

Original article

Allergenicity assessment of transgenic mustard (*Brassica juncea*) expressing bacterial *codA* gene

Background: Assessing the allergenicity and toxicity of genetically modified (GM) crops is essential before they become a regular part of our food supply. The present study aimed to assess the allergenicity of *Brassica juncea* (mustard) expressing choline oxidase (*codA*) gene from *Arthrobacter globiformis* that provides resistance against abiotic stresses.

Methods: SDAP, Farrp, and Swiss-Prot databases were used to study allergenicity of choline oxidase. Digestibility of choline oxidase was assessed in simulated gastric fluid (SGF). Specific immunoglobulin E (IgE) reactivity of native and GM mustard was compared by using enzyme-linked immunosorbent assay (ELISA) and skin tests in respiratory-allergic patients. Allergenicity of GM and native mustard proteins was compared in Balb/c mice.

Results: Choline oxidase showed no significant homology with allergenic proteins in SDAP and Farrp databases. Cross-reactive epitope search showed a stretch similar to Hev b 6 having some antigenic properties. Purified choline oxidase showed complete degradation with SGF. Skin prick test of native and GM mustard extract on respiratory allergic patients showed significant correlation ($P < 0.05$). ELISA with 96 patients' sera showed comparable IgE reactivity. Balb/c mice immunized with native and GM mustard proteins showed low IgE response. Presensitized mice on intravenous challenge with *Brassica* extract showed no anaphylactic symptoms unlike ovalbumin (OVA) sensitization that showed anaphylactic reaction in mice. Lung histology of OVA-sensitized mice showed narrowing of airway and large eosinophilic infiltration, whereas native and GM *Brassica* extract showed normal airway.

Conclusion: Genetically modified mustard with the *codA* gene possessed allergenicity similar to that of native mustard and no enhancement of IgE binding was observed due to genetic manipulation.

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Agricultural biotechnology has potential to improve the food supplies required to feed the growing world population. The classical breeding method, where large amount of DNA is transferred, is replaced by the use of biotechnological approach and a specific trait is introduced into the host. Major traits being introduced in crops are improved tolerance to pests (1), herbicides (2, 3), drought (4), salt (5) and nutritional quality (6). Genetically modified (GM) crops can also be exploited for development of oral vaccines (7). However, critics have raised concern about the safety, allergenicity and toxicity of these crops. There are very few studies which provide information about allergenic potential of GM crops. FAO/WHO/OECD has proposed certain guidelines for safety and allergenicity evaluation of GM crops

(8, 9). These guidelines recommends bioinformatics approach, digestibility studies, animal model, specific sera screening, etc. to study allergenicity of GM crops (10). However, the animal models are scarce to predict allergenic potential of GM crops.

The incidence of food allergy is 1–2% in adults and 6–8% in children (11). Food induced anaphylaxis is reported in 3/100 000 individual per year (12). Food allergens are mostly proteins which may be modified upon processing. GM foods likely to be introduced might have enhanced allergenicity due to the presence of cross-reactive epitopes (13). GM soybean containing Brazil nut allergen has improved protein content but retained its allergenicity (14). GM corn with *cry9c* gene has allergenic properties but no immunoglobulin E (IgE) was detected in patients' sera (15). Therefore, extensive investigations are required for allergenicity assessment of GM food.

Mustard is a worldwide crop and leaves/seeds are consumed in various food forms. Mustard containing

Abbreviations: *codA*, choline oxidase gene; GM, genetically modified; PBS, phosphate-buffered saline; SGF, simulated gastric fluid; SPT, skin prick test.

choline oxidase provides abiotic stress tolerance in plants by introducing glycinebetaine pathway and was expressed in leaves/seeds. Transgenic *Brassica juncea* expressing choline oxidase gene from *Arthrobacter globiformis* was evaluated for allergenicity using bioinformatic approach, *in vitro* digestibility, immuno screening and an ovalbumin (OVA) animal model.

Materials and methods

Sequence homology of choline oxidase

Sequence homology study was done using Structural Database of Allergenic Proteins (SDAP) and Food Allergy Resources and Research Program (Farrp) to identify any allergen presenting a 35% amino acid identity through a window of 80 amino acids with choline oxidase. Swiss-Prot database was used to study cross-reactive epitopes in a six-amino acid match according to FAO/WHO (2001) guidelines.

Digestibility studies

The digestibility of the purified choline oxidase protein produced from *A. globiformis* was examined, as described by Astwood et al., with slight modifications (16). Briefly, choline oxidase protein (400 µg) was dissolved in 400 µl of simulated gastric fluid [SGF: 0.32% w/v pepsin, 0.03 M NaCl, pH 1.2]. Digestion proceeded at 37°C with continuous rocking, and an aliquot (30 µl) was periodically withdrawn. The reaction was completed by adding 26 µl of sample buffer (containing 2% 2-mercaptoethanol and 4% sodium dodecyl sulphate (SDS) together with Na₂CO₃ (200 mM). The mixture was then boiled for 5 min and loaded onto 12% SDS-polyacrylamide gel electrophoresis (PAGE). Resolved proteins were silver stained (17) or Western blotted (18).

Plant material and protein extraction

Leaves of *B. juncea* expressing *codA* gene isolated from *A. globiformis* and native species were used for the present study (19). Leaves were frozen in liquid nitrogen, crushed to a fine powder and defatted with repeated changes of diethyl ether. Proteins were extracted in 50 mM ammonium bicarbonate buffer (pH 8.0) containing 2 mM EDTA and 1 mM phenyl methyl sulfonyl fluoride for 6 h at 4°C. The supernatant was centrifuged at 12 000 g for 30 min at 4°C, lyophilized, and stored.

Immunoblot

Genetically modified and native mustard proteins were resolved onto 12% SDS-PAGE. Resolved proteins were electrophoretically transferred onto nitrocellulose membrane, blocked with defatted milk, and incubated with antibodies raised in rabbit against choline oxidase (1:1000 v/v) and after washing with phosphate-buffered saline (PBS) further incubated with anti-rabbit IgG-horse radish peroxidase (HRP) (1:1000 v/v) and developed (18).

Skin prick tests and sera collection

Skin prick tests (SPT) were performed with GM and native mustard on 96 (61 males and 35 females) allergy patients aged 15–45 at the outpatient department (OPD), V.P. Chest Institute, Delhi. It is a

referral chest hospital and receives patients from different parts of the country for diagnosis and treatment of allergy and asthma. For the present study, the diagnosis of asthma was ascertained in cases following the guideline of the American Thoracic Society (20). The patients having any two of the symptoms, viz. sneezing, rhinorrhea, nasal blockage, postnasal drip, etc. were diagnosed as having rhinitis (21).

For SPT, a drop of the glycerinated extract (50%) was placed on the volar aspect of the forearm, pricked with a sterile needle and skin was slightly raised to allow antigen to enter. The reactions were graded after 20 min in comparison with weal size of positive control, i.e. histamine diphosphate (5 mg/ml). Weal diameter equal to positive control or more (>3 mm) were considered as marked positive skin reactions (22). Sera were collected from SPT-positive patients and normal healthy volunteers ($n = 10$) for immunoassays with GM and native mustard. Skin tests and sera collection were carried out with the patient's consent and the study protocol was approved by the Human Ethics Committee of the institute.

Specific IgE estimation

Specific IgE levels against GM and native mustard was estimated by using enzyme-linked immunosorbent assay (ELISA) (23). Briefly, microtiter plates (Maxisorp; NuncTM Immunomodule, Roskilde, Denmark) were coated with 1 µg protein in 100 µl per well in carbonate buffer (pH 9.6). Nonspecific sites were blocked with 3% defatted milk, washed, and incubated with the serum of the individual patient (1:10 v/v). The serum from nonallergic individuals was tested as negative controls. After washing, the plates were incubated for 2 h with anti-human IgE-HRP (1:1000 v/v; Sigma Chemical Co., St Louis, MO, USA) in PBS. Color was developed and the absorbance was read at 492 nm.

Animal studies

Six-week-old Balb/c mice were randomly divided into seven groups of six mice each. Group 1 mice were fed daily with PBS. Groups 2 and 3 were given OVA by the oral and intraperitoneal route, respectively. Groups 4 and 5 were given native mustard protein and group 6 and 7 were given GM mustard protein via the oral and intraperitoneal route, respectively. Mice were fed orally with 100 µg protein in 100 µl PBS daily for 42 days or injected i.p. with 100 µg protein in 100 µl PBS once a week for 7 weeks. Blood samples were collected from tail vein to measure serum IgE, IgG1 and IgG2a antibodies at different time intervals. Two mice in each group were challenged i.v. on day 60 with 3, 6 and 12 mg of OVA, GM or native protein in PBS and the symptoms were scored (24).

Challenged mice in each group were killed. Lungs were taken out for histology and fixed in 10% formalin in PBS. The sections (2–3 µm) were cut, stained with hematoxylin and eosin, and visualized by using light microscopy for antigen-induced peribronchial and perivascular inflammation. Animal study protocol was cleared by the institute's Ethics Committee and approved by the Biosafety Committee for handling of GM food material.

Measurement of OVA/native/GM-specific antibodies

Serum antibodies specific for OVA, native, or GM protein were measured by using ELISA (23). Briefly, the microtiter plates (Nunc) were coated with 250 ng of OVA or with 1 µg of native or GM mustard proteins in carbonate buffer (pH 9.6). The plates were incubated with mice sera for IgE estimation (1:10), IgG1 and IgG2a

(1:500). After washing, rat antimouse IgE (1:1000; Bethyl Laboratories, Montgomery, TX, USA), rat antimouse IgG1 (1:1000; BD Pharmingen, San Diego, CA, USA), rat antimouse IgG2a (1:1000; BD Pharmingen) in PBS were added and developed.

Statistical analysis

Specific IgE levels against GM and native mustard in mice and food-hypersensitive patient's sera were compared by using the paired *t*-test. Skin prick test results were compared by Wilcoxon test. $P < 0.05$ was considered as statistically significant.

Results

Amino acid sequence homology

Choline oxidase sequence was compared with the allergenic protein databases. The SDAP database showed maximum similarity of 8% with *Candida albicans* allergen Cand a 1 (25). As per FAO/WHO 2001 guidelines, 35% or more sequence similarity can be considered as allergenic. In the Farrp allergen database, choline oxidase demonstrated an *e*-value of 0.49 with glycinin allergen (26), which was higher than the significant value of 0.02 for two homologous proteins. Cross-reactive epitopes were studied for six consecutive amino acid homology with known allergens using the Swissprot database. Choline oxidase sequence exhibited one stretch of six amino acids identical to that of latex allergen Hev b 6. This stretch showed no antigenic index in choline oxidase when the DNA Star software was used and falls under hydrophobic region. In addition, the stretch showed three cleavage sites for proteinase K and thermolysin.

Genetically modified mustard expressing *codA* gene

The expression of the integrated *codA* gene was confirmed in GM mustard leaves by Western blot with antibodies raised in rabbit against purified choline oxidase. No such immunoreactive polypeptide was observed in native mustard leaf extract (Fig. 1).

SGF digestibility

The purified choline oxidase protein was completely digested by SGF within 5 s. Figure 2A shows the SDS-PAGE of the choline oxidase protein digestion. No protein fragment was detected in the silver-stained gel after 5 s. Immunoblotting with antibodies raised in rabbit against choline oxidase showed no fragment after 5 s of SGF digestion (Fig. 2B).

In vivo and *in vitro* IgE reactivity

Skin prick tests were carried out with GM and native *B. juncea* leaf extracts on 96 respiratory allergy patients with a history of sensitization to inhalant and food

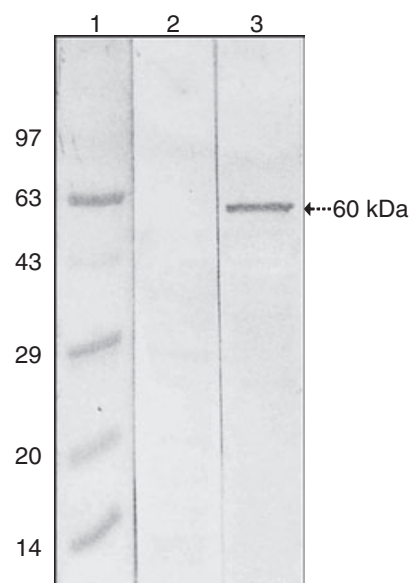


Figure 1. Immunoblot analysis of choline oxidase: soluble fractions of leaf protein extract immunoblotted with choline oxidase antibodies raised in rabbit. Lane 1: marker; lane 2: native mustard; lane 3: GM mustard.

allergens. Of these, six patients showed marked positive skin reactions to GM and native mustard extracts (Table 1).

Table 1 shows the specific IgE value for native and GM proteins in sera of mustard-positive patients. Ten patients show specific IgE levels, 2.5–3 times of negative control, exhibiting comparable value against GM and native proteins.

Immune response to OVA/native/GM protein

Oral sensitization with GM and native proteins showed comparable IgE response in Balb/c mice (Fig. 3A). Both the proteins elicited low IgE response on day 15 and it continued till day 59. In contrast, OVA stimulated high IgE titer in mice on day 15, which further increased on day 43. Intraperitoneal sensitization with native and GM mustard protein showed low IgE level on day 15 that remained unchanged till day 59. OVA immunization showed high allergenic response through the intraperitoneal, compared with the oral, route (Fig. 3B).

Oral sensitization with native and GM proteins showed similar IgG1 response (Fig. 4A). Interestingly, on intraperitoneal sensitization, both native and GM mustard showed vigorous but comparable IgG1 response (Fig. 4B). Mice sensitized orally with native and GM mustard showed an early IgG2a response by day 15, which further increased on day 43 but reduced considerably by day 59 (Fig. 4C). On intraperitoneal sensitization, both the native and GM mustard showed high IgG2a

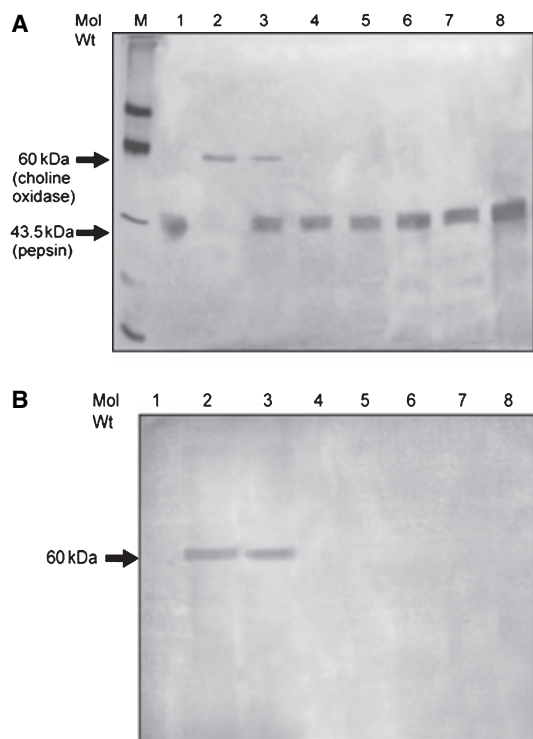


Figure 2. Simulated gastric fluid (SGF) degradation of choline oxidase: choline oxidase protein incubated in SGF for 0, 5, 30, 60, 120, and 300 s. After electrophoresis, the proteins that were separated were visualized by (A) silver staining and (B) Western blot analysis with antibodies raised in rabbit against choline oxidase protein. Lane 1: pepsin; lane 2: choline oxidase pure; lanes 3–8: choline oxidase + SGF for 0, 5, 30, 60, 120, and 300 s.

Table 1. Clinical and immunologic analysis of GM and native mustard on respiratory allergic patients

Patient no.	Age/sex	Symptoms	SPT		Sp. IgE (OD)	
			Native	GM	Native	GM
1*	16/F	AR/A	2+	2+	0.322	0.313
2*	29/M	AR/A	2+	2+	0.257	0.259
3*	26/F	AR/A	2+	2+	0.303	0.316
4*	29/F	AR/A	2+	1+	0.189	0.196
5*	41/M	AR/A	2+	2+	0.222	0.235
6*	17/M	A	2+	2+	0.270	0.254
7	33/M	AR/A	–	–	0.228	0.213
8	17/M	AR/A	–	–	0.289	0.303
9	43/M	A	–	–	0.156	0.161
10	22/M	A	–	–	0.313	0.323
11	19/F	AR/A	–	–	0.298	0.306
12	26/M	AR/A	–	–	0.258	0.237
13	37/M	AR	–	–	0.263	0.254
14	31/M	AR	–	–	0.265	0.271
15	26/F	AR/A	–	–	0.258	0.237

M, male; F, female; sp. IgE, specific immunoglobulin E; SPT, skin prick test; A, asthma; AR, allergic rhinitis; GM, genetically modified. Patient no. 7–15: mustard-positive patients.

*Patients showing positive SPT to native and GM mustard leaf extract.

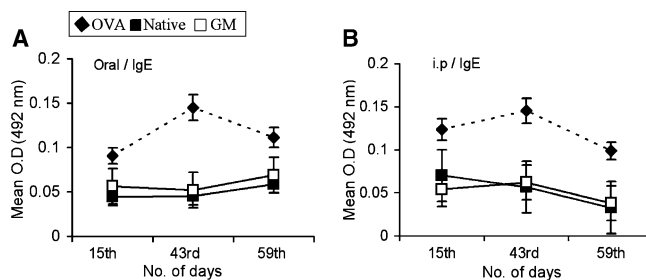


Figure 3. Specific IgE response in Balb/C mice: serum antibody following administration of ovalbumin, native and GM mustard proteins (A) orally and (B) intraperitoneally. Data are reported as mean $\pm$ SE.

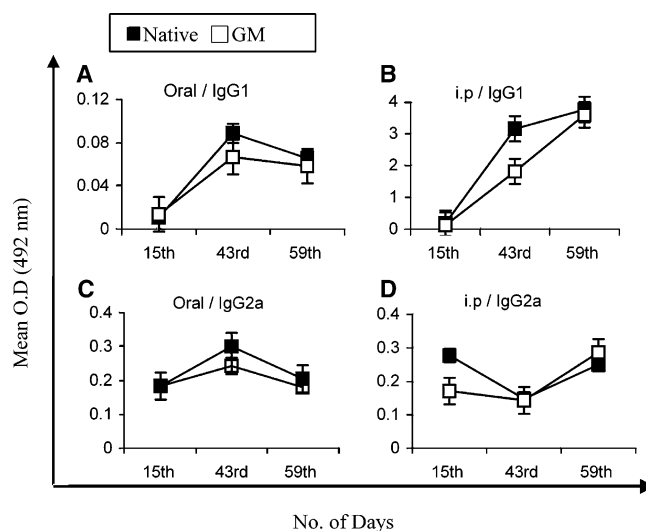


Figure 4. Immunogenic response in Balb/c mice: serum antibody following administration of native and GM mustard protein. Specific IgG1 response in (A) oral and (B) intraperitoneal routes. Specific IgG2a response in (C) oral and (D) intraperitoneal administration. Data are reported as mean $\pm$ SE.

level by day 15 that reduced by day 43. However, on day 59, there was some increase in IgG2a response (Fig. 4D). No significant difference was observed between the IgG1 and IgG2a antibody response induced by native and GM mustard proteins by oral and intraperitoneal routes.

Antigen challenge of sensitized mice

Oral and intraperitoneally sensitized mice were challenged intravenously with different doses of antigen. Native and GM mustard protein challenge did not show anaphylactic response in both oral and intraperitoneally sensitized mice even with a dose of 12 mg antigen. Mice challenged with OVA demonstrated anaphylactic response with low dose of 3 mg antigen. The severity of

reaction was more pronounced in intraperitoneal sensitization when compared with oral challenge in mice (Table 2).

Histology of lungs

Ovalbumin (oral) sensitized and challenged mice lung showed perivascular and peribronchial inflammatory cell infiltrate, with loss of normal lung structure (Fig. 5A). Lungs from intraperitoneally sensitized/challenged mice were severely congested, with disruption of alveolar structure and a profound cellular infiltration

Table 2. Presensitized mice challenged with different doses of protein

Mice no./protein dosage (mg)	OVA		Native mustard		GM mustard	
	Oral	i.p.	Oral	i.p.	Oral	i.p.
1/12	5	5	0	0	1	0
2/12	4	5	1	0	0	0
3/6	4	5	0	0	0	0
4/6	5	4	0	0	0	0
5/3	3	5	0	0	0	0
6/3	4	4	0	0	0	0

Symptom 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; and 5: mortality (death).

throughout the tissue (Fig. 5B). In contrast, native and GM protein-sensitized/challenged mice showed normal lung structure with defined bronchi and alveoli, irrespective of the route of sensitization (Fig. 5C–F). There were no inflammatory cells in the lung tissue. The lungs of native and GM protein sensitized mice were similar to that of control mice, sensitized with PBS (Fig. 5G).

Discussion

Genetically modified crops have reached the market and others are under development. This scenario necessitates the evaluation of safety and allergenicity of GM foods. GM food is assessed by genetic modification, safety analysis of new protein, occurrence and implication of unintended effects, gene transfer to gut micro flora, allergenicity of new protein, role of new food in the diet, influence of food processing, etc. (27). The present study was aimed to assess the allergenicity of transgenic mustard (*B. juncea*) expressing bacterial *codA* gene according to FAO/WHO guidelines. The *codA* gene has also been introduced in rice, tobacco, tomato, *Arabidopsis* (28–31), etc. but none of the plant products has been evaluated for allergenicity.

Bioinformatic analysis of choline oxidase sequence showed no homology with allergens listed in the SDAP and Farrp databases. Further studies identified cross-

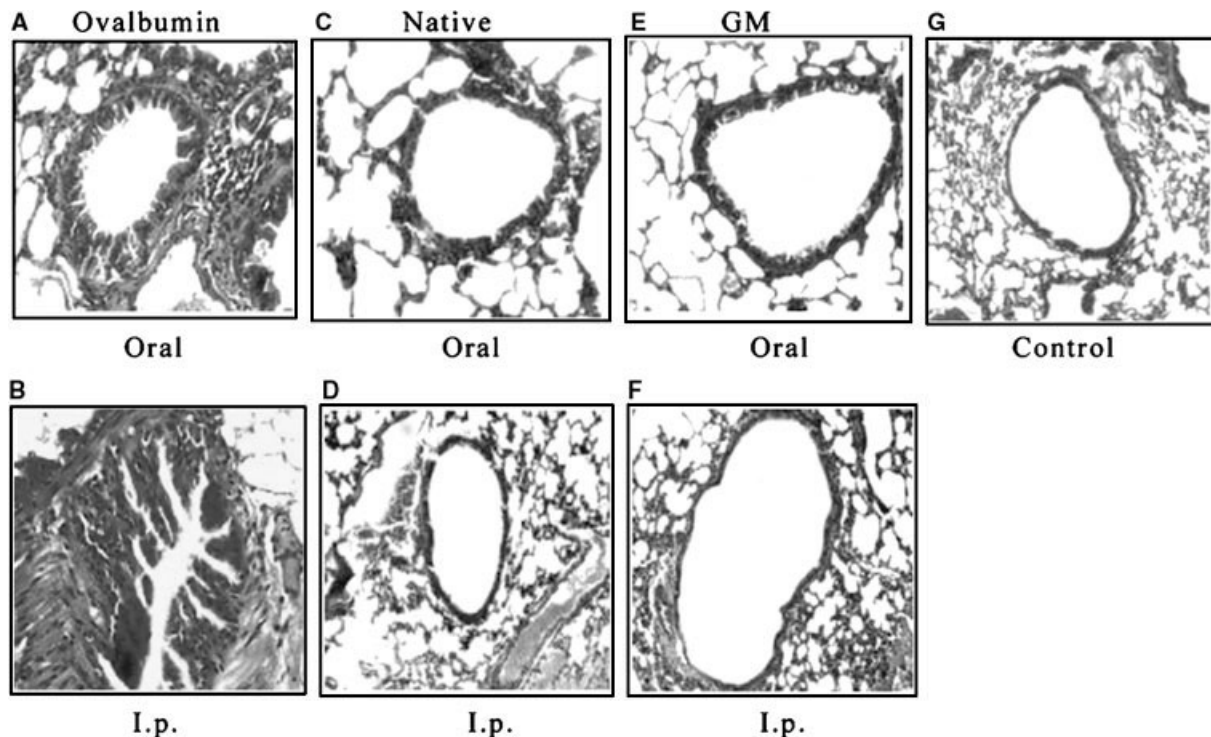


Figure 5. Lung histology of sensitized/challenged mice: H&E staining of lungs. (A) Ovalbumin oral administration, (B) ovalbumin intraperitoneal administration, (C) native mustard oral administration, (D) native mustard intraperitoneal administration, (E) GM mustard oral administration, (F) GM mustard intraperitoneal administration, and (G) PBS injected (control).

reactive epitope 'VGGGSA' similar to a latex allergen Hev b 6. This stretch is hydrophobic with less antigenicity in choline oxidase but more antigenicity in Hev b 6. Furthermore, the stretch was studied with 'peptide cutter' software showed three protease cleavable sites (<http://us.expasy.org/tools/peptidecutter>; accessed 29 December 2005). Thus the epitope seems to be susceptible to disruption by protease. Studies of structural biology of allergens have proposed that the α -helix region contains IgE-binding epitopes (32). The six amino acid stretch of choline oxidase falls in the β -sheet, indicating that its chances of being an epitopic region is low. Hev b 6 stretch falls under the β -coil region and this may not be epitopic region. An earlier study revealed that the six amino acid stretch analysis shows many random and irrelevant matches (33).

Food allergens are required to exhibit sufficient gastric stability to reach the intestinal mucosa where absorption and sensitization can occur. Many allergens such as milk β -lactoglobulin, peanut Ara h2, soybean β -conglycinin, etc. were stable to digestion with SGF (16). Purified choline oxidase protein was rapidly digested by SGF, thereby minimizing its potential for absorption by the intestinal mucosa and the chances of this to behave as an allergen are low.

Skin tests on allergic rhinitis and asthma patients with GM and native mustard extracts showed similar results ($P < 0.05$). Specific IgE values obtained with GM and native mustard in patients' sera show no variation ($P < 0.05$). Batista et al. monitored IgE response to GM maize and soya and showed that none of the individuals reacted differently (34).

The intrinsic property of allergenic protein is to induce IgE antibody production. In our study, OVA induced high IgE antibody response both with the oral and the intraperitoneal route in mice models. IgE antibody response with GM and native mustard was low and showed no significant difference ($P < 0.05$). Thus, both the extracts have similar allergenic property and intro-

duction of the *codA* gene did not enhance the IgE response of the GM mustard. Dearman et al. showed that intraperitoneal administration of OVA elicited allergenic response (35). Further, Knippels et al. demonstrated OVA allergenicity by oral administration (36).

Asthma is an immune inflammatory disease characterized by airway hyper-responsiveness. Inflammatory infiltrates are present in the bronchial walls containing eosinophils with elevated serum IgE levels (37). In the present study, presensitized mice with GM and native mustard on challenge with the same proteins showed no visible signs of anaphylaxis, but OVA induced severe anaphylaxis leading to mortality. Lungs histology further confirmed that exposure to GM and native mustard protein via the oral or the intraperitoneal route did not induce airway remodeling, whereas OVA increased eosinophils and caused airway constriction.

Avoidance of allergen(s) is the best therapeutic approach for food allergy. Therefore, testing of all new foods including GM crops should be made mandatory prior to release in the market. Researchers can think about hypoallergenic crops by silencing certain genes, epitope modification and/or alteration of secondary structure. The second-generation GM crops with reduced toxicity, allergenicity and no antibiotic resistance gene may be developed in the future to meet the growing food requirement.

In conclusion, both GM and native mustard showed same allergenicity with no enhancement in IgE binding due to genetic manipulation.

Acknowledgments

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References

1. Stewart CNJ, Adang MJ, All JN, Boerma HR, Cardineau G, Tucker D et al. Genetic transformation, recovery, and characterization of fertile soybean transgenic for a synthetic *Bacillus thuringiensis* cryIaC gene. *Plant Physiol* 1996;**112**:121–129.
2. Padgett SR, Kolacz KH, Delannay X, Re DB, LaVallee BJ, Tinius CN et al. Development, identification, and characterization of a glyphosate-tolerant soybean line. *Crop Sci* 1995;**35**:1451–1461.
3. Song JD, Lee MH, Chang HS, Lee CH. Quick screening of phosphinothricin-resistant transgenic tobacco plants using chlorophyll fluorescence. *Mol Cells* 1996;**6**:405–410.
4. Yeo ET, Kwon HB, Han SE, Lee JT, Ryu JC, Byun MO. Genetic engineering of drought resistant potato plants by introduction of the trehalose-6 phosphate synthase (*tsp1*) gene from *Saccharomyces cerevisiae*. *Mol Cells* 2000;**11**:263–268.
5. Jeong MJ, Park SC, Byun MO. Improvement of salt tolerance in transgenic plants by glyceraldehyde-3 phosphate dehydrogenase gene transfer. *Mol Cells* 2001;**12**:185–189.
6. Datta K, Baisakh N, Oliva N, Torrizo L, Abrigo E, Tan J et al. Bioengineered 'golden' indica rice cultivars with β -carotene metabolism in the endosperm with hygromycin and mannose selection systems. *Plant Biotechnol J* 2003;**1**:81–90.
7. Tacket CO. Plant-derived vaccines against diarrheal diseases. *Vaccine* 2005;**23**:1866–1869.

8. Evaluation of Allergenicity of Genetically Modified Foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, 22–25 January 2001. Rome: Food and Agriculture Organisation of the United Nations, 2001. <http://www.ftp://ftp.fao.org/es/esn/food/gmreport.pdf> [accessed on 6 June 2005].
9. Organization for Economic cooperation and Development. Report of the OECD Workshop on the Toxicological and Nutritional Testing of Novel Foods, Aussois, France, 5–8 March 1997. France: Organization for Economic cooperation and Development, 1998. [http://www.oilis.oecd.org/oils/1998doc.nsf/LinkTo/sg-icgb\(98\)1-final](http://www.oilis.oecd.org/oils/1998doc.nsf/LinkTo/sg-icgb(98)1-final) [accessed on 6 June 2005].
10. Lehrer SB, Bannon GA. Risks of allergic reactions to biotech proteins in foods: perception and reality. *Allergy* 2005;**60**:559–564.
11. Sampson HA. Update on food allergy. *J Allergy Clin Immunol* 2004;**113**:805–819.
12. Burks AW, Fuchs RL. Assessment of the endogenous allergens in glyphosate-tolerant and commercial soybean varieties. *J Allergy Clin Immunol* 1995;**96**:1008–1010.
13. Gavrovic-Jankulovic M, Cirkovic T, Burazer L, Vuckovic O, Jankov RM. IgE cross-reactivity between meadow fescue pollen and kiwi fruit in patients' sera with sensitivity to both extracts. *J Investig Allergol Clin Immunol* 2002;**12**:279–286.
14. Nordlee JA, Taylor SL, Townsend JA, Thomas LA, Bush RK. Identification of a Brazil-nut allergen in transgenic soybeans. *New Engl J Med* 1996;**334**:688–692.
15. Bucchini L, Goldman LR. Starlink corn: a risk analysis. *Environ Health Perspect* 2002;**110**:5–13.
16. Astwood JD, Leach JN, Fuchs RL. Stability of food allergens to digestion in vitro. *Nat Biotechnol* 1996;**14**:1269–1273.
17. Wayne W, Teni B, Virginia PW, Ronald H. Silver staining of proteins in polyacrylamide gels. *Anal Biochem* 1981;**118**:197–203.
18. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;**76**:4350–4354.
19. Prasad KVSK, Sharmila P, Pardha Saradhi P. Enhanced tolerance of transgenic *Brassica juncea* to choline confirms successful expression of the bacterial *codA* gene. *Plant Sci* 2000;**159**:233–242.
20. American Thoracic Society. Lung function testing: selection of reference values and interpretative strategies. *Am Rev Respir Dis* 1991;**144**:1202–1218.
21. Adomopoulos G, Monolopoulos L, Giotakis I. A comparison of the efficacy and the patient's acceptability of budesonide and beclomethasone dipropionate aqueous nasal sprays in patients with perennial rhinitis. *Clin Otolaryngol* 1995;**20**:340–344.
22. Aggarwal S, Chhabra SK, Saxena RK, Agarwal MK. Heterogeneity of immune responses to various *Aspergillus* species in patients with allergic respiratory diseases. *Indian J Chest Dis Allied Sci* 2000;**42**:249–258.
23. Voller A, Bidwel DE, Barlett A. Enzyme-linked immunosorbent assay. Manual of clinical immunology. Washington: American Society of Microbiology, 1980.
24. Li XM, Serebrisky D, Lee SY, Huang CK, Bardina L, Schofield BH et al. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol* 2000;**106**:150–158.
25. Shen HD, Choo KB, Yu KW, Ling WL, Chang FC, Han SH. Characterization of a monoclonal antibody (RJ5) against the immunodominant 41-kD antigen of *Candida albicans*. *Int Arch Allergy Appl Immunol* 1991;**96**:142–148.
26. Beardslee TA, Zeece MG, Sarath G, Markwell JP. Soybean glycinin G1 acidic chain shares IgE epitopes with peanut allergen Ara h 3. *Int Arch Allergy Immunol* 2000;**123**:299–307.
27. Kuiper HA, Kleter GA, Noteborn HPJM, Kok EJ. Assessment of the food safety issues related to genetically modified foods. *Plant J* 2001;**27**:503–528.
28. Sakamoto A, Alia, Murata N. Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. *Plant Mol Biol* 1998;**38**:1011–1019.
29. Parvanova D, Ivanov S, Konstantinova T, Karanov E, Atanassov A, Tsvetkov T et al. Transgenic tobacco plants accumulating osmolytes show reduced oxidative damage under freezing stress. *Plant Physiol Biochem* 2004;**42**:57–63.
30. Park EJ, Jeknic Z, Sakamoto A, DeNoma J, Yuwansiri R, Murata N et al. Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. *Plant J* 2004;**40**:474–487.
31. Sakamoto A, Valverde R, Alia, Chen TH, Murata N. Transformation of *Ara-bidopsis* with the *codA* gene for choline oxidase enhances freezing tolerance of plants. *Plant J* 2000;**22**:449–453.
32. Karisola P, Mikkola J, Kalkkinen N, Airenne KJ, Laitinen OH, Repo S et al. Construction of Hevein (Hev b 6.02) with reduced allergenicity for immunotherapy of latex allergy by commutation of six amino acid residues on the conformational IgE epitopes. *J Immunol* 2004;**172**:2621–2628.
33. Hileman RE, Silvanovich A, Goodman RE, Rice EA, Holleschak G, Astwood JD et al. Bioinformatic methods for allergenicity assessment using a comprehensive allergen database. *Int Arch Allergy Immunol* 2002;**128**:280–291.
34. Batista R, Nunes B, Carmo M, Cardoso C, Jose HS, de Almeida AB et al. Lack of detectable allergenicity of transgenic maize and soya samples. *J Allergy Clin Immunol* 2005;**116**:403–410.
35. Dearman R, Kimber I. Determination of protein allergenicity: studies in mice. *Toxicol Lett* 2001;**120**:181–186.
36. Knippels LM, Houben GF, Spanhaak S, Penninks AH. An oral sensitization model in Brown Norway rats to screen for potential allergenicity of food proteins. *Methods* 1999;**19**:78–82.
37. Holt PG, Macaubas C, Stumbles PA, Sly PD. The role of allergy in the development of asthma. *Nature* 1999;**402**:B12.