 g serving of green Rb papaya would intake only 8.6 mg of papain which is much lower than the papain found in most of the dietary supplements consumed by humans. Thus, although the papain content in mature green fruit of Rb is higher compared to that of the non-transgenic control, it is still at a level that does not seem to pose any threat to human consumption.

3.4. Benzyl isothiocyanate (BITC)

BITC is a volatile compound naturally found in many fruits and vegetables. A higher level of BITC is present in papaya seed as compared to papaya pulp (Ettlinger and Hodgkins, 1956; Tang, 1971). Papaya fruit at the unripe, immature green stage generally contain a higher level of BITC than that observed in ripened fruits (Tang, 1971). The BITC levels of individual samples ranged from 0.014 to 0.084 mg and from 0.010 to 0.150 mg/100 g FW in Rb and Hyb papaya fruit, respectively, and averaged 0.040–0.061 mg/100 g FW of papaya (Table 4). No significant differences were detected ($P > 0.05$) in BITC content between transgenic and non-transgenic papaya fruit.

The changes in BITC level between mature green/color break fruit and ripe fruit were statistically insignificant ($P > 0.05$) and within the range of other published reports for papaya (Roberts et al., 2008; Tang, 1971). Isothiocyanate concentrations in ripe Rb fruits were about 1000 times lower than the values reported in other crops, particularly in *Brassica* (Josefsson, 1967). The level of BITC in Rb papaya was not altered by the transgene and was within the range found in other papaya and thus poses no special concern for human health.

4. Conclusions

As part of food safety requirements for Japan's deregulation measures, a comprehensive study comparing transgenic Rb with a non-transgenic cultivar of similar pedigree showed that the contents of nutrients, papain and BITC of the two varieties were the same, except for calcium and vitamin A. Plant-to-plant variation even within a particular papaya cultivar grown under the same conditions is common and could possibly explain this variation observed between Rb and the non-transgenic control. However, the differences identified between transgenic Rb and the non-transgenic control in calcium and vitamin A were small, and along with the values of other tested components at various ripening stages in Rb papaya, were within the ranges previously reported for papaya (Roberts et al., 2008; USDA, 2008; Wall, 2006). Compositional analysis is one of the important aspects assessed in determining substantial equivalence, a criterion used internationally for risk assessment of transgenic food. Substantial equivalence refers to the finding of insignificant differences between two or more compared goods or products (Codex Alimentarius Commission, 2003; FAO/WHO, 1996, 2000, 2002; OECD, 1993).

In addition to this study, molecular characterization of the transgene insertion in the Rb or Su papaya genome and analysis of the whole Su papaya genome sequence showed that transgenesis did not disrupt the function of any papaya genes (Ming et al., 2008; Suzuki et al., 2008). Collectively, these data make Rb papaya the most extensively characterized transgenic tropical fruit crop. Although Rb papaya has been widely consumed since 1998 without any reported adverse health effects, to date, Rb and Su remain the first and only widely commercially cultivated transgenic papaya varieties. Several other laboratories have reported developing transgenic papaya resistant to PRSV. However, apart from perhaps few exceptions, these transgenic plants have been restricted to the laboratory and are facing deregulation challenges, a major hurdle that limits them from benefiting small farmers and industry.

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