

Safety assessment of leaf curl virus resistant tomato developed using viral derived sequences

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Abstract Genetic engineering of food crops has significantly influenced the agricultural productivity over the past two decades. It has proved a valuable tool, offering crops with higher yields, improved nutritional quality, resistance against pesticides, herbicides and tolerance against abiotic stresses. However, the safety assessment of genetically engineered (GE) crops is prerequisite before introduction into human food chain. The present study was aimed to assess the toxicity and allergenicity of leaf curl virus resistant GE tomato compared to its wild-type species. Balb/c mice fed with genetically engineered or wild-type tomato did not show significant differences in growth, body weight ($P > 0.05$) and food consumption when compared with control mice. Values for serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase, urea and cholesterol were comparable in GE and wild-type tomato fed mice. Mice immunized with GE or wild-type tomato extract showed low IgE response. Lung histology of ovalbumin fed mice showed bronchoconstriction with eosinophilic infiltration whereas GE

or wild-type tomato showed no cellular infiltration with normal airways. Genetically engineered and wild-type tomato sensitized mice demonstrated similar IL-4 release in splenic cell culture supernatant. GE and wild tomato extract on ELISA showed comparable IgE binding ($P > 0.05$) with food allergic patients' sera. In conclusion, genetically engineered tomato showed no toxicity in mice and allergenicity is similar to the wild-type tomato.

Keywords Allergenicity · Animal feeding studies · GE food safety · siRNA tomato · Toxicity

Abbreviations

ELISA	Enzyme linked immunosorbent assay
GE	Genetically engineered
H&E	Hematoxylin-eosin
IFN- γ	Interferon- γ
IgE	Immunoglobulin E
IL-4	Interleukin-4
i.p.	Intraperitoneal
i.v.	Intravenous
PBS	Phosphate buffer saline
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase

Introduction

Agriculture biotechnology has provided methods for developing genetically engineered (GE) crops with

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better yield to feed the growing world population. The benefits include, crops with improved nutritional value, resistance against pesticides, herbicides and tolerance against abiotic stresses. Genetic engineering involves introduction of a foreign gene into the plant genome to achieve desired trait. However, it is advisable to ascertain that the introduced gene does not evoke any adverse reaction in humans and animals on consumption of the crop product. Moreover, the safety assessment of genetically engineered crops is prerequisite before introduction into human food chain (Purchase 2005).

Safety assessment of GE crops is based on the concept of substantial equivalence to wild-type species as directed by OECD (1993a, 1998) and elaborated further by FAO/WHO (2001). These organizations have evolved concept of comparative approach and embody the idea that existing traditionally produced foods can serve as a reference to evaluate the safety of the genetically engineered foods (Kuiper et al. 2002). GE glyphosate tolerant soybean was observed equivalent to non-GE soybean in terms of composition and nutrients (Padgett et al. 1996). However, GE soybean expressing a sulphur rich protein from a Brazil nut gene was potentially allergenic to humans (Nordlee et al. 1996). A study with Bt potato demonstrated abnormal and excessive cell growth in the small intestine of mice (Fares and El-Sayed 1998). Recently, transgenic expression of bean α -amylase inhibitor in peas resulted in altered structure and immunogenicity (Prescott et al. 2005). These reports suggest safety and allergenicity evaluation of GE crops before they are recommended for human or animal consumption.

Tomato is one of the important vegetable crops cultivated world over for its fleshy fruit. A major threat to tomato crop is an infection with “*Tomato leaf curl virus*” that induces several annihilative changes in plant ruining the crop. A transgenic tomato variety has been developed that employs RNA mediated resistance for viral control (Praveen et al. 2005a). Small RNA hold promise for developing viral resistance and careful selection of viral gene helps in effective silencing. Targeting AC1 (the only viral encoded protein required for viral replication), via RNAi machinery is supposed to be effective silencing strategy. The potent siRNAs were filtered based on the off-target sequences but for their accessibility to the secondary structure of target mRNA. The plants

transformed with this construct inherit resistance to diseases for subsequent generations (Praveen et al. 2005b). In the present study, toxicity and allergenicity assessment of transgenic tomato was undertaken in Balb/c mice. Ovalbumin was used to develop the allergic mice model and hypersensitive patients’ sera were used to analyze tomato extract for IgE binding.

Methods

Test material

Fruits of transgenic *Lycopersicon esculentum* (eng. tomato) with the replicase gene from “*Tomato leaf curl virus*” (Praveen et al. 2005a) and a non-transgenic wild-type variety of same species were used for toxicity and allergenicity assessment. The handling of GE food material was approved by Biosafety committee of the Institute of Genomics and Integrative Biology, Delhi.

Toxicity assessment

Animal feeding

Balb/c mice were segregated into three groups of ten mice each. Mice were kept on standard recommended chow diet, allowed free access to water and maintained on 12:12 hours light: dark cycle in an environmentally controlled chamber. The temperature and humidity were maintained at $23 \pm 2^\circ\text{C}$ and $55 \pm 10\%$, respectively. Group 1 mice were gavaged daily with PBS (control group), group 2 with wild-type tomato and group 3 with GE tomato for 42 days. The wild-type and GE tomato (whole fruit) were freeze dried and grounded to fine powder. The dosage of wild-type and GE tomato was administered 100 mg/kg body weight (EFSA report 2007). The animals were observed daily for activity, fur color, food consumption and excretion. Body weight was measured every week for each mouse.

Blood biochemistry

Blood samples from test animals and controls were collected on day 42 for hematological analysis. Serum biochemical indices were determined by standard clinical method (Singh et al. 2008). Total

plasma protein, urea, creatinine, serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) levels were determined for liver and kidney functioning using commercial kits (Span Diagnostics Pvt. Ltd, Surat, India). Total and HDL cholesterol levels were measured for normal cardiac functioning (Monozyme Pvt. Ltd, Secunderabad, India).

Histological studies

The gut tissues were collected from the sacrificed mice and fixed in 10% neutral-buffered formalin (v/v) overnight (Singh et al. 2006). The tissues were embedded in paraffin, sliced to 4 µm sections and stained with Hematoxylin-eosin (H&E) for analysis of antigen induced inflammation. Sections were scanned under a light microscope and images of these fields were captured by an in-line camera (Nikon TE 2000-S, NY, USA) and assembled into multi-panel figure using Photoshop software (Adobe version 7.0).

Allergenicity assessment

Sequence analysis

The introduced antisense strand of the viral replicase protein was translated into protein sequence using expasy tool (<http://www.expasy.ch/tools/dna.html>). The translated sequence was studied in all the three frames for homology with allergens using SDAP (Structural database of allergenic protein; http://fermi.utmb.edu/SDAP/sdap_who.html), FARRP (<http://allergenonline.com>) and Swiss-Prot databases.

Protein extraction

The tomato fruits were freeze dried and ground to a fine powder. The powdered material was defatted with repeated changes of diethyl ether for 36 h at 4°C. The protein was extracted from defatted powder 1:20 (w/v) in 50 mM ammonium bicarbonate buffer (pH 8.0) containing 2 mM ethylene diamine tetra acetic acid (EDTA) and 1 mM phenyl methyl sulfonyl fluoride (PMSF), by continuous stirring for 5 h at 4°C (Kumari et al. 2006). The supernatant was collected after centrifugation at 12,000g for 30 min at 4°C. The proteins from wild type and GE tomato fruits were

separately extracted, freeze dried and stored at −20°C.

Animal studies

Six weeks old Balb/c mice were segregated into four groups of six mice each. Group 1 mice were injected daily with phosphate buffer saline (PBS) and group 2 with ovalbumin for 42 days. Group 3 and 4 mice were given wild-type and GE tomato protein extract, respectively, via intraperitoneal route. Mice were injected (i.p.) 100 µg protein in 100 µl PBS once a week for 7 weeks. Blood samples were collected to measure serum IgE, IgG1 and IgG2a antibodies on day 15, 30 and 43. The sensitized mice were challenged i.v. on day 45 with 3, 6 and 12 mg of ovalbumin, wild-type or GE tomato protein and symptoms were recorded (symptoms score: 0. no symptoms; 1. scratching and rubbing around the nose and head; 2. puffiness around the eyes and mouth, pilar erecti, diarrhea, and reduced activity or standing still with an increased respiratory rate; 3. wheezing, labored respiration, and cyanosis around the mouth; 4. symptoms as in no. 3 with loss of consciousness, tremor, and/or convulsion; 5. death). The challenged mice were sacrificed on day 45 for splenic cell culture and lung tissue histology.

Measurement of ovalbumin/wild-type/GE tomato specific antibodies

Serum antibodies specific for ovalbumin, wild-type or GE proteins were measured by enzyme linked immunosorbent assay (ELISA) (Singh et al. 2006). Briefly, the microtitre plates (Maxisorp, Denmark) were coated with 250 ng of ovalbumin or with 1 µg of wild-type or GE tomato extracts in carbonate buffer (pH 9.6). The plates were incubated with mice sera for IgE (1:10), IgG1 and IgG2a (1:500) estimations. After washing, rat antimouse IgE-horse raddish peroxidase (HRP) (1:1000, Bethyl laboratories, USA), rat antimouse IgG1-HRP (1:1000, BD Pharmingen) and rat antimouse IgG2a-HRP (1:1000, BD Pharmingen) in PBS were added to the respective wells. After washing with PBST and PBS, the color was developed using *o*-phenylenediamine in citrate-phosphate buffer and H₂O₂. The reaction was stopped with 4N H₂SO₄ and the absorbance read at 492 nm.

Splenic cell culture

Spleens were removed aseptically on day 45 from three mice in each group, cells were isolated and cultured to study cytokines profile (Singh et al. 2008). Single cell suspensions were prepared depleted of red blood cells by lysis buffer. For in vitro experiments, cells were resuspended in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA), 10 U/ml penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO, USA). Five million cells/ml were seeded in 96 well flat bottomed culture plates. For stimulation of the cells, OVA, wild-type or GE tomato extract (100 µg/ml) were added to cultures. Phytohaemeagglutinin (5 µg/ml) was used as a positive control and no stimulant was added to negative control. Cells were cultured for 72 h at 37°C in a humidified incubator with 5% CO₂ environment and supernatant harvested for cytokines estimation by ELISA.

Cytokines estimation

IL-4 and IFN- γ levels were determined in culture supernatants by sandwich ELISA following manufacturer's instructions (BD Pharmingen, San Diego, USA). Capture antibody (1:250 v/v) for each cytokine was coated separately on microtiter plates in carbonate buffer, pH 9.6 and incubated overnight at 4°C. After washing, the wells were blocked with PBS containing 10% fetal bovine serum at 37°C for 1 h. After washing, 100 µl of standards (seven serial dilutions), diluted sample (1:2 v/v) and controls were added to wells and incubated at 37°C for 3 h. The plates were washed again and incubated with biotinylated detector antibody labeled with avidin HRP at 37°C for 1 h. The substrate was added after seven washings and plates were incubated in dark at 37°C for 30 min. The reaction was stopped with 2N H₂SO₄ and read at 450 nm. The cytokine concentrations were deduced from the standards. The detection limit for IL-4 and IFN- γ was 7.8 and 31.3 pg/ml, respectively.

Lung histology

Lungs were taken out and fixed immediately in 10% neutral-buffered formalin for histology. The sections (3 µm) were cut, stained with hematoxylin and eosin and visualized under light microscope (Nikon TE

2000-S, USA) for antigen induced peribronchial and perivascular inflammation. The study protocol was approved by Animal Ethics Committee of the Institute of Genomics and Integrative Biology, Delhi.

Skin-tests and sera collection

The patients suffering from asthma, rhinitis or both aged 15–45 years were skin prick tested with aeroallergen (pollen, fungi and insects), common food/vegetable extracts including tomato at Out Patient Department, V.P. Chest Institute, Delhi. The diagnosis of asthma was ascertained by American Thoracic Society guidelines (ATS 1991) with 12% reversibility or 200 ml increase in FEV1 with 200 µg of salbutamol. The patients having two of the symptoms namely sneezing, rhinorrhea, nasal blockage, post natal drip, etc. over the past 2 years were diagnosed as rhinitis (Adomopoulos et al. 1995). Histamine diphosphate (5 mg/ml) and glycerinated phosphate buffer saline (PBS) were used as positive and negative controls, respectively (Kumari et al. 2006). Skin tests were graded based on wheal size of positive control. The skin reactions equal or more to size of positive control (3 mm or more) were graded as marked positive. The blood was collected from patients ($n = 40$) and healthy subjects ($n = 5$) and sera separated for immunoassay. Informed written consent of subjects was obtained for participation in the study and the protocol was approved by human ethics committee of the Institute.

Enzyme linked immunosorbent assay

Specific IgE level against wild-type or GE tomato extract was estimated in patients' sera by indirect ELISA. Briefly, the microtitre plates (Maxisorp, NuncTM immunomodule, Denmark) were coated with protein (1 µg/100 µl) in carbonate buffer (pH 9.6). Individual patient's serum (1:10 v/v) was added to the wells and epsilon specific antihuman IgE linked to horseradish peroxidase (Sigma, 1:1000 v/v) was used to detect the bound IgE. The serum from non-allergic individuals was tested as control.

Statistical analysis

Student t-test (unpaired) was used for statistical analysis of the data and $P < 0.05$ was considered as statistically significant.

Results

Toxicity evaluation of GE tomato

Blood and clinical parameters

The mice fed with wild-type or GE tomato for 42 days appeared healthy and normal in terms of growth (Fig. 1), development, activity, food consumption, excretion (data not shown). The average body weight gained per week showed no significant difference ($P > 0.05$) between animals fed with wild-type or GE tomato and those fed with PBS (control).

The mean levels of various hematological and biochemical indices are presented in Table 1. Total plasma protein and urea levels were similar in GE tomato or wild-type tomato fed mice and control mice. Serum creatinine level was also similar for wild-type and GE tomato fed mice with no statistically significant difference ($P > 0.05$). SGPT and SGOT levels were comparable in GE tomato, wild-type and PBS control group mice indicating normal liver functioning. Total and HDL cholesterol level in GE tomato fed mice were slightly higher than those fed with wild-type tomato but levels were in the range of control mice. There is no statistically significant difference ($P > 0.05$) in these two indices suggesting normal cellular metabolism for mice fed with GE or wild-type tomato.

Gut histology

Histological analysis of hematoxylin and eosin stained gastric tissues of GE and wild-type tomato

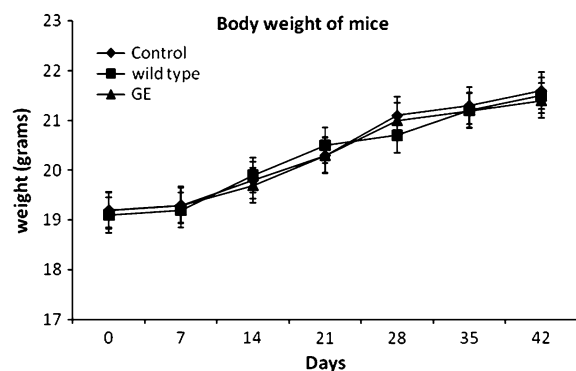


Fig. 1 Body weight of Balb/c mice: weight records of mice fed with PBS (◆) wild-type tomato (■) and GE tomato extract (▲) during the 6 weeks study. Weight was recorded every week. Data are presented as mean $\pm$ SE

fed mice revealed normal structure with no distortion in gut lining. Parietal cells were smooth indicating no infiltrations of eosinophils in surrounding tissues (Fig. 2a–c). Duodenal villi revealed normal surface area with numerous absorptive enterocytes cells without any sign of ulcer, bleeding or disruption in the villus (Fig. 2d–f). Gut histology of GE and wild-type tomato fed mice was comparable with that of PBS control mice. Liver sections showed normal appearance of hepatic cells similar to control (Fig. 2g–i).

Allergenicity studies

Bioinformatics analysis

Computational approach was used to determine allergenicity in all the three frames of the anti-sense sequence introduced in genetically engineered tomato. The possible sequence was deduced and searched in various databases. The sequence homology studies showed no significant homology with allergenic sequences in SDAP allergen database. The maximum homology is 7.76% with *Penicillium citrinum* allergen Pen c 3, which is below 35% suggested by the guidelines. In the Farrp allergen database, it has demonstrated an *e*-value of 2.0 with honey bee allergen Api m 1, which was 100 times higher than the significant value of 0.02 for two homologous proteins. Six consecutive amino acid search using Swissprot database revealed no matches.

Immune response to ovalbumin, GE and wild-type tomato proteins

Intraperitoneal sensitization with wild-type and GE tomato protein produced low IgE titer on day 15 that remained unchanged till day 43 (Fig. 3a). Ovalbumin immunization produced high IgE response by the intraperitoneal route. Compared to ovalbumin, intraperitoneal sensitization with both wild-type and GE tomato showed high IgG1 response (Fig. 3b). Both wild-type and GE tomato produced similar IgG2a level on day 15 that increased further by day 30 (Fig. 3c). No significant difference ($P > 0.05$) was observed between the IgG1 and IgG2a antibody response induced by wild-type and GE tomato proteins.

Table 1 Serum biochemistry and hematological indices for mice fed with wild type or GE tomato

Serum chemistry	Control	Wild type tomato	GE tomato
Plasma protein (g/dl)	7.11 ± 2	7.62 ± 1.0	6.99 ± 1.9
Creatinine (mg/dl)	0.21 ± 2	0.19 ± 2	0.19 ± 1.0
Urea (mg/100 ml)	56.2 ± 3.7	46.2 ± 6.9	55.1 ± 5.2
Nitrogen (mg/100 ml)	22.2 ± 2.6	24.4 ± 5.7	26.9 ± 7.1
SGPT (IU/l)	53.0 ± 2.3	51.6 ± 6.2	54.0 ± 5.3
SGOT (IU/l)	78.6 ± 7.1	75.0 ± 5.1	81.6 ± 8.6
Total cholesterol (mg/dl)	227 ± 11	212.3 ± 9	229 ± 14
HDL cholesterol (mg/dl)	18.9 ± 10	18 ± 7	22.1 ± 6
Hematological values			
Lymphocytes (%)	58.6	60.1	59.3
Eosinophils (%)	1.2	0.9	1.0
Monocytes (%)	3.1	2.8	2.9
Neutrophils (%)	37.1	36.2	36.8

Mean ± SEM; control mice were given PBS

Antigen challenge in sensitized mice

Intraperitoneally sensitized mice were challenged i.v. with different doses of pre-sensitized antigen. Mice challenged with ovalbumin demonstrated anaphylactic response frequently with 12 mg antigen. Even low doses of 6 or 3 mg ovalbumin protein caused death in mice due to anaphylaxis. Wild-type and GE tomato sensitized mice on challenge with 3, 6 or 12 mg protein did not show anaphylactic response. Control group mice showed no signs of anaphylaxis after PBS challenge.

Cytokine levels in spleen cell culture

IL-4 and IFN- γ were determined by ELISA in spleen cell culture supernatant of sensitized mice. Ovalbumin sensitized mice showed four times increased levels of Th2 cytokine, i.e. IL-4 (29 ± 5 pg/ml) than control (7 ± 2 pg/ml). The level of IL-4 was low in cell culture supernatant in wild-type and GE tomato protein sensitized mice (8 pg/ml), similar to control. The level of IFN- γ did not differ between the wild-type (110 ± 5 pg/ml) and GE (115 ± 8 pg/ml) tomato sensitized mice. Ovalbumin sensitized mice showed lower IFN- γ (70 ± 5 pg/ml) response compared to control mice sensitized with PBS (120 ± 2 pg/ml).

Histology of lungs

Lungs of ovalbumin sensitized and challenged mice showed perivascular and peribronchial inflammatory

cell infiltrate with loss of normal lung structure (Fig. 4a). In contrast, both wild-type and GE tomato protein-sensitized/challenged mice showed normal lung structure with defined bronchi and alveoli, irrespective of the route of sensitization (Fig. 4b–c). There were no inflammatory cells infiltrate in the lung tissue of wild-type and GE tomato protein sensitized mice.

IgE binding of GE and wild-type tomato extract with patients' sera

The patients of stable asthma, allergic rhinitis or both with history of food allergy ($n = 40$) were included in the present study. ELISA was done with patients' sera to determine any change in IgE binding with GE tomato compared to wild-type extract. Figure 5 shows the specific IgE binding to the wild-type and GE tomato protein with food allergic patients' sera. Of the 40 patients' sera tested, six showed three times or more IgE binding to both GE and/or wild-type tomato extracts (0.35–0.57 OD) as compared to control (0.05–0.07 OD) group (Table 2). Wild-type and GE tomato protein showed no significant difference in IgE binding with hypersensitive patients' sera (Fig. 5, correlation coefficient, $R^2 = 0.84$).

Discussion

The safety assessment of GE crops involves consideration of possible health effects, such as allergenicity,

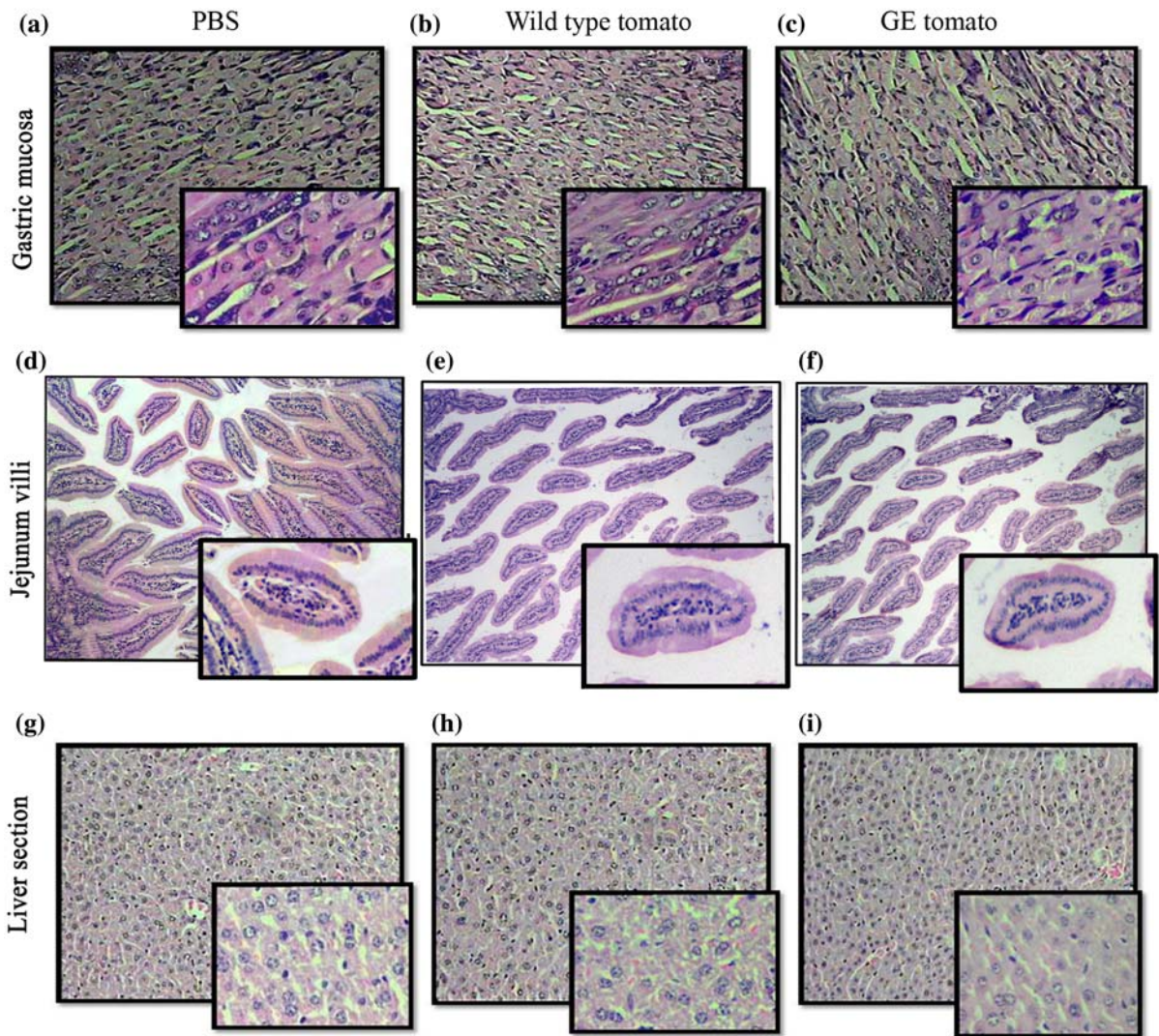


Fig. 2 Histological analysis of gut tissues: hematoxylin and eosin staining of gastric mucosa: **a** PBS, **b** wild type tomato, **c** GE tomato fed mice: sections showing jejunum villi **d** PBS, **e**

wild type tomato, **f** GE tomato fed mice. Sections showing liver sections **g** PBS, **h** wild type tomato, **i** GE tomato fed mice

toxicity and nutritional imbalance due to their consumption. Several studies have demonstrated that GE products are substantially equivalent with wild-type species (Li et al. 2007; Batista et al. 2005; Takagi et al. 2006). GE sweet pepper and tomato expressing coat protein gene of *Cucumber mosaic virus* (CMV) showed no detrimental effect on growth and development of mice (Chen et al. 2003). GE glyphosate tolerant soybean was equivalent to non-GE soybean in terms of composition and nutrition (Hammond et al. 1996). There was no significant abnormality in the mucosa of the small intestine of animals fed either with

genetically modified or non GE soybean (Teshima et al. 2000). GE mustard (*Brassica juncea*) expressing choline oxidase protein demonstrated allergenicity similar to wild-type mustard and showed no enhancement of IgE binding due to genetic modification (Singh et al. 2006). However, GE peas expressing bean α -amylase induced Th2 immune response and allergic lung damage in mice (Prescott et al. 2005). Such results require safety assessment of transgenic crops in comparison to wild-type species. In the present study, toxicity and allergenicity assessment of transgenic *L. esculentum* (English name: tomato) was carried out

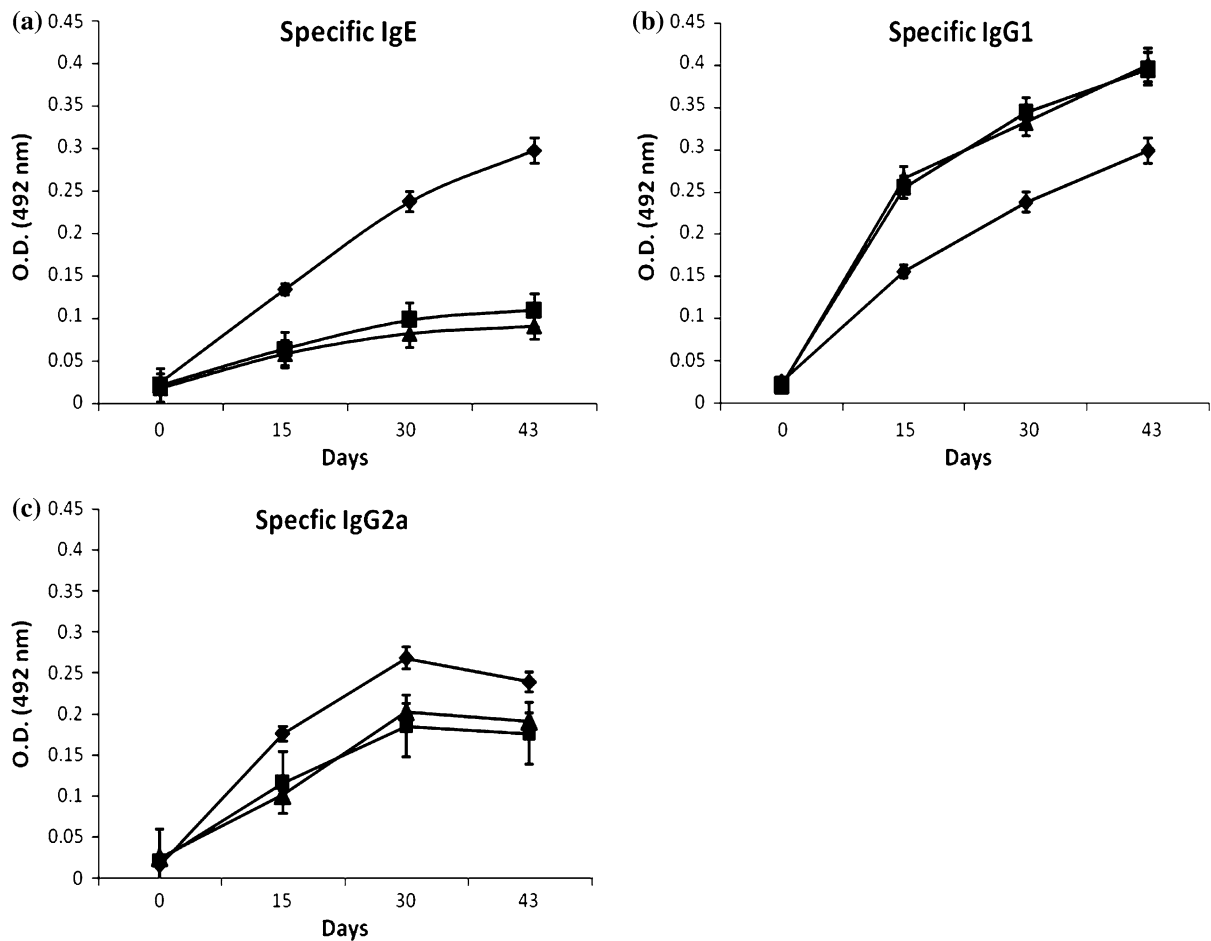


Fig. 3 Specific antibody response in Balb/C mice: serum antibody following i.p. administration of ovalbumin (◆), wild-type (■) and GE (▲) tomato protein extract. **a** Specific IgE; **b**

specific IgG1; **c** specific IgG2a. Pre-immune sera was taken as control and the values were subtracted before plotting. Data are reported as mean $\pm$ SE

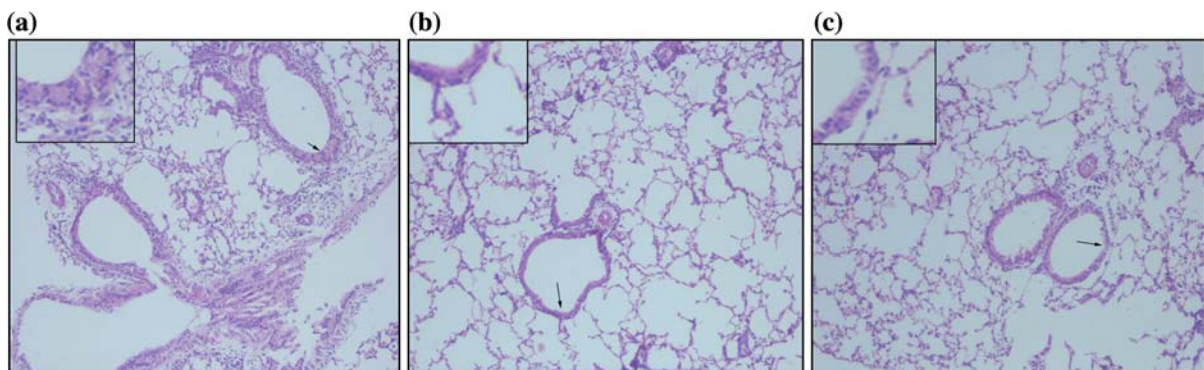


Fig. 4 Lung histology of mice: hematoxylin and eosin staining ($\times 10$) of lungs: **a** Ovalbumin fed mice, **b** wild-type tomato and **c** GE tomato fed mice. Black arrow showed infiltration of eosinophils in airway cavity and magnified at $\times 40$ as seen in the box

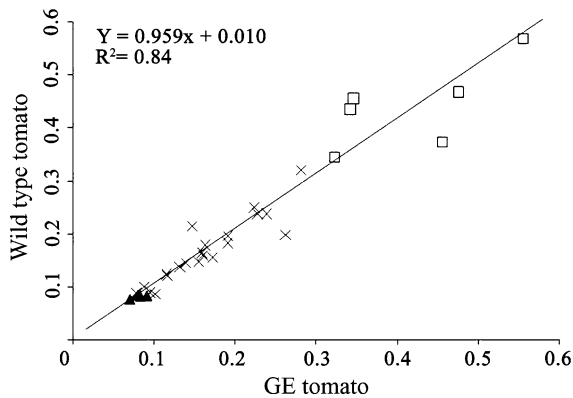


Fig. 5 Evaluation of IgE binding: relationship between IgE binding of wild-type and genetically engineered (GE) tomato protein by ELISA. IgE reactivity of GE and wild type tomato extract showed a linear regression with normal human sera (▲); tomato positive patients' sera (□) and other food allergic patients' sera (×)

Table 2 Clinical characteristics of patients showing IgE binding with GE and wild-type tomato extracts than controls

Patient No.	Age/sex	Symptoms	Specific IgE (OD)	
			Wild-type	GE
1	17/M	AR	0.34	0.32
2	32/F	AR/BA	0.41	0.36
3	23/F	AR/BA	0.43	0.37
4	37/M	AR	0.36	0.43
5	28/M	BA	0.45	0.47
6	33/F	AR/BA	0.56	0.57
7*	18/F	NHS	0.05	0.06
8*	24/M	NHS	0.06	0.07
9*	27/F	NHS	0.05	0.07

Patient No. 1–6: tomato positive patients' sera; of the 40 patients sera tested, six showed ≥ 3 times IgE binding than control by ELISA

M male, F female, AR allergic rhinitis, BA bronchial asthma

* NHS: normal human sera (control)

using Balb/c mice and food allergic patients' sera. Antisense construct (siRNA) chosen for the silencing of leaf curl virus showed no property to silence other mouse or human gene (Praveen et al. 2007).

For comprehensive safety assessment of GE tomato, the animal feeding experiments were conducted with respect to toxicity and metabolism. In an earlier study, Schröder et al. (2007) showed that feeding rats with GE rice expressing *cry 1* Ab protein showed no adverse effect. Transgenic maize grain fed

to rats showed no clinical sign of toxicity (Malley et al. 2007). However, mice fed with genetically engineered potato expressing *cry 1* gene showed mild adverse changes in the ileac compartment as compared to the control group (Fares and El-Sayed 1998). In the present study, Balb/c mice fed with GE or wild-type tomato extract showed no significant difference ($P > 0.05$) in body weight (Fig. 1) and food consumption. Hematology and blood biochemical indices in both the groups of test animals were similar to control mice (Table 1). GE and wild-type tomato fed mice demonstrated normal appearance of gut tissue with no infiltration of inflammatory cells. Liver sections showed normal appearance of hepatic cells similar to control mice (Fig. 2). It demonstrates that consumption of GE tomato has no detrimental effect on the growth and metabolic functions of mice.

Bioinformatics analysis offers initial prediction about allergenic nature of proteins. There is no previous report showing that antisense construct make functional proteins. However, to check for any possibility, the computational analysis of translated protein of antisense sequence in all the three reading frames was performed. The translated protein showed no significant homology with allergens listed in the SDAP or Farp databases. The six consecutive aminoacid homology for epitope matching revealed no significant match indicating absence of cross-reactive epitope.

The intrinsic property of allergenic protein is to induce immunoglobulin E production. Thus assessment of the immunoglobulin levels after antigen administration allows determination of allergenic potential. Gizzarelli et al. (2006) showed that sensitization with both GE and wild-type soybean extracts induced high levels of antigen-specific IgE, IgG1 and low levels of specific IgG2a in Balb/c mice. Singh et al. (2006) observed comparable immune response (IgE and IgG1) for transgenic and wild-type mustard in Balb/c mice. In the present study, GE and wild-type tomato protein extracts showed no significant difference in IgE response in Balb/c mice (Fig. 3). Spleen cells culture supernatant showed comparable IL-4 levels in both GE and wild-type tomato sensitized mice. IFN- γ levels were also comparable in GE and wild-type tomato sensitized mice ($P > 0.05$). Pre-sensitized mice challenged with GE or wild-type tomato extracts showed no signs of anaphylaxis and no enhancement of the Th2 cytokines. Singh et al.

(2006) also reported that pre-sensitized mice with GE and/or wild-type mustard on challenge with the same proteins showed no visible signs of anaphylaxis. Lung histology of sensitized mice further explained that exposure to GE or wild-type tomato protein via the intraperitoneal route did not induce airway remodeling whereas sensitization with ovalbumin increased eosinophils infiltration and caused airway constriction (Fig. 4).

The potential allergenicity of transgenic plants can be determined to a reasonable degree using specific screening with sera of subjects allergic to the source of gene (Taylor 2006). Previously, analysis of the IgE response to GE maize and soya demonstrated that none of the individuals reacted differently than their wild type species (Batista et al. 2005). IgE sensitization rate to GE soybean extract was identical to that of wild-type soybean extract in allergy patients by skin prick test, ELISA, or IgE immunoblot (Kim et al. 2006). In the present study, Specific IgE values obtained against GE and wild-type tomato proteins with food allergic patients' sera showed no variation ($P > 0.05$; Table 2; Fig. 5).

Studies suggest that no single criterion is sufficient for assessing the consequences after consumption of GE crop products. Hence, several different tests continue to be used, as a weight-of-the-evidence approach. In the present study, genetically engineered tomato with replicase gene was assessed along with wild-type tomato species for toxicity and allergenicity in Balb/c mice and screening with patients' sera. The results suggest that genetically engineered tomato with replicase gene is safe with respect to toxicity and allergenicity.

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