

The impact of transgenic papaya (TPY10-4) fruit supplementation on immune responses in ovalbumin-sensitised mice

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Abstract

BACKGROUND: A transgenic papaya line (TPY10-4) that is resistant to both papaya ringspot virus (PRSV) and papaya leaf distortion mosaic virus (PLDMV) has been developed in Taiwan. This study investigated the immunomodulatory properties of transgenic TPY10-4 and its native (TCK) papaya fruits using an ovalbumin (OVA)-sensitised mouse model. Both green and ripe papaya fruits at low (0.2 g powder kg⁻¹ body weight (BW)) and high (1.6 g powder kg⁻¹ BW) doses were administered to experimental mice by intragastric gavage for 5 weeks. Changes in serum total immunoglobulin A (IgA), IgE, IgG and IgM levels, OVA-specific IgE, IgG1 and IgG2a titres and Th1/Th2 cytokine secretions using splenocytes were determined.

RESULTS: Transgenic TPY10-4 or native TCK papaya fruit supplementation did not significantly affect body, visceral organ and relative tissue weights, total IgE antibody levels, OVA-specific IgE and IgG1 antibody titres or OVA-stimulated interferon- γ (IFN- γ), interleukin-2 (IL-2), IL-4, IL-5 and IL-10 secretions using splenocytes. However, transgenic papaya fruits markedly increased serum total IgM levels.

CONCLUSION: This study suggests that transgenic TPY10-4 papaya fruits do not increase the allergenic potential of OVA by oral administration but may have a protective immunity via increasing the serum total IgM level.

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Keywords: ovalbumin (OVA)-sensitised mouse model; papaya leaf distortion mosaic virus (PLDMV); papaya ringspot virus (PRSV); serum antibody levels; Th1/Th2 cytokines; transgenic papaya

INTRODUCTION

Papaya (*Carica papaya* L.) fruit has been consumed for centuries and is one of the most important fruits in tropical and subtropical areas. However, papaya is susceptible to various plant viruses, including papaya ringspot virus (PRSV) and papaya leaf distortion mosaic virus (PLDMV), both of which belong to the genus *Potyvirus*. The presence of ringspot blemishes on papaya fruits is the diagnostic symptom for PRSV worldwide.¹ Recently, the technology for developing PRSV-resistant transgenic papaya has been reported. Two transgenic varieties, 'Rainbow' and 'SunUp', were released commercially in Hawaii in 1998 and successfully saved Hawaii's papaya industry from PRSV disaster.² However, papaya is often infected by PLDMV as well.³ PLDMV, causing leaf distortion and similar symptoms in papaya fruits to PRSV, is now the most destructive factor of papaya production in Taiwan and Japan. Virus diseases are the most limiting factor to papaya production. The papaya cultivated in Taiwan still suffers from both PRSV and PLDMV. Recently, a novel transgenic papaya line (TPY10-4) that is resistant to both PRSV and PLDMV has been successfully developed in Taiwan. The developed transgenic papaya line TPY10-4, carrying coat protein genes of target viruses, has shown great potential for controlling a number of PRSV and PLDMV strains in Taiwan and elsewhere.⁴ The antiviral effect of the developed transgenic papaya line occurs via RNA-mediated post-transcriptional gene

silencing, since there are no transgene proteins in the papaya pulp. Even though it is hardly ever reported, the genetic modification process may change the properties of native crops, such as resulting in unintentional allergenicity in genetically modified crops.⁵ To identify any changes in immunomodulatory function of the transgenic papaya line TPY10-4, possible changes between transgenic TPY10-4 and its native (TCK) papaya fruits were evaluated according to immunological theory using an established ovalbumin (OVA)-sensitised murine model.⁶

OVA is recognised as an allergen and generally selected to induce adaptive immunity, including T cell-mediated immune responses and humoral immune responses in murine experimental

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models. Among T cell-mediated immune responses, the T helper (Th) lymphocyte subclasses Th type 1 (Th1) and Th2 are major components of acquired (adaptive) immunity.⁷ Th1 cells participate mainly in cell-mediated immune responses and commonly express cytokines such as interferon- γ (IFN- γ), interleukin-2 (IL-2) and tumour necrosis factor (TNF). Th2 cells are mainly involved in humoral immune responses and commonly secrete granulocyte macrophage colony-stimulating factor (GM-CSF), IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and macrophage-derived chemokines.^{8,9} However, Th1 and Th2 cell function imbalances may result in many different disorders. A Th1-skewed pattern might be the major cause of type 1 diabetes, an autoimmune disease resulting from Th1-mediated destruction of pancreatic beta cells.¹⁰ A Th2-skewed pattern may play a triggering role in the activation and/or recruitment of immunoglobulin E (IgE) antibody-producing B cells, mast cells and eosinophils and ultimately may induce allergic inflammation.¹¹ Therefore the immunomodulatory properties of novel foods may be judged based on changes in antigen-specific or total antibody titres in sera as well as the balance between Th1 and Th2 cells.

The transgenic papaya TPY10-4 has been developed in Taiwan.⁴ However, the genetic engineering process might result in unintended effects causing changes in immunomodulatory function. To evaluate any immunomodulatory changes between transgenic TPY10-4 and native TCK papaya fruits, an OVA-sensitised BALB/c mouse model was used in this study.

MATERIALS AND METHODS

Preparation of papaya fruit samples

There are no known allergenic proteins in papaya, so both green and ripe papaya fruits were assessed. Two varieties of *C. papaya* L., transgenic papaya line TPY10-4 and its parent line TCK (non-transgenic papaya, as a control), were chosen and bred in an isolated area of central Taiwan (Taiwan Agricultural Research Institute, Taichung, Taiwan). Fruit from the transgenic papaya line TPY10-4, which is resistant to both PRSV and PLDMV and was successfully developed by Dr Shyi-Dong Yeh (National Chung Hsing University, Taichung, Taiwan),⁴ was selected as the experimental material to assess any immunomodulatory changes. Both green and ripe fruits from TPY10-4 and TCK papaya were provided by Dr Ying-Huey Cheng (Virus Disease Laboratory, Plant Pathology Division, Taiwan Agricultural Research Institute, Taichung, Taiwan). The fresh papaya fruit samples were harvested and peeled and the seeds were removed. The fruit flesh was weighed, lyophilised, ground into powder and stored at -20°C for later use. The moisture contents of fresh papaya fruit and its lyophilised powder were *ca* 870 and 0 g kg^{-1} respectively.

Experimental animals and grouping

Female BALB/c mice (6 weeks old, 120 mice in total) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories (National Science Council, Taipei, Taiwan) and maintained in the Department of Food Science and Biotechnology, National Chung Hsing University (College of Agriculture and Natural Resources, Taichung, Taiwan). The animal room was kept on a 12 h light/12 h dark cycle. Constant temperature ($25 \pm 2^{\circ}\text{C}$) and humidity were maintained. The mice were randomly housed in stainless steel cages and kept on a chow diet (laboratory standard diet, Diet MF 18, Oriental Yeast Co., Osaka, Japan) to acclimatise for 2 weeks. After this equilibration

period the animals were randomly divided into ten groups, including non-sensitised control (treated i.p. with phosphate-buffered saline (PBS) and aluminium hydroxide ($\text{Al}(\text{OH})_3$, AL), coded as PBS/AL), dietary control (coded as OVA/AL) and low-dose ($4\text{ mg}/0.5\text{ mL day}^{-1}$, 0.2 g kg^{-1} body weight (BW)) and high-dose ($32\text{ mg}/0.5\text{ mL day}^{-1}$, 1.6 g kg^{-1} BW) papaya fruit-supplemented groups. A quantity of 95 g fresh fruit day^{-1} is suggested as an appropriate supplement for humans. Thus the high supplementary dose of papaya fruit ($32\text{ mg lyophilised powder day}^{-1}$) given to mice is equivalent to 95 g fresh fruit day^{-1} for humans using a conversion ratio of 1:387.9 between mice (20 g) and humans (70 kg).¹²

The experimental mice were divided into non-sensitised control (treated with PBS and AL, 0 mg papaya fruit powder/ $0.5\text{ mL deionised water day}^{-1}$ per mouse, coded as PBS/AL), dietary control (treated with OVA and AL, 0 mg papaya fruit powder/ $0.5\text{ mL deionised water day}^{-1}$ per mouse, coded as OVA/AL), OVA/AL-TCK-L (treated with OVA and AL, supplemented with a low dose (4 mg day^{-1} per mouse, 0.2 g kg^{-1} BW) of green or ripe fruits of TCK papaya), OVA/AL-TPY10-4-L (treated with OVA and AL, supplemented with a low dose (4 mg day^{-1} per mouse, 0.2 g kg^{-1} BW) of green or ripe fruits of TPY10-4 papaya), OVA/AL-TCK-H (treated with OVA and AL, supplemented with a high dose (32 mg day^{-1} per mouse, 1.6 g kg^{-1} BW) of green or ripe fruits of TCK papaya) and OVA/AL-TPY10-4-H (treated with OVA and AL, supplemented with a high dose (32 mg day^{-1} per mouse, 1.6 g kg^{-1} BW) of green or ripe fruits of TPY10-4 papaya) groups. Each group consisted of 12 mice. Four sets of three mice in each group were respectively earmarked and housed in separate stainless steel cages. The experimental mice were randomised over space in the cages. During the study period, grouped mice respectively received 0.5 mL extra of deionised water or lyophilised papaya fruit powder suspended in deionised water by intragastric gavage using a stainless steel feeding syringe for five consecutive weeks from day 0 to day 35. The body weight of each mouse was measured twice a week. The experimental mice were bled to collect approximately 100 μL serum samples on days -1 , 7 and 21. The experimental mice were sensitised with OVA/AL on days 0, 14 and 28. All experimental mice were sacrificed on day 35. At study completion the mice were weighed, anaesthetised with 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane (Sigma-Aldrich, St Louis, MO, USA) and immediately bled by retro-orbital venous plexus puncture to collect blood for later determination of non-specific antibody titres, including IgA, IgG, IgM and IgE, as well as OVA-specific IgE, IgG1 and IgG2a. The blood was collected into a 1.5 mL vial, allowed to stand for 2 h at room temperature and centrifuged at $12\,000 \times g$ for 15 min at 4°C to separate the serum. The sera were collected and stored at -30°C for later analysis. Immediately after serum collection the animals were sacrificed using CO_2 inhalation for tissue studies. The thoracic and abdominal cavities were opened aseptically and the internal organs (spleen, liver, kidney, heart, lung and thymus) were removed and weighed. The visceral organ weights in each experimental group were expressed as absolute and relative weights of visceral organs respectively. The animal use protocol of this study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University, ROC.

OVA/AL sensitisation treatment

To test the effects of transgenic papaya fruit supplementation on OVA-sensitised mice, the mice (8 weeks old) were sensitised with OVA i.p. to induce systemic allergy. The allergic mouse

model was used in our previous studies.^{13,14} In brief, the mice were sensitised using an i.p. dose of 0.1 mL of AL-precipitated antigen containing 8 µg of OVA (albumin chicken egg grade III, Sigma-Aldrich) and 2 mg of AL (Sigma-Aldrich) to induce primary immunity at day 0. Two booster injections of this AL/OVA mixture were given 14 and 28 days later respectively. Non-sensitised control mice received AL/PBS (137 mmol L⁻¹ NaCl, Wako Pure Chemical Industries, Osaka, Japan), 2.7 mmol L⁻¹ KCl (Sigma-Aldrich), 8.1 mmol L⁻¹ Na₂HPO₄ (Sigma-Aldrich) and 1.5 mmol L⁻¹ KH₂PO₄ (Sigma-Aldrich) (pH 7.4, 0.2 µm filtered) only.

Preparation of splenocyte cultures

Single spleen cells were prepared by lysing the red blood cells in the OVA-sensitised mouse model. The splenocytes were prepared by aseptically removing spleens from the experimental BALB/c mice. Spleens were homogenised in TCM (tissue culture medium, a defined commercial serum replacement, Celox Laboratories, Lake Zurich, IL, USA) medium (10 mL of TCM, 500 mL of RPMI 1640 medium (Atlanta Biologicals, Norcross, GA, USA) and 2.5 mL of 100× antibiotic/antimycotic solution (Biological Industries, Kibbutz Beit Haemek, Israel) containing 10 000 units mL⁻¹ penicillin, 10 000 µg mL⁻¹ streptomycin and 25 µg mL⁻¹ amphotericin B in 8.5 g L⁻¹ saline (Atlanta Biologicals)). Single spleen cells were collected and treated by lysing the red blood cells with RBC lysis buffer (0.017 mol L⁻¹ Trizma base (Sigma-Aldrich), 0.144 mol L⁻¹ ammonium chloride (Sigma-Aldrich), pH 7.2, 0.2 µm filtered). Splenocytes were isolated from each animal and adjusted to 1 × 10⁷ cells mL⁻¹ in TCM medium with a haemocytometer using the trypan blue dye exclusion method. The splenocytes (0.50 mL per well) were respectively cultured in the absence or presence of specific allergen (0.50 mL per well, 60 µg OVA mL⁻¹). The plates were incubated at 37 °C for 48 h in a humidified incubator with 5% (v/v) CO₂ and 95% (v/v) air. The cultures were centrifuged at 200 × *g* for 10 min. The supernatant was collected and stored at -70 °C for cytokine assay.

Non-specific antibody quantification by enzyme-linked immunosorbent assay (ELISA)

Serum total IgA, IgE, IgG and IgM antibody levels were analysed using mouse IgA, IgE, IgG and IgM ELISA quantitation kits (E90-103, E90-115, E90-131 and E90-101 respectively, Bethyl Laboratories, Montgomery, TX, USA). Briefly, microtitre plate wells (flat bottom, high binding, Costar, Corning, NY, USA) were coated with 100 µL aliquots of goat anti-mouse IgA, IgE, IgG and IgM affinity purified antibodies (Bethyl Laboratories) at a concentration of 10 µg mL⁻¹ carbonate buffer (0.05 mol L⁻¹ sodium carbonate (Wako Pure Chemical Industries), pH 9.6) for 60 min at room temperature. After the plate wells had been washed twice with washing buffer (50 mmol L⁻¹ Tris (Sigma-Aldrich), pH 8, 0.1 mol L⁻¹ NaCl, 0.5 g L⁻¹ Tween 20 (Wako Pure Chemical Industries)), 200 µL aliquots of blocking solution (10 g L⁻¹ bovine serum albumin (BSA, Sigma-Aldrich), 50 mmol L⁻¹ Tris, pH 8, 0.15 mol L⁻¹ NaCl) were post-coated on each well for 30 min at room temperature. Serum samples collected from all animals in different test groups were diluted to 1:5 for IgE and 1:5000 for IgA, IgG and IgM determinations. After the plate wells had been washed twice with washing buffer, 100 µL aliquots of purified mouse IgA, IgE, IgG and IgM standards (Bethyl Laboratories) were diluted serially in diluent (10 g L⁻¹ BSA, 50 mmol L⁻¹ Tris, pH 8, 0.15 mol L⁻¹ NaCl, 0.5 g L⁻¹ Tween 20) and the pre-diluted serum samples were added to the wells. The standards, but not samples, were loaded in

duplicate on the plates. The plates were then incubated for 60 min at room temperature. After the plate wells had been washed twice with washing buffer, 100 µL aliquots of horseradish peroxidase-conjugated goat anti-mouse IgA, IgE, IgG and IgM antibodies (Bethyl Laboratories) were added to the wells for 60 min. After washing three more times, the plate wells were developed by adding 100 µL aliquots of substrate solution containing 3,3',5,5'-tetramethylbenzidine (Clinical Science Products, Mansfield, MA, USA) and incubated for about 30 min for colour development.¹⁵ The reaction was stopped by adding a 100 µL aliquot of 2 mol L⁻¹ H₂SO₄ per well and the absorbance at 450 nm was measured with an ELISA plate reader (ASYS Hitech, Eugendorf, Austria). The antibody levels were determined using a seven-point standard.

OVA-specific IgE, IgG1 and IgG2a assay

An ELISA protocol was used to determine the OVA-specific IgE, IgG1 and IgG2a antibody levels in collected sample sera. Aliquots (200 µL per well) of OVA (10 µg mL⁻¹ dissolved in 0.1 mol L⁻¹ NaHCO₃ (Wako Pure Chemical Industries), pH 8.2)) were pipetted into 96-microwell plates (Nunc, Thermo Fisher Scientific, Roskilde, Denmark). The plates were incubated overnight at 4 °C and then carefully washed three times with washing buffer (0.5 g L⁻¹ Tween 20 in PBS). Serum samples were diluted to 1:4 for IgE, 1:100 for IgG2a and 1:5000 for IgG1 determinations before they were added to the plate wells. Aliquots (100 µL) of diluted serum samples were added to the wells. Pooled sera from non-sensitised and OVA-sensitised mice were also included in each assay to serve as negative and positive controls respectively. OVA-specific levels were determined using biotin-conjugated rat anti-mouse IgE, IgG1 and IgG2a monoclonal antibodies (BD PharMingen, San Diego, CA, USA). The procedure was carried out according to the manufacturer's instructions. The plates were finally read using an ELISA plate reader (ASYS Hitech). The absorbance (*A*) at 450 nm was measured with the reader and optical densities were converted into arbitrary ELISA units,^{14,16} where ELISA unit = (*A*_{sample} - *A*_{blank})/(*A*_{positive} - *A*_{blank}).

Measurement of Th1/Th2 cytokine levels secreted by splenocyte cultures using ELISA

Both Th1 (IFN-γ and IL-2) and Th2 (IL-4, IL-5 and IL-10) cytokine levels were determined using sandwich ELISA kits. The IFN-γ, IL-2, IL-4, IL-5 and IL-10 concentrations were assayed using a mouse DuoSet ELISA development system (R&D Systems Inc., Minneapolis, MN, USA). The samples used in the cytokine measurements were undiluted and the sensitivity of the assays was <15.6 pg mL⁻¹. The Th1 and Th2 cytokine concentrations were assayed according to the cytokine ELISA protocol in the manufacturer's instructions. The plates were finally measured on an ELISA plate reader (ASYS Hitech) to obtain the absorbance at 450 nm. The IFN-γ, IL-2, IL-4, IL-5 and IL-10 levels in the cell cultures were determined using a seven-point standard.

Statistical analysis

Values are expressed as mean ± standard deviation (SD). Data were analysed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Differences between control and dietary experimental groups were considered statistically significant at *P* < 0.05. Statistical tests were performed using SPSS Version 12.0 (SPSS Inc., Chicago, IL, USA).

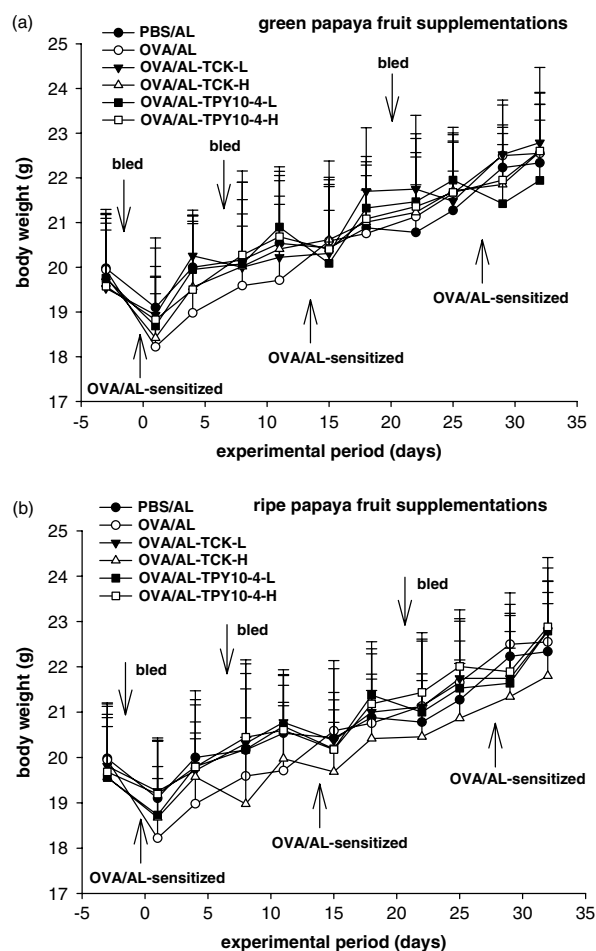


Figure 1. Effects of (a) green and (b) ripe papaya fruit supplementations by tube gavage on body weight changes in OVA/AL-sensitized BALB/c mice over 5 weeks. Values are mean $\pm$ SD ($n = 12$). There are no significant differences among groups at the same experimental point assayed using one-way ANOVA and Duncan's multiple range tests. TCK, non-transgenic papaya; TPY10-4, transgenic papaya; L, low dose ($4 \text{ mg}/0.5 \text{ mL day}^{-1}$, $0.2 \text{ g kg}^{-1} \text{ BW}$); H, high dose ($32 \text{ mg}/0.5 \text{ mL day}^{-1}$, $1.6 \text{ g kg}^{-1} \text{ BW}$).

RESULTS

Body weight changes and visceral organ weights

The changes in body weight together with the procedures applied during the experimental period are shown in Fig. 1. The mean body weight of all mice increased slightly as the experimental period progressed. Additionally, there were no significant differences between groups administered either green (Fig. 1(a)) or ripe (Fig. 1(b)) fruits. The results suggest that both non-transgenic TCK and transgenic TPY10-4 papaya fruit administration at either low or high dose did not significantly affect the weight gain of the experimental mice. To further clarify the effects of OVA/AL treatment and papaya fruit supplementation on the absolute and relative visceral organ weights of female BALB/c mice, six visceral organs (spleen, liver, kidney, heart, lung and thymus) were assessed at the end of the 5 week experimental period. The results showed that OVA/AL sensitisation treatment did not significantly affect any of the six visceral organ weights but slightly increased the spleen weight compared with PBS/AL treatment (Tables 1 and 2). However, there were no significant differences between groups administered either green (Table 1) or ripe (Table 2) papaya fruits. The results suggest that both non-transgenic TCK and transgenic TPY10-4 papaya fruit administration at either low or high dose did not significantly affect the six visceral organ weights of the

experimental mice compared with those of the dietary control (OVA/AL) group.

Serum total (non-specific) antibody levels

The changes in serum total IgE, IgA, IgG and IgM levels of mice supplemented with green and ripe papaya fruits are presented in Table 3. The results showed that OVA/AL sensitisation treatment significantly increased the serum total IgE level (from 0.09 ± 0.05 to $0.15 \pm 0.07 \mu\text{g mL}^{-1}$) but did not significantly affect serum total IgA, IgG and IgM levels. However, supplementations with green or ripe papaya fruits, either TCK or TPY10-4 at low or high dose, did not significantly affect serum IgE, IgA and IgG levels compared with those of the dietary control (OVA/AL) group. Interestingly, supplementation with ripe transgenic TPY10-4 papaya fruit at high dose for 5 weeks significantly increased the serum total IgM level.

Serum OVA-specific antibody titres

The serum OVA-specific IgE, IgG1 and IgG2a titres in mice supplemented with green and ripe papaya fruits throughout the 5 week experimental period are given in Table 4. The results showed that OVA/AL sensitisation treatment markedly ($P < 0.05$) increased OVA-specific IgE, IgG1 and IgG2a titres compared with those of the PBS/AL group (Table 4). Interestingly, green

Table 1. Effects of green papaya fruit supplementations by tube gavage on absolute and relative weights of visceral organs of OVA/AL-sensitised female BALB/c mice after 5 weeks

Organ	Group	OVA/AL	OVA/AL-TCK-L	OVA/AL-TPY10-4-L	OVA/AL-TCK-H	OVA/AL-TPY10-4-H	PBS/AL
Spleen	ATW	0.20 ± 0.03ab	0.17 ± 0.02b	0.20 ± 0.02a	0.21 ± 0.04a	0.18 ± 0.02ab	0.18 ± 0.03ab
	RTW	9.2 ± 1.5a	7.8 ± 0.6b	9.2 ± 1.0a	9.7 ± 1.7a	8.5 ± 1.1ab	9.0 ± 1.0ab
Liver	ATW	1.17 ± 0.08	1.21 ± 0.12	1.17 ± 0.08	1.16 ± 0.12	1.14 ± 0.08	1.15 ± 0.12
	RTW	54.5 ± 2.9ab	56.7 ± 2.9a	54.8 ± 2.8ab	54.3 ± 3.6ab	52.6 ± 2.6b	55.1 ± 3.6ab
Kidney	ATW	0.28 ± 0.02	0.29 ± 0.03	0.28 ± 0.02	0.28 ± 0.02	0.29 ± 0.02	0.28 ± 0.04
	RTW	13.0 ± 0.8	13.6 ± 1.0	13.1 ± 0.6	13.2 ± 0.6	13.2 ± 0.5	13.5 ± 1.1
Heart	ATW	0.11 ± 0.00abc	0.11 ± 0.01ab	0.10 ± 0.01bc	0.11 ± 0.01a	0.10 ± 0.00abc	0.10 ± 0.01a
	RTW	4.9 ± 0.2abc	5.1 ± 0.1ab	4.8 ± 0.2c	5.2 ± 0.2a	4.8 ± 0.2bc	5.0 ± 0.4abc
Lung	ATW	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
	RTW	5.5 ± 0.3	5.5 ± 0.4	5.8 ± 0.7	5.7 ± 0.3	5.9 ± 0.4	5.7 ± 0.3
Thymus	ATW	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
	RTW	2.0 ± 0.3	1.8 ± 0.4	2.1 ± 0.3	1.9 ± 0.4	1.8 ± 0.4	1.9 ± 0.4

Values are mean ± SD ($n = 12$). Values within the same row not sharing common letters are significantly different ($P < 0.05$) from each other. Data were analysed using one-way ANOVA followed by Duncan's multiple range tests. TCK, non-transgenic papaya; TPY10-4, transgenic papaya; L, low dose (4 mg/0.5 mL day⁻¹, 0.2 g kg⁻¹ BW); H, high dose (32 mg/0.5 mL day⁻¹, 1.6 g kg⁻¹ BW); ATW, absolute tissue weight (g); RTW, relative tissue weight (g kg⁻¹ BW).

Table 2. Effects of ripe papaya fruit supplementations by tube gavage on absolute and relative weights of visceral organs of OVA/AL-sensitised female BALB/c mice after 5 weeks

Organ	Group	OVA/AL	OVA/AL-TCK-L	OVA/AL-TPY10-4-L	OVA/AL-TCK-H	OVA/AL-TPY10-4-H	PBS/AL
Spleen	ATW	0.20 ± 0.03ab	0.24 ± 0.09a	0.18 ± 0.03b	0.20 ± 0.02ab	0.22 ± 0.07ab	0.18 ± 0.03b
	RTW	9.2 ± 1.5ab	11.2 ± 4.0a	8.7 ± 1.1b	9.2 ± 1.2ab	10.1 ± 2.9ab	9.0 ± 1.0b
Liver	ATW	1.17 ± 0.08	1.12 ± 0.11	1.11 ± 0.09	1.16 ± 0.10	1.14 ± 0.09	1.15 ± 0.12
	RTW	54.5 ± 2.9	52.4 ± 4.1	53.2 ± 2.2	53.4 ± 3.7	52.7 ± 3.4	55.1 ± 3.6
Kidney	ATW	0.28 ± 0.02	0.29 ± 0.03	0.28 ± 0.02	0.28 ± 0.02	0.29 ± 0.01	0.28 ± 0.04
	RTW	13.0 ± 0.8	13.6 ± 1.0	13.2 ± 0.7	12.7 ± 0.7	13.4 ± 0.8	13.5 ± 1.1
Heart	ATW	0.11 ± 0.00	0.10 ± 0.01	0.10 ± 0.00	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
	RTW	4.9 ± 0.2	4.7 ± 0.2	4.9 ± 0.4	4.9 ± 0.3	4.8 ± 0.3	5.0 ± 0.4
Lung	ATW	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
	RTW	5.5 ± 0.3ab	5.7 ± 0.3ab	5.6 ± 0.3ab	5.4 ± 0.4b	5.6 ± 0.4ab	5.7 ± 0.3a
Thymus	ATW	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
	RTW	2.0 ± 0.3	2.0 ± 0.4	2.0 ± 0.5	1.7 ± 0.3	1.9 ± 0.3	1.9 ± 0.4

Values are mean ± SD ($n = 12$). Values within the same row not sharing common letters are significantly different ($P < 0.05$) from each other. Data were analysed using one-way ANOVA followed by Duncan's multiple range tests. TCK, non-transgenic papaya; TPY10-4, transgenic papaya; L, low dose (4 mg/0.5 mL day⁻¹, 0.2 g kg⁻¹ BW); H, high dose (32 mg/0.5 mL day⁻¹, 1.6 g kg⁻¹ BW); ATW, absolute tissue weight (g); RTW, relative tissue weight (g kg⁻¹ BW).

fruit supplementation with non-transgenic TCK papaya at high dose (1.6 g kg⁻¹ BW) significantly decreased the OVA-specific IgE titre, suggesting that green papaya fruit supplementation may alleviate an allergic status. Ripe fruit supplementation with non-transgenic TCK papaya at both low (0.2 g kg⁻¹ BW) and high (1.6 g kg⁻¹ BW) doses significantly increased the OVA-specific IgG2a titre, suggesting that ripe papaya fruit supplementation may also alleviate an allergic status *in vivo*. However, green and ripe fruit supplementations with transgenic TPY10-4 papaya at low (0.2 g kg⁻¹ BW) or high (1.6 g kg⁻¹ BW) dose did not significantly affect OVA-specific IgE, IgG1 and IgG2a titres compared with those of the OVA/AL group (Table 4), suggesting that transgenic TPY10-4 papaya fruit supplementation did not increase the allergenicity of OVA *in vivo*.

Effects of green and ripe papaya fruit supplementations on Th1/Th2 cytokine secretions by splenocytes in OVA/AL-sensitised BALB/c mice

Tables 5 and 6 show the levels of Th1 (IFN- γ and IL-2) and Th2 (IL-4, IL-5 and IL-10) cytokines secreted by splenocytes in OVA/AL-sensitised mice supplemented with green and ripe papaya fruits respectively at the end of the 5 week experimental period. The results showed that OVA/AL sensitisation treatment increased both Th1 and Th2 cytokine (including IL-2 and IL-10) secretions by splenocytes in either spontaneous or OVA-stimulated cultures compared with those of the PBS/AL group. However, neither green nor ripe papaya fruit supplementations *in vivo* significantly affected Th1 and Th2 cytokine secretions by splenocytes in OVA/AL-sensitised mice during the 5 week experimental period.

Table 3. Effects of papaya fruit supplementations by tube gavage on serum total antibody titres from OVA/AL-sensitised female BALB/c mice after 5 weeks

Treatment	Group	Serum total antibody titre ($\mu\text{g mL}^{-1}$)			
		IgE	IgA	IgG	IgM
Green fruit	OVA/AL	0.15 $\pm$ 0.07a	1665 $\pm$ 376ab	788 $\pm$ 370	1583 $\pm$ 381
	OVA/AL-TCK-L	0.12 $\pm$ 0.03ab	1355 $\pm$ 357b	575 $\pm$ 122	1415 $\pm$ 286
	OVA/AL-TPY10-4-L	0.11 $\pm$ 0.03ab	1713 $\pm$ 623ab	698 $\pm$ 520	1381 $\pm$ 211
	OVA/AL-TCK-H	0.11 $\pm$ 0.04ab	1481 $\pm$ 513b	721 $\pm$ 159	1361 $\pm$ 243
	OVA/AL-TPY10-4-H	0.12 $\pm$ 0.04ab	1338 $\pm$ 301b	674 $\pm$ 110	1445 $\pm$ 278
Ripe fruit	PBS/AL	0.09 $\pm$ 0.05b	1934 $\pm$ 311a	696 $\pm$ 279	1573 $\pm$ 394
	OVA/AL	0.15 $\pm$ 0.07b	1665 $\pm$ 376ab	788 $\pm$ 370ab	1583 $\pm$ 381b
	OVA/AL-TCK-L	0.14 $\pm$ 0.06ab	1211 $\pm$ 315c	728 $\pm$ 132b	1537 $\pm$ 264b
	OVA/AL-TPY10-4-L	0.13 $\pm$ 0.03ab	1465 $\pm$ 343bc	922 $\pm$ 287ab	1484 $\pm$ 263b
	OVA/AL-TCK-H	0.15 $\pm$ 0.06b	1447 $\pm$ 206bc	764 $\pm$ 254ab	1483 $\pm$ 267b
	OVA/AL-TPY10-4-H	0.15 $\pm$ 0.06b	1547 $\pm$ 585bc	1028 $\pm$ 328a	2062 $\pm$ 476a
	PBS/AL	0.09 $\pm$ 0.05a	1934 $\pm$ 311a	696 $\pm$ 279b	1573 $\pm$ 394b

Values are mean $\pm$ SD ($n = 12$). Values within the same column and same treatment not sharing common letters are significantly different ($P < 0.05$) from each other. Data were analysed using one-way ANOVA followed by Duncan's multiple range tests. TCK, non-transgenic papaya; TPY10-4, transgenic papaya; L, low dose ($0.2 \text{ g kg}^{-1} \text{ BW}$); H, high dose ($1.6 \text{ g kg}^{-1} \text{ BW}$).

Table 4. Effects of papaya fruit supplementations by tube gavage on serum antibody titres of OVA-specific IgE, IgG1 and IgG2a from OVA/AL-sensitised female BALB/c mice after 5 weeks

Treatment	Group	OVA-specific antibody titre (ELISA units)		
		IgE	IgG1	IgG2a
Green fruit	OVA/AL	0.08 $\pm$ 0.05a	0.20 $\pm$ 0.14	0.10 $\pm$ 0.07bc
	OVA/AL-TCK-L	0.07 $\pm$ 0.03ab	0.18 $\pm$ 0.04	0.14 $\pm$ 0.07ab
	OVA/AL-TPY10-4-L	0.07 $\pm$ 0.03ab	0.22 $\pm$ 0.06	0.15 $\pm$ 0.10ab
	OVA/AL-TCK-H	0.04 $\pm$ 0.03b	0.21 $\pm$ 0.08	0.22 $\pm$ 0.21b
	OVA/AL-TPY10-4-H	0.06 $\pm$ 0.04ab	0.17 $\pm$ 0.06	0.05 $\pm$ 0.01bc
Ripe fruit	PBS/AL	0.01 $\pm$ 0.01c	ND	0.01 $\pm$ 0.01c
	OVA/AL	0.08 $\pm$ 0.05a	0.20 $\pm$ 0.14	0.10 $\pm$ 0.07bc
	OVA/AL-TCK-L	0.07 $\pm$ 0.03a	0.22 $\pm$ 0.07	0.26 $\pm$ 0.13a
	OVA/AL-TPY10-4-L	0.06 $\pm$ 0.02a	0.18 $\pm$ 0.07	0.10 $\pm$ 0.04bc
	OVA/AL-TCK-H	0.08 $\pm$ 0.04a	0.18 $\pm$ 0.06	0.24 $\pm$ 0.15a
	OVA/AL-TPY10-4-H	0.07 $\pm$ 0.04a	0.21 $\pm$ 0.05	0.11 $\pm$ 0.04b
	PBS/AL	0.01 $\pm$ 0.01b	ND	0.01 $\pm$ 0.01c

Values are mean $\pm$ SD ($n = 12$). Values within the same column and same treatment not sharing common letters are significantly different ($P < 0.05$) from each other. Data were analysed using one-way ANOVA followed by Duncan's multiple range tests. ELISA unit = $(A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{positive}} - A_{\text{blank}})$; TCK, non-transgenic papaya; TPY10-4, transgenic papaya; L, low dose ($0.2 \text{ g kg}^{-1} \text{ BW}$); H, high dose ($1.6 \text{ g kg}^{-1} \text{ BW}$); ND, not detectable. To determine OVA-specific antibody titres, the collected sera were diluted to 1 : 4 for IgE detection, 1 : 100 for IgG2a detection and 1 : 5000 for IgG1 detection.

These results indicate that neither non-transgenic TCK nor transgenic TPY10-4 papaya fruits significantly affected Th1/Th2 cytokine secretion profiles, suggesting that the fruits of transgenic TPY10-4 papaya do not modify the response to OVA.

DISCUSSION

The present study showed that neither non-transgenic TCK nor transgenic TPY10-4 papaya fruit administration significantly affected the six visceral organ weights of the experimental mice compared with those of the dietary control (OVA/AL) group (Tables 1 and 2). These results suggest that papaya fruit supplementation, even at high dose ($1.6 \text{ g kg}^{-1} \text{ BW day}^{-1}$ in mice, equivalent to $95 \text{ g fresh papaya fruit day}^{-1}$ in humans) for 5 weeks,

is safe and has no toxic side effects on body weight change and visceral organ weights. In this study, OVA/AL sensitisation treatment increased the spleen weight slightly compared with PBS/AL treatment (Tables 1 and 2), suggesting that OVA treatment indeed sensitised the experimental mice and may induce an inflammatory response in the spleen. Therefore the established OVA-sensitised murine model was suitable for investigating the immunomodulatory changes in adaptive immunity between transgenic TPY10-4 and native TCK papaya fruits.⁶

Different antibody isotypes participate in different humoral immune responses in the body. B lymphocytes proceed with isotype switching *in vivo* and switch the immunoglobulin class from IgM to IgG, IgE or IgA isotype during an immune response.¹⁷ IgM antibodies can form a pentamer and frequently

Table 5. Effects of green papaya fruit supplementations by tube gavage on Th1/Th2 cytokines (pg mL⁻¹) secreted using splenocytes from OVA/AL-sensitised BALB/c mice after 5 weeks

Cytokine	Group	Treatment	
		Spontaneous	OVA (30 µg mL ⁻¹)
IFN- γ	OVA/AL	ND	ND
	OVA/AL-TCK-L	ND	ND
	OVA/AL-TPY10-4-L	ND	ND
	OVA/AL-TCK-H	ND	ND
	OVA/AL-TPY10-4-H	ND	ND
	PBS/AL	ND	ND
IL-2	OVA/AL	17.8 $\pm$ 11.6ab	14.9 $\pm$ 4.6
	OVA/AL-TCK-L	20.1 $\pm$ 17.9a	13.0 $\pm$ 9.8
	OVA/AL-TPY10-4-L	10.2 $\pm$ 5.1ab	10.8 $\pm$ 5.3
	OVA/AL-TCK-H	14.8 $\pm$ 10.0ab	16.3 $\pm$ 13.1
	OVA/AL-TPY10-4-H	21.5 $\pm$ 15.1a	14.7 $\pm$ 8.4
	PBS/AL	6.6 $\pm$ 6.2b	0.8 $\pm$ 1.5
IL-4	OVA/AL	1.6 $\pm$ 2.4	2.0 $\pm$ 3.1
	OVA/AL-TCK-L	ND	ND
	OVA/AL-TPY10-4-L	2.4 $\pm$ 3.4	1.5 $\pm$ 3.1
	OVA/AL-TCK-H	ND	ND
	OVA/AL-TPY10-4-H	3.1 $\pm$ 3.4	ND
	PBS/AL	2.2 $\pm$ 2.9	ND
IL-5	OVA/AL	6.5 $\pm$ 8.6	ND
	OVA/AL-TCK-L	0.8 $\pm$ 1.1	ND
	OVA/AL-TPY10-4-L	4.2 $\pm$ 3.8	6.4 $\pm$ 8.3
	OVA/AL-TCK-H	7.2 $\pm$ 8.4	ND
	OVA/AL-TPY10-4-H	5.7 $\pm$ 7.7	4.1 $\pm$ 6.7
	PBS/AL	4.9 $\pm$ 5.7	ND
IL-10	OVA/AL	30.4 $\pm$ 20.6	39.0 $\pm$ 18.8a
	OVA/AL-TCK-L	23.7 $\pm$ 11.3	27.3 $\pm$ 20.1ab
	OVA/AL-TPY10-4-L	32.9 $\pm$ 23.7	30.5 $\pm$ 17.3ab
	OVA/AL-TCK-H	19.5 $\pm$ 10.8	37.5 $\pm$ 19.2a
	OVA/AL-TPY10-4-H	30.6 $\pm$ 12.5	43.5 $\pm$ 21.3a
	PBS/AL	20.7 $\pm$ 18.0	17.6 $\pm$ 13.5b

Values are mean $\pm$ SD ($n = 12$). Values within the same column and same treatment not sharing common letters are significantly different ($P < 0.05$) from each other. Data were analysed using one-way ANOVA followed by Duncan's multiple range tests. TCK, non-transgenic papaya; TPY10-4, transgenic papaya; L, low dose (0.2 g kg⁻¹ BW); H, high dose (1.6 g kg⁻¹ BW); ND, not detectable. The sensitivity of these cytokine assays was <15.6 pg mL⁻¹.

Table 6. Effects of ripe papaya fruit supplementations by tube gavage on Th1/Th2 cytokines (pg mL⁻¹) secreted using splenocytes from OVA/AL-sensitised BALB/c mice after 5 weeks

Cytokine	Group	Treatment	
		Spontaneous	OVA (30 µg mL ⁻¹)
IFN- γ	OVA/AL	ND	ND
	OVA/AL-TCK-L	ND	ND
	OVA/AL-TPY10-4-L	ND	ND
	OVA/AL-TCK-H	ND	ND
	OVA/AL-TPY10-4-H	ND	ND
	PBS/AL	ND	ND
IL-2	OVA/AL	17.8 $\pm$ 11.6a	14.9 $\pm$ 4.6
	OVA/AL-TCK-L	14.6 $\pm$ 5.4ab	10.7 $\pm$ 7.8
	OVA/AL-TPY10-4-L	13.8 $\pm$ 8.6ab	14.5 $\pm$ 11.2
	OVA/AL-TCK-H	20.9 $\pm$ 9.7a	8.7 $\pm$ 6.9
	OVA/AL-TPY10-4-H	20.5 $\pm$ 12.1a	13.5 $\pm$ 12.0
	PBS/AL	6.6 $\pm$ 6.2b	0.8 $\pm$ 1.5
IL-4	OVA/AL	1.6 $\pm$ 2.4	2.0 $\pm$ 3.1
	OVA/AL-TCK-L	6.2 $\pm$ 5.5	5.1 $\pm$ 4.3
	OVA/AL-TPY10-4-L	4.7 $\pm$ 3.6	5.2 $\pm$ 5.6
	OVA/AL-TCK-H	ND	1.5 $\pm$ 2.1
	OVA/AL-TPY10-4-H	8.9 $\pm$ 8.8	2.2 $\pm$ 2.0
	PBS/AL	2.2 $\pm$ 2.9	ND
IL-5	OVA/AL	6.5 $\pm$ 8.6ab	ND
	OVA/AL-TCK-L	12.1 $\pm$ 5.6ab	18.5 $\pm$ 12.9
	OVA/AL-TPY10-4-L	12.9 $\pm$ 6.5a	26.3 $\pm$ 11.4
	OVA/AL-TCK-H	8.3 $\pm$ 6.0ab	8.8 $\pm$ 5.9
	OVA/AL-TPY10-4-H	10.5 $\pm$ 9.3ab	15.1 $\pm$ 7.6
	PBS/AL	4.9 $\pm$ 5.7b	ND
IL-10	OVA/AL	30.4 $\pm$ 20.6ab	39.0 $\pm$ 18.8a
	OVA/AL-TCK-L	46.0 $\pm$ 14.4a	23.5 $\pm$ 21.9ab
	OVA/AL-TPY10-4-L	33.6 $\pm$ 10.9ab	24.8 $\pm$ 13.1ab
	OVA/AL-TCK-H	28.9 $\pm$ 11.8ab	18.4 $\pm$ 10.6b
	OVA/AL-TPY10-4-H	37.1 $\pm$ 25.0ab	32.8 $\pm$ 19.5ab
	PBS/AL	20.7 $\pm$ 18.0b	17.6 $\pm$ 13.5b

Values are mean $\pm$ SD ($n = 12$). Values within the same column and same treatment not sharing common letters are significantly different ($P < 0.05$) from each other. Data were analysed using one-way ANOVA followed by Duncan's multiple range tests. TCK, non-transgenic papaya; TPY10-4, transgenic papaya; L, low dose (0.2 g kg⁻¹ BW); H, high dose (1.6 g kg⁻¹ BW); ND, not detectable. The sensitivity of these cytokine assays was <15.6 pg mL⁻¹.

recognise repetitive epitopes such as those on bacterial cell wall polysaccharides. The IgE antibody *in vivo* plays an important role in resistance to parasite infection.¹⁸ However, a higher serum IgE level is also an indicator of allergy. Our results showed that transgenic papaya fruit administration did not significantly affect the IgE titre but had a protective immunity via increasing the total serum IgM level (Table 3). This study further suggests that transgenic TPY10-4 papaya fruit supplementation, even at high dose, does not induce overall allergic skewing in OVA/AL-sensitised mice. Green and ripe papaya fruits (epicarp extracts) are traditionally used to treat many diseases, such as skin diseases, in Asia.¹⁹ Interestingly, this study further evidenced that papaya fruit supplementation enhanced humoral immunity, especially the IgM level in OVA/AL-sensitised mice (Table 3). Papaya fruit products may have the potential for future development as immunomodulatory health foods. In

fact, ripe transgenic papaya fruit slightly, but not significantly ($P > 0.05$), increased the serum IgG level in a dose-dependent manner compared with that of the OVA/AL group (Table 3). This study suggests that ripe transgenic papaya fruit significantly ($P < 0.05$) increases humoral immunity but slightly affects isotype switching *in vivo*.

IgE and IgG1 antibodies are classified as Th2-type immunoglobulins, while the IgG2a antibody is regarded as a Th1-type immunoglobulin.⁶ The results from this study indicated that non-transgenic TCK papaya fruit supplementation significantly decreased the OVA-specific IgE titre with green fruit but increased the OVA-specific IgG2a titre with ripe fruit (Table 4). These results suggest that supplementation with native TCK papaya fruits is effective in modulating Th1 and Th2 antibody titres via isotype

switching *in vivo*. However, the immunomodulatory mechanism of the papaya fruit remains to be further clarified.

Cytokines such as IL-4, IL-5, IL-6 and TNF- α (mostly Th2 cytokines) can direct murine class switching to IgG1 and IgE and human class switching to IgG4 and IgE, while IL-2, IFN- γ , IFN- α and transforming growth factor β (TGF- β) (mostly Th1 cytokines) promote murine IgG2a production but inhibit IgE production.^{17,20,21} IgE production is critically dependent on the ratio in which Th1 (IL-2 and IFN- γ) and Th2 (IL-4 and IL-5) cytokines are elicited *in vivo*. In Tables 5 and 6 there are some low and undetectable levels of Th1/Th2 cytokines when OVA is added to the splenocyte cultures. Our results indicated that papaya fruit supplementation did not significantly affect either Th1 or Th2 cytokine secretions. Low and undetectable levels of Th1/Th2 cytokines secreted by OVA-stimulated splenocytes may cause a bias in judging the Th1/Th2 balance. Evidently, a higher OVA sensitisation dose (20 μ g per mouse i.p.) and OVA stimulation concentration (final concentration 100 μ g mL⁻¹) to splenocytes *in vitro* produce higher Th1/Th1 cytokine secretion levels (nanogram quantities).²² This study suggests that a higher OVA sensitisation dose (20 μ g per mouse i.p.) and OVA stimulation concentration (final concentration 100 μ g mL⁻¹) to splenocytes *in vitro* are a better treatment in the OVA-sensitised mouse model.

There are no widely recognised validated animal models for predicting allergenicity of novel foods. However, Ladics *et al.*²³ imply that some animal models have been validated. Most of the developed animal models assess the induced antibody of known allergenic proteins in the test groups, such as IgE or IgG1 in mice or IgG2a in rats, but there is still some lack of reproducibility and predictive value.²³ The present study attempted to detect any immunomodulatory changes due to transgenic TPY10-4 papaya fruit supplementation in OVA-sensitised BALB/c mice. The results suggest that transgenic TPY10-4 papaya fruits do not increase the allergenic potential of OVA by oral supplementation in OVA-sensitised mice.

CONCLUSION

The results showed that oral supplementation with either green or ripe transgenic TPY10-4 papaya fruits did not significantly ($P > 0.05$) affect the serum total IgE antibody level or OVA-specific IgE and IgG1 antibody titres in OVA-sensitised mice. This study suggests that transgenic TPY10-4 papaya fruits do not increase the allergenic potential of OVA by oral administration but may have a protective immunity via increasing the serum total IgM level.

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