

Allergenicity Assessment of Genetically-modified Tobacco Expressing Salt Tolerance *cbl* Gene

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Abstract It is mandatory to assess the allergenic potential of genetically modified (GM) crops before their commercialization. Recently, a transgene [Calcineurin B-like (CBL) protein] has been introduced into tobacco plant to make the crop salt resistance. Therefore, it was felt necessary to assess the allergenic potential of the *cbl* gene product, which was introduced and expressed in *Nicotiana tabacum* (tobacco) plant and compared the allergenic effects with the wild-type (WT) counterpart. Bioinformatic analysis revealed that there was no significant sequence homology with known allergens. Also, no difference between the protein digestibility profiles of GM and WT tobacco was found. Rapid digestion of CBL protein (Mol Wt 35 kDa) by simulated gastric fluid (SGF) indicated reduced chances of this protein to induce allergenicity. In addition, BALB/c mice sensitized by intraperitoneal administration of WT and GM tobacco protein showed comparable levels of clinical score, specific IgE, IgG1, histamine level, similar effect on different organs as well as IgE binding

proteins. These findings indicate that insertion of *cbl* gene in tobacco did not cause any additional allergic risk to consumer and the GM and native tobacco proteins behave similarly in both *in vitro* and *in vivo* situations even after genetic modification.

Keywords Allergy · GM crops · *cbl* gene · Simulated gastric fluid · Murine model

Abbreviations

Mol Wt	Molecular weight
WT	Wild type
CBL	Calcineurin B-like
GM	Genetically modified
SGF	Simulated gastric fluid

Introduction

Tobacco is one of the important commercial agricultural products processed from the leaves of plants in the genus *Nicotiana*. Tobacco has been used in many forms such as organic pesticides and as a topical treatment on insect stings inspite of being a controlled substance [1]. In recent years, many transgenic varieties of tobacco are being developed to increase its productivity. These transgenic plants offer improved agronomic characteristics and enhanced consumer benefits.

Drought and salinity are the critical abiotic environmental factors having major impact on the development and productivity of plants, leading to serious agricultural yield losses [2]. Excess salinity, however, is becoming a major problem to crop production throughout the world. The deleterious effects of salt stress is the consequence of global climate changes and inappropriate irrigation practices such as increased use of salt-

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rich water and/or insufficient drainage, use of fertilizers and soil salinization, etc. [3].

It is now possible to develop salt-tolerant plants using genetic modification to increase productivity in salt rich soils and this approach may subsequently contribute to enhance the economy of the country. This approach will become more powerful as more candidate genes associated with salinity tolerance are identified and widely utilized [3, 4]. Recently, a *cbl* gene from a pea plant was inserted into tobacco plant to provide salt tolerance properties. It is largely accepted that calcium (Ca^{2+}) plays a crucial role as a second messenger to respond to various types of stimuli including abiotic stresses [5–7]. Calcium sensors transmit signals to downstream signaling. A new family of calcium sensors has been identified in plants known as calcineurin B-like proteins (CBLs) [8, 9]. Four elongation factor domains are identified in CBL proteins for Ca^{2+} binding, and they also interact with another group designated as CBL-interacting protein kinases (CIPKs), which are basically Serine/Threonine protein kinases [10–12]. Thus, the CBL family represents a unique group of calcium sensors in plants that interact with and regulate protein kinase family CIPK by decoding calcium signals. Before release for public consumption in the market, it is mandatory that the GM crops may be assessed for safety issues [13, 14]. Therefore, the aim of the present study was to investigate the safety including allergenicity of the transgenic tobacco in comparison to native ones using bioinformatic tools, physico-chemical parameters and *in vivo* allergenicity assessment methods.

Materials and Methods

Preparation of Crude Protein Extract (CPE)

Leaves of GM tobacco and wild-type (WT) tobacco were provided by International Centre for Genetic Engineering and Biotechnology (ICGEB) New Delhi, India. Proteins from the WT and GM tobacco leaves were extracted with a P-PER kit (Thermo Scientific, Rockford, USA) as per manufacturers' instructions.

Bioinformatics Analysis of *cbl* Gene

Sequence homology analysis was performed for the *cbl* gene using AllergenOnline, Algpred and Allergen Database for Food Safety (ADFS) software (Supplementary information S1.1).

Protein Profiling of GM and WT Tobacco

Proteins of GM and WT tobacco leaves (CPE) were resolved via SDS-PAGE as well as 2D-gel electrophoresis [15] (Supplementary information S1.2).

Simulated Gastric Fluid (SGF) Assay of GM and WT Tobacco Protein

Pepsin-resistant proteins in tobacco were identified by SGF assay. Tobacco CPE was digested with SGF [6.4 mg pepsin was dissolved in 1 ml of 34 mM NaCl, 0.7 % HCl (pH 2.0)] at different time points (0.25, 4, 15, 60 min) (Supplementary information S1.3).

Stability of *cbl* Gene Product in SGF

Expression of the *cbl* gene in GM tobacco leaves was analyzed by Western blot analysis using antibodies raised in rabbit against CBL protein only (Supplementary Information S1.4).

In Vivo Treatments

Female Balb/c mice (6–8 weeks of age, 20 ± 3 g) were procured from the animal breeding colony of CSIR-Indian Institute of Toxicology Research, Lucknow, India. This study was carried out after the approval of the Institutional Ethics Committee (ITRC/IAEC/07/2009). Peanut protein was chosen as the positive control as the allergenicity of peanut protein is well established [16]. Detail of animal treatment is provided in Supplementary information S1.5 and Supplementary Fig. S1. Mice were sensitized according to earlier described method [17].

Systemic Anaphylaxis Score

Anaphylactic reactions were scored using method previously described by Li et al. (1999) [17]. The grading of clinical score was done on scale 1–5 (Supplementary information S1.6).

IgE Immunoblotting and Measures of Specific IgE and IgG₁ Antibody

Immunoblotting was performed to detect allergenic proteins in CPE by analyzing their IgE binding affinity using the sera that was obtained from GM and WT tobacco-treated mice using the method described by Misra et al. [18].

Specific IgE (1:20 dilution) and IgG₁ (1:1000 dilution) levels in response to GM and WT tobacco CPE treated mice were estimated using enzyme-linked immunosorbent assays (ELISA) according to the method of Misra et al. [19].

Histamine Release Assay

Plasma histamine levels were determined using an immunoassay kit (SPI-BIO, Montigny le Bretonneux, France) according to manufacturers' protocols.

Histopathology

The lungs, intestine and spleen taken at necropsy were fixed in 10 % formalin. Sections (3–5- μ m thick) were then stained with hematoxylin and eosin and examined using a light microscope (Leica Microscope, Tokyo, Japan).

Statistical Analysis

All data are expressed as mean $\pm$ SEM and analyzed using Instat version 3.0 software (Graphpad, San Diego, CA, USA; <http://www.graphpad.com>). Differences between values were compared using one-way ANOVA and a Bonferroni *post-hoc* test.

Results and Discussion

International regulatory agencies suggest that all the GM crops must be assessed for their safety including allergenic potential before their release into the market. Therefore, the aim of the present study was to assess the allergenicity of GM tobacco expressing *cbl* gene using bioinformatic *in vitro* and *in vivo* approaches to ensure the safety for the consumer population.

In silico evaluation of protein allergenicity by bioinformatics method is considered a primary criterion to predict the possible allergenicity of inserted protein. AllergenOnline, Algpred as well as ADFS softwares were used for allergenicity prediction. Bioinformatics analysis of inserted *cbl* gene did not show sequence homology with any known allergen. Search for full-length alignments (e value=0.001) by FASTA revealed matches with two known allergens. When e value cut off was set at zero, no matching with any known allergen was found that indicated very low probability of inducing allergenicity by *cbl* gene (Supplementary

Table S1). Three parameters of Algpred software, *i.e.*, IgE epitope mapping, MAST result, and allergen representative peptide (ARP), CBL protein did not qualify as an allergen. However, SVM module, which is based on amino-acid and dipeptide composition, CBL protein was found as an allergen. A combination of above parameters, *i.e.*, hybrid approach of Algpred Software indicated that the *cbl* gene product may or may not be allergenic. Moreover, a motif-based method used for allergen prediction (through ADFS software) indicated a non-allergic nature of the *cbl* gene product (Supplementary Table S1). When using these tools only, it was difficult to conclude about the nature of *cbl* gene. To confirm these findings, further experiments were required. Therefore, physico-chemical and *in vivo* analysis were performed.

To observe any difference in the protein profile of GM and WT tobacco CPE, we analyzed GM and WT tobacco proteins by SDS-PAGE and 2D electrophoresis. Protein profiles of both groups were found similar on SDS-PAGE whereas some extra protein spots were observed in GM tobacco CPE upon 2D gel electrophoresis (Fig. 1a and b). These spots may be of *cbl* gene product present in the GM tobacco.

On comparing the pepsin digestibility profile of both GM and WT tobacco protein, it was found that the profiling of both were very similar or comparable on SDS-PAGE. All proteins got digested in SGF except one protein of approximate 20 kDa. This 20 kDa protein was found to be abundant in tobacco CPE, which also increases the probability of allergenic responses (Fig. 2a and b). Densitometry analysis of the pepsin digestibility profile (SGF assay) showed that 20 kDa protein from WT tobacco remained undigested nearly 90, 81, 78 and 70 % while 20 kDa protein from the GM tobacco showed 83, 71, 62, and 55 % digestibility after 0.25, 4, 15 and 60 min, respectively (Fig. 2c and d). As we know, proteins that are non-digestible in SGF assay are considered to have chances to interact with the intestinal immune system and may induce allergenic symptoms in the gastrointestinal tract of exposed individual [18–23]. As per the DBT, India

Fig. 1 Protein profile of WT and GM tobacco protein extract (a) WT and GM tobacco protein extract separated on 12 % SDS-PAGE profile (b) Two-dimensional (2D) SDS-PAGE profile of WT and GM tobacco CPE, separated by isoelectric focusing (pH 4–7) followed by SDS-PAGE (12 %). M indicates molecular weight marker and encircled spots denote CBL protein expression in GM tobacco

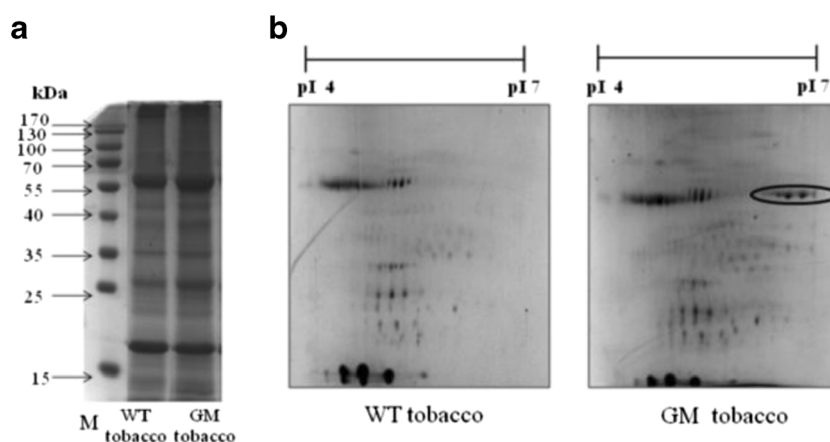
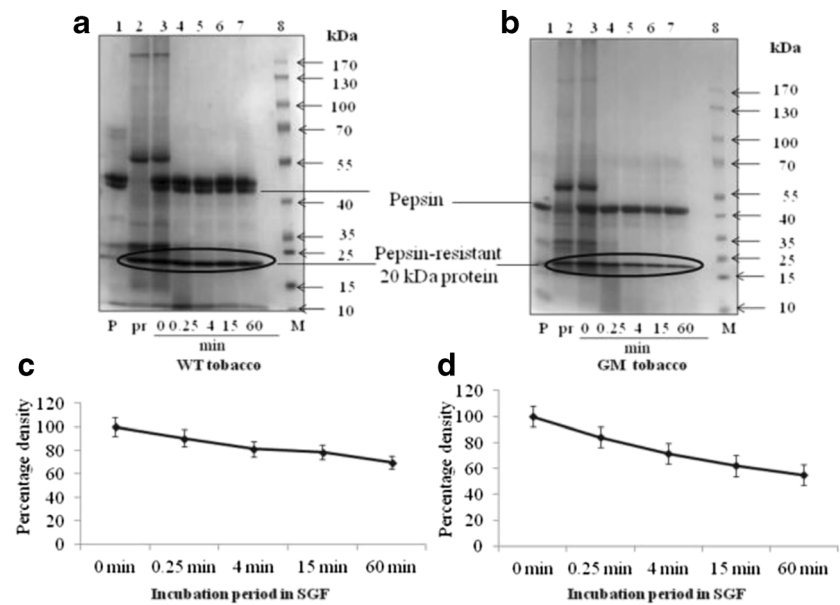


Fig. 2 Pepsin digestion profile of WT and GM tobacco proteins (a) Pepsin digestibility profile of WT tobacco at different time points at 0, 0.25, 4, 15 and 60 min (b) Pepsin digestibility profile of GM tobacco at different time points at 0, 0.25, 4, 15 and 60 min and (c, d) densitometry analysis of the pepsin digestibility profile of WT and GM tobacco CPE (P indicates pepsin, Pr denotes protein, M for molecular weight marker and SGF indicates simulated gastric fluid



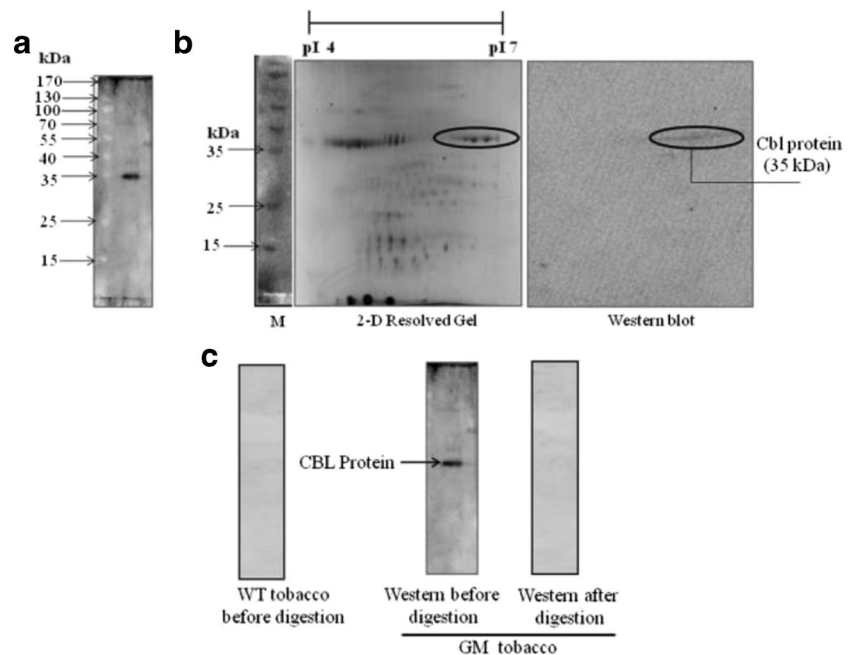
guidelines,, proteins with more than 10 % stainable full-length protein band remaining at 60 min may be considered stable and these protein may have the allergenic potential [24].

To confirm the presence of CBL protein in GM tobacco, antibody specific to CBL protein was used and found a single band at 35 kDa that was specific for CBL protein whereas no such protein expression was observed in WT tobacco extract (Fig. 3a, b). It was hypothesized that if CBL protein is non-digestible (probably allergenic tobacco proteins) then it should be recognized by anti-CBL antibody after SGF digestion. Therefore, we incubated GM tobacco protein in SGF for

different time-points and looked for *cbl* protein using western blot but no band was recognized at any time-point to bind with anti-CBL antibody, which indicated that CBL protein got completely digested within 15 s (Fig. 3c). It also indicates extremely low chance of its absorption by the gastrointestinal tract and subsequently no enhancement in allergenicity because of *cbl* gene product exposure.

It is required to assess the allergenicity potential of any allergen under *in vivo* scenario as the results need to be extrapolated to human situations. Therefore, *in vivo* experiments in Balb/c were performed; where comparable

Fig. 3 (a, b) The expression of the *cbl* gene product (Mol Wt 35 kDa) in GM tobacco leaves was identified by Western blot analysis with antibodies raised in rabbit against CBL protein. (c) Stability of CBL protein was assessed in SGF (simulated gastric fluid) in digested and non-digested conditions using CBL antibody raised in rabbits. CBL protein got digested within 15 s in SGF



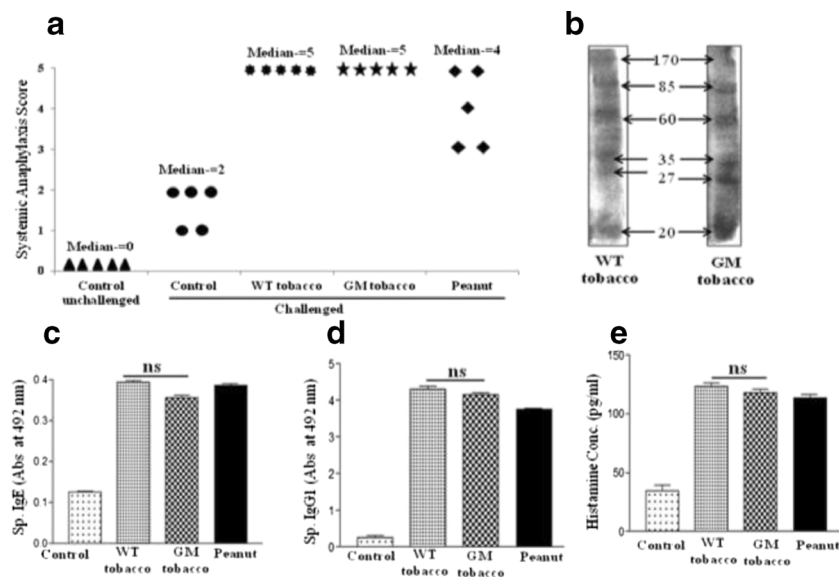


Fig. 4 Assessment of different allergic parameters in Balb/c mice following treatment (a) Systemic anaphylactic symptoms scored after 10 min following protein challenge, presented as median value for unchallenged control (0), challenged control group (2), WT tobacco treated (5) GM tobacco treated (5) and peanut (4) treated mice respectively (b) IgE immunoblotting with WT and GM tobacco sensitized mice

sera. Similar IgE binding proteins were detected in both groups (c) measurement of specific IgE (d), specific IgG1 in serum collected on day 43 (e) histamine levels were estimated in plasma collected after challenge (ns = non significant). Data were expressed as means $\pm$ SEM of 3 replicates. Values represented were non-significant among WT and GM tobacco treated groups at $*p < 0.05$

immunological responses in both GM and WT tobacco treated mice were found.

To ascertain potential allergenicity induced by *cbl* gene, GM and WT tobacco extract sensitized mice were challenged with their respective CPE (5 mg/mouse) on the 60th day. Pre-sensitized mice with either GM or WT tobacco protein on challenge showed no differential response in systemic anaphylaxis score. Sensitized mice from both the GM and WT tobacco groups died within 10 min after challenge (clinical score 5) (Fig. 4a). Two mice even from control group after challenge displayed scratching and rubbing around the snout and head (score 1), while the remaining three of the exposed group had diarrhea and increased respiration rates (score 2). None of the control mice died. Control group mice showed

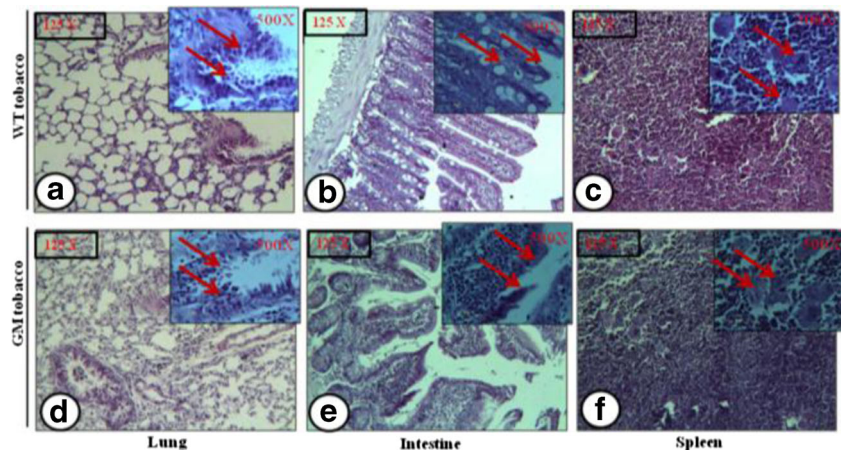
clinical score of 1 and 2 upon challenge that might be due to presence of other toxic substance of unknown nature in tobacco leaves, but it still needs experimental evidences.

IgE binding proteins in both GM and WT tobacco extracts were also compared where multiple IgE binding proteins were identified in each tobacco extract in the range of 20–170 kDa (20, 27, 35, 60, 85 and 170 kDa) (Fig. 4b). These results indicated that there is no addition to allergenic risk associated with GM tobacco due to *cbl* gene insertion when compared to WT tobacco.

GM and WT tobacco CPE sensitized mice showed enhanced generation of specific IgE/IgG₁ as compared to the control. However, levels of specific IgE/IgG₁ were comparable between the GM and WT tobacco CPE groups (Fig. 4c and

Fig. 5 Histopathological analysis of different organs following WT and GM tobacco proteins exposure in mice.

Histopathological changes in (a, b, c) lung, intestine and spleen of WT tobacco treated mice (d, e, f) lung, intestine and spleen of GM tobacco treated mice



d). Th2 cells produce IgE and IgG1 antibodies that favour the development of allergic reactions. The level of specific IgE demonstrated the participation of specific allergen in inducing allergic reaction [25, 26]. The level of histamine in the plasma of the sensitized mice after challenge was increased ~3–4 fold in both GM and WT tobacco treated mice as compared to control mice (Fig. 4e). Histamine, a prominent mediator of anaphylaxis, plays a crucial role in pathogenesis of allergy, where upon cross-linking of mast cell-bound IgE releases histamine and other allergic mediators [27]. Comparable histamine level in both GM and WT tobacco treated mice again confirmed the fact that the allergenic potential of GM tobacco was similar to the counterpart.

Prominent histopathological changes in the lungs, intestines, and spleen were observed in both GM and WT tobacco-treated mice. Lungs from the GM and WT tobacco-sensitized mice showed exfoliation of the epithelial lining at the bronchioles and an infiltration of inflammatory cells within/around the bronchioles. The alveoli showed signs of mild thickening. The intestines of GM and WT treated mice showed a denudation of villi as well as moderate goblet cell hyperplasia. Spleens of these mice evidenced lymphoid hyperplasia, megakaryocytosis, and increase in numbers of large megakaryocytes (Fig. 5). Histopathological examination of the lungs, intestine and spleen revealed that exposure to tobacco protein recruits inflammatory cells in the lungs and intestine, as well as airway blockage in lungs. Airway hyper-responsiveness and recruitment of inflammatory cells indicated the inflammatory symptoms as observed in the case of asthma [28]. Mucosal exfoliation observed in intestine is a characteristic feature of diarrhea, commonly reported in case of anaphylactic reactions. The lymphoid hyperplasia observed in spleen in the present study may be due to massive proliferation and differentiation of T lymphocytes reflects the main histological signs of chronic inflammation in the lymphoid organ [29]. Both GM and WT tobacco induced similar changes in histology that indicated the role of tobacco protein in allergic exacerbations and responses of GM tobacco proteins were not different from WT tobacco protein in spite of having inserted *cbl* gene product.

Conclusion

In the present study, allergenic responses to both GM and WT tobacco were comparable. The presence of similar IgE binding proteins, rapid digestion of the transgenic CBL protein in SGF, similar pepsin digestibility profiles and lack of differences among the immune responses support substantially equivalent responses of GM tobacco with WT tobacco (Supplementary Fig. S2). We still do not know what would be the toxic manifestations of tobacco leaf proteins in tobacco chewers. Development of other new varieties with improved

characteristic will be helpful to meet the requirement of consumer, but before their commercialization, risk assessment is necessary for safety purpose.

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Conflict of Interest The authors declare no conflict of interest.

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Supplementary Information

S1.1. Bioinformatics Analysis of *cbl* Gene

A sequence homology analysis was performed for the *cbl* gene using 'Allergen-Online' software (<http://www.allergenonline.com>) to compare the sequence of this gene with ~~any~~all ~~the~~ known allergens, which are recorded in the database list. This sequence homology analysis helps in assessing the probable allergenicity of any newly-expressed transgenic protein. Identification of exact matches of any 8-amino acid long sequence between the CBL protein sequence and any well-known allergen was performed, then 80-amino acid sub-sequences of the CBL protein using a sliding window of 80-amino acid size was done. Further, Full FASTA search was performed by comparing long regions of CBL proteins with known allergen.

Prediction of allergenic potential of inserted *cbl* gene was also done by Algpred software and Allergen Database for Food Safety (ADFS). Algpred is available at www.imtech.res.in/raghava/algepred/. This system predicts allergenicity of protein sequences using different approaches, namely IgE epitope mapping, MAST result, Support Vector Machine based on amino acids and dipeptide composition, allergen representative peptide (ARP) methods, and hybrid approaches. [ADFS, available at http://allergen.nihs.go.jp/ADFS/](http://allergen.nihs.go.jp/ADFS/), is a Web server database system that is used as allergenicity prediction tools. This ADFS was launched as a project of the Division of Biochemistry and Immunochemistry of National Institute of Health Sciences (Japan). Sequence similarity between CBL proteins sequence with known allergen will predict the chances of GM crop to be allergenic or non allergenic.

Supplementary Table S1: Bioinformatics analysis of inserted gene *cbl* product using AllergenOnline, Algpred and ADFS databases

A. Bioinformatics analysis of inserted <i>cbl</i> gene product on AllergenOnline								
Access No.	Name of protein		Amino acid length		80-mer	8-mer	FULL FASTA	
AAW73072.1	Calcineurin B-like protein		225		0	0	0 (E-Value Cutoff =0.00)	
AAW73072.1	Calcineurin B-like protein		225		0	0	2 (E-Value Cutoff =0.001)	
B. Prediction of allergenic potential of inserted <i>cbl</i> gene by Algpred Software								
Name of inserted protein	MAST result	Number of IgE epitopes in allergen	SVM module based on amino-acid composition (threshold= −0.4)		SVM module based on dipeptide composition (threshold = −0.2)		Blast search on allergen representative peptides	Hybrid–hybrid approach (E-Value Cutoff =0.001)
CBL Protein	Non allergen	No IgE epitope	Score 0.6654176	Result Potential allergen	Score 0.3816007	Result Potential allergen	Non allergen	Non allergen/allergen
C. Prediction of allergenic potential of inserted <i>cbl</i> gene by Allergen Database for Food Safety								
Name of inserted protein	FAO/WHO		Method		Motif-based method			
	(E-Value Cutoff =0.001)				Motif result	BLAST result	Total	
	80 mer	6 mer						
Calcineurin B-like protein	0	1			Negative	Positive	Positive	
			(Globin CTT-VIII)			(Troponin-like protein)		

- 4
- 5 Results shown describe possibility of allergenicity induced by inserted Calcineurin B-like
- 6 protein (using indicated databases).
- 7 Where 6/8/80-mer =Peptide of 6/8/80 amino acids
- 8 BLAST=Basic Local Alignment Search Tool
- 9 SVM=Support Vector Machine
- 10 FAO /WHO= Food and Agriculture Organization/ World Health Organization

S1.2. Protein Profiling of WT and GM Tobacco

Proteins were resolved on SDS-PAGE and 2 D gel electrophoresis. For the latter experiment, protein samples were processed by Trichloroacetic Acid (TCA) precipitation to remove small interfering species (salts, detergents) and to concentrate the samples for downstream applications. Briefly, equal volume of 10% TCA was added to protein samples and incubated on ice for 30 min., followed by centrifugation at 10,000 g for 10 min. Supernatants were removed and 300 µl cold acetone was added to protein pellet. After centrifugation at 10,000 g for 5 min., supernatant was discarded and pellet dissolved in 2-D rehydration buffer [7 M Urea, 2 M Thiourea, 2% CHAPS, 65 mM Dithiothreitol (DTT)]. The protein extract in rehydration buffer was then resolved over a linear IPG strip (pH 4-7; GE Healthcare Life Sciences, USA) and focused up to 20,000 VhT with a maximum of 4500 V, at 20°C using Ettan IPGPhor 3 (GE Healthcare, USA). Resolution of protein was further performed using 12% SDS-PAGE gels.

S1.3. Simulated Gastric Fluid (SGF) Assay of WT and GM Tobacco Protein

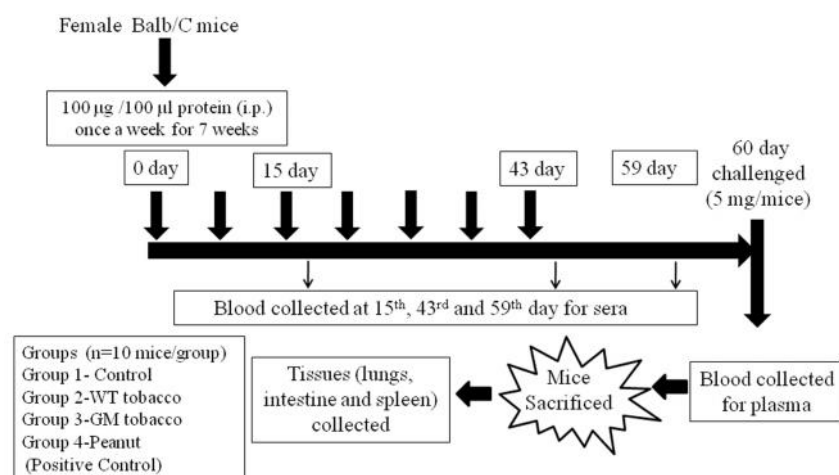
Identification of pepsin-resistant protein in tobacco was performed using SGF assay. In brief, 6.4 mg pepsin was dissolved in 1 ml of 34 mM NaCl, 0.7% HCl (pH 2.0). The pepsin:protein ratio in the incubation mixture was 1:1. The reaction was stopped at different time points (0.25, 4, 15, 60 min) by addition of 60 µl of the incubation mixture to 20 µl of 6X stopping solution [200 mM DTT, 4% Sodium Dodecyl Sulphate, 0.2% Bromophenol Blue, 20% Glycerol, 100 mM Tris (pH 8.8)]. Protein samples were resolved on 12% SDS-PAGE gels at 22 mA for 4.5 hr [15] and stained with Coomassie Brilliant Blue (G-250). Images were captured using Syngene Bio Imaging System (Syngene, Cambridge, UK) and densitometry was performed by the Gene tools software (Syngene, Cambridge, UK).

S1.4. Stability of *cbl* Gene Product in SGF

For determination of the stability of CBL protein, GM and native tobacco CPE were digested with SGF for different time periods, resolved in 12% SDS-PAGE gels and transferred onto Polyvinylidene Difluoride (PVDF) membranes. Blots were incubated with antibodies raised in rabbits against *cbl* gene (1:2000 ratio) for overnight incubation at 4°C. Anti-rabbit IgG-horseradish peroxidase (HRP)-conjugated antibody (1:1000 ratio) used as a secondary antibody. The blots were analyzed using a chemiluminescence method (ECL, Amersham Pharmacia Biotech, UK), according to manufacturers' protocols.

S1.5. *In vivo* Treatments

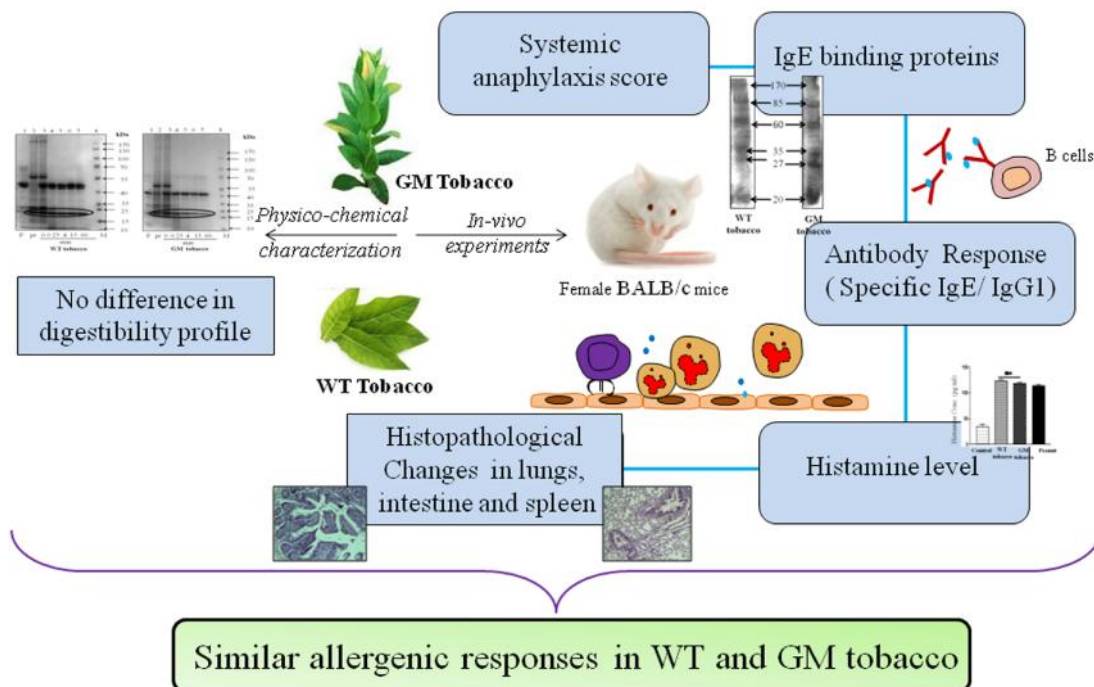
The mice were randomly divided into four groups (10 mice each). Mice in groups 1, 2, 3 and 4 were given PBS (vehicle), 100 µg of WT tobacco CPE, GM tobacco CPE, or peanut CPE respectively by intraperitoneal (IP) injection once a week for 7 weeks following a method as described earlier [17]. Blood samples were collected from retro-orbital sinus to measure levels of serum specific IgE and IgG1 antibodies. Mice in each group were challenged with 5 mg respective protein on day 60 and blood was collected for plasma. Lungs, intestine and spleen were taken out from each group of challenged mice for histopathology.



Supplementary Fig. S1. Diagrammatic representation of animal treatment protocol.

S1.6. Systemic Anaphylaxis Score

Anaphylactic reactions were scored using a scoring system previously described by Li et al (1999) [18]. Scoring was: 0 = no symptoms; 1 = scratching and rubbing around the snout and head; 2 = puffiness around the eyes and snout, pilar erection, 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 #3 with loss of consciousness, tremors, and/or convulsion; and, 5 = death.



Supplementary Fig. S2. Summary of allergic responses compared between WT and GM tobacco proteins.