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1 **Comparison of Allergenicity at Gly m 4 and Gly m Bd 30K of soybean after the**
2 **genetic modification**

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Abstract

Despite rapid growth of the genetic modified (GM) crops, effective evaluations of genetic modification on allergenicity are still lacking. Gly m Bd 30 K is cross-reactive with cow milk protein casein, Gly m 4, and with birch pollen allergen Bet v 1. Here we compared the allergenicity between GM and non-GM soybeans with respect to the foci Gly m 4 and Gly m Bd 30K. Recombinant allergens of Gly m Bd 30K and Gly m 4 were generated and polyclonal antibodies raised to identify these two allergenic components in soybeans. GM soybean was first PCR-confirmed using 35S-promoter. A total of 20 soybeans (half GM, half non-GM) obtained from food market were used to assess their allergenicity based on IgE-binding and histamine release. The concentrations of Gly m Bd 30K and Gly m 4 in soybeans were then determined. Most soybean-allergic patients (9 out of 10) showed IgE-positive reactions to the allergen of 30kDa in molecular weight. That allergen turned out to be *Glycine max* Gly m Bd 30K based on LC/MS/MS analyses. Gly m Bd 30K is therefore the major allergen in the soybean. An increase in the transcription of both the Gly m 4 (stress-induced protein SAM22) and Gly m Bd 28K (soybean allergen precursor) was found after GM. The protein concentrations of Gly m 4 and Gly m Bd 30K were no statistically significant differences between Non-GM and GM soybeans. There were also no statistical significances between them in the tests of IgE binding and histamine release. In conclusions, soybeans showed similar concentrations of Gly m Bd 30K and Gly m 4 regardless of genetically modified or not. The allergenicity of both Gly m Bd 30K and Gly m 4 were therefore not altered after genetic modification. Patients showing hypersensitivity to soybeans and who had pre-existing allergy to birch pollen and cow milk casein might not further increase their allergic reactions following exposures to the GM soybeans.

Keywords: genetic modified (GM); soybean allergy; Gly m Bd 30K; Gly m 4; GM soybean

Introduction

Genetic modified (GM) crops are a dominant agricultural food product worldwide owing to their superior productivity. The food allergy is an important medical and social issue. The incidence of food allergy appears to be increasing in the last few years¹. The popularity of GM crops whether affect or increase together the incidence of food allergy remains unclear. The rapid growth of the GM crops created controversies in many regions, including the European Union, Africa, Argentina, Korea, Japan, United States and Brazil²⁻¹². Soybean, which belongs to the “Big-8 allergenic foods” may act as an aeroallergen causing occupational allergy in rural workers and those involved in soybean processing²⁻³. To mitigate these controversies, effective evaluation of allergenicity of GM soybean is in demand.

The identification and characterization of the allergenic components in soybean is essential for the development of immuno-intervention strategies⁴. DNA-based methods such as real-time quantitative PCR (qPCR) have been successfully applied to GM crop detection for the past two decades¹³⁻¹⁴. However, the rapid development of new GM crops demands more effective methods. Based on IgE-mediated immune responses, three soybean proteins, Gly m Bd 30 K/P34, Gly m Bd 28 K/P28 and Gly m 5, have been identified as the major soybean allergens⁵⁻⁷. Gly m Bd 30 K/P34 is a soybean seed allergen and possesses cysteine protease activity belonging to the papain-superfamily^{8,15}. While the prevalence is low for hypersensitivity to soybeans, soy allergy may occur through cross-reaction sensitization¹⁶. Co-sensitization of Gly m Bd 30K to bovine casein in cow-milk allergy was reported in Korea and Argentina¹⁶⁻¹⁷. In Europe, it was also reported IgE-mediated allergic symptoms in patients with birch pollen allergy after ingestion of soybean as a result from cross-reactivity of Bet v 1 to homologous pathogenesis-related proteins (PR-10)-SAM22¹⁸. The severe oral allergy syndrome and anaphylactic reactions caused by a Bet v 1-related PR-10 protein in soybean SAM22 was reported¹⁸. Allergic reactions, ranging from mild to severe anaphylactic shock, found after digesting soybean in birch pollen allergic patients,

are attributed to Bet v 1-Gly m 4 cross-reactivity¹⁸⁻¹⁹. However, the allergenic component Gly m 4-specific IgE is rarely detected in soybean-allergic patients with anaphylaxis. Gly m 4 is a stress-induced protein, which be classified as PR-10, its mRNA is expressed on young leaves under stress conditions such as starvation or wounding¹⁹. Since Gly m 4 is a stress-related protein and can be upregulated in a stress environment. Whether it can be upregulated by genetic modification remains unclear. The cross-reactivity between the soybean protein P34 and bovine caseins has been demonstrated¹⁶.

Gly m Bd 30K, an IgE-binding protein with a molecular weight of 30 kD, was identified in soybean extracts by Western IgE-immunoblot analysis²⁰. IgE binding to sections of soybean seeds showed dense staining throughout the vacuolar bodies, localizing the allergens in protein storage vacuoles of seed cotyledons and 65.2% of the patients tested bound to this protein. Since Gly m Bd 30K and Gly m 4 were reported to be cross-reactive with milk casein and Bet v 1^{16, 19}. To answer whether genetic modification increases the allergenic risk of soybeans, here we compared the allergenicity and IgE-reactive components of wild-type and GM soybean extracts in relation to Gly m Bd 30K and Gly m 4. Gly m Bd 30K was identified by Western IgE-immunoblotting and its allergenicity by histamine release.

Materials and Methods

Soybean extracts preparation

Soybean seeds were obtained from Taiwan Food Industry Research and Development Institute. The genetic background of GM soybean was with transgenes (5-enolpyruvylshikimate-3-phosphate) *EPSPS* gene and *CaMV 35S* promoter. The standard of GM and non-GM soybeans used as positive control and negative control for gene confirmation were provided from Food and Drug Administration, Republic of China, Taiwan. The soybean plants were grown in the United States. The soybean seeds were harvested at the R8 mature stage (full maturing) and imported from U. S. to Taiwan. The year of soybean seeds was sampled within one year. Soybean seeds of Organic, Non-GM and GM were ground to fine powder using a grinding machine. The resulted soybean flour was extracted with PBS buffer and stirred overnight at 4°C. Protein concentration was measured with the Bio-Rad protein assay kit. All extracts were stored at -20°C before analysis.

Patient subjects

The Institutional Review Board of Taichung Veterans General Hospital approved this study (IRB Nos. SE14183). Written informed consent was obtained from each participant before they were enrolled in the study. We recruited a total of 12 allergic patients who attended the Allergy Clinic at Taichung-Veterans General Hospital. Serum samples obtained from 10 patients were sensitive to soybean. Soybean-specific IgE levels were measured in a UniCAP system (Thermo Fisher Scientific, Uppsala, Sweden), and 2 patients insensitive to soybean served as controls. Blood samples were drawn, and serum stored in aliquots at -20°C. IgE antibodies to the soybean protein extract were detected with immunoblotting.

Immunoblotting

Total extracts (15µg) of Non-GM and GM soybeans were separated by SDS-PAGE, and transferred onto Immobilon-P PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk, and incubated overnight with diluted human serum samples (1:4 dilution in Tris-buffered saline, pH 7.4, containing 0.05% Tween-20 and 1% skim milk) at 4°C. Human IgE antibodies were probed for one hour with horseradish peroxidase-conjugated monoclonal anti-human IgE antibodies (Southern Biotechnology Associates Inc.) at room temperature. Detection was performed with enhanced chemiluminescence (ECL) reagents (Millipore, Massachusetts, USA), and chemiluminescence detected with LAS 3000. Band intensity was analyzed by Multi Gauge software V 3.0.

Plants genomic DNA preparation and polymerase chain reaction (PCR) amplification

Genomic DNA was isolated from soybean samples using a GeneJET™ Plant Genomic DNA Purification Mini kit (Thermo Scientific, Lithuania, EU) as instructed by the manufacturer. The DNA served as a template in the PCR (G-Storm PCR system). PCR amplification targets are the *rbcL* gene (non-GM) and the transgene element (CaMV35S promoter region). The forward and reverse primers were as follows: 35S, 5'-GCT CCT ACA AAT GCC ATC A-3' and 5'-GAT AGT GGG ATT GTG CGT CA-3'; *rbcL*, 5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3', 5'-GTA AAA TCA AGT CCA CCR CG-3'. PCR thermocycler was run according to the following program: 95 °C for 5 min followed by 95 °C for another 30 sec, 54 °C for 1 min, and 72 °C for 1 min (a total of 35 cycles was performed), and finally at 72 °C for 10 min. PCR products were put through electrophoresis on 2% agarose gel. Products of electrophoresis were visualized by ethidium bromide staining.

Generation of recombinant Gly m Bd 30K and Gly m 4 and the production of polyclonal

antibodies

cDNA covering the entire coding region of Gly m Bd 30K and Gly m 4 was amplified from RNAs taken at the leaf of soybean by reverse-transcription (RT)-PCR. cDNA encoding the mature Gly m Bd 30K (777 bp) and Gly m 4 (477 bp) polypeptide was fused in frame to the N-terminal sequence of His-tagged, and the plasmid constructs were confirmed by sequencing. The recombinant polypeptide expressed in *E. coli* DH5 α was affinity purified, under denaturing condition from a Ni-NTA resin column (Novegen). Purified protein from recombinant Gly m Bd 30K and Gly m 4 was used to raise polyclonal antibody in mouse and rabbits. The polyclonal antibody against recombinant Gly m Bd 30K protein was generated by AllBio Science Inc., Ltd. The polyclonal antibody against recombinant Gly m 4 protein was prepared in Rabbit as follows. Rabbit was injected with protein antigen 250 μ g/mL phosphate buffered saline (PBS) buffer mixed with an equal volume of the Complete Freund's adjuvant (CFA). For booster injection, protein antigen (250 μ g/mL PBS buffer) and equal volume of the Incomplete Freund's adjuvant (IFA) were used. After ELISA test, whole blood was taken and the serum separated. The serum samples were stored in aliquots at -20°C before use.

Determination soybean allergen by MALDI-TOF-Mass Spectrometry (MS)

In order to clarify the peptide sequences, the protein were separated by SDS-PAGE and presented by Coomassie Brilliant Blue staining. The predicted sizes of soybean allergenic components were taken from the gel and performed the protein digestion²¹. In briefly, protein samples were excised from stained gel and washed with $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (1:1, v/v) containing 25 mM ammonium bicarbonate to remove the staining dye. The gel plug was dehydrated with 100% acetonitrile, and was dried under vacuum and incubated overnight at 37°C with 20 μ L of 10 μ g/mL porcine trypsin in 20 mM ammonium bicarbonate. The extract was vacuum dried and the pellet was dissolved in $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (1:1, v/v) and 0.1% trifluoroacetic acid. Then, these samples were sent for

implementation at the Proteomics Research Core Laboratory of National Cheng Kung University (NCKU) to identify the unknown protein, performed by MALDI-TOF LC/MS/MS analysis²¹. A Voyager DETM-STR MALDI-TOF mass spectrometer (Thermo Fisher Scientific, Framingham, MA) operated in positive ion reflector mode was used to analyze tryptic peptides.

Enzyme-linked immunosorbent assay (ELISA)

Microtiter 96-well plates (EIA/RIA plates, Costar) were coated overnight at 4 °C each well with 100 µl of Gly m Bd 30K at different concentrations (from 781 to 50000 pg/ml) and Gly m 4 (from 156 to 10000 pg/ml) as the standard. Soybean crude extracts were coated in duplicate at the protein concentration of 0.4 µg/ml to determine the allergen concentration. Polyclonal antibodies were incubated with crude extract at room temperature for an hour followed by rat monoclonal anti-mouse immunoglobulin G antibodies conjugated with HRP. The color was developed with TMB substrate and the reaction was stopped by adding 1M H₃PO₄. Absorbance was measured at 450/540 nm.

Soybean RNA Isolation

Soybean leaves were grown from seeds of Organic, Non-GM and GM. Total leaf RNA was extracted by Trizol® Reagent (Invitrogen, USA) according to the instruction manual. The purified RNA was quantified at OD₂₆₀ nm using a ND-1000 spectrophotometer (Nanodrop Technology, USA) and qualified with a Bioanalyzer 2100 (Agilent Technology, USA) using an RNA 6000 labchip kit (Agilent Technologies, USA).

Library Preparation & Sequencing

All procedures were carried out according to the manufacture's protocol from Illumina. Library construction of all samples were done using the Agilent's SureSelect Strand Specific RNA Library

Preparation Kit for 75SE bp (Single-End or Paired-End) sequencing on the Solexa platform. The sequence was directly determined using sequencing-by-synthesis technology via the TruSeq SBS Kit. Raw sequences were obtained from the Illumina Pipeline software bcl2fastq v2.0 and expected to generate 10M (million reads or Gb) per sample.

RNA Sequence Analysis

Initially, the sequences generated were filtered to obtain the qualified reads. Trimmomatics was used to trim or remove the reads based on the quality score. After filtering low-quality data qualified reads were analyzed using TopHat/Cufflinks to estimate the gene expression²². The gene expression level was calculated as FPKM (Fragments Per Kilobase of transcript per Million mapped reads). For differential gene expression profile, the CummeRbund statistical software was used. The reference genome and gene annotations were retrieved from Ensembl database²².

Basophil Histamine Release Assay

The serum samples from soybean-allergic patients and control subjects were analyzed in the basophil histamine release assay. Washed polymorphonuclear cells were obtained from the venous blood of non-atopic donors using Polymorphpret™ solution (Axis-Shield, Oslo, Norway). Cells were resuspended in RPMI-1640 medium after adjusted to 2×10^6 cells/ml using the trypan blue assessment. Passive sensitization of basophils was achieved with specific IgE from the subject serum samples for 4 hr at 37 °C. Sensitized cells were triggered with 50 µg/ml of allergen for 30 min at 37 °C, and the supernatant was collected and reacted with O-phthalaldehyde (OPA, 5 mM) for 7 min. The reaction was stopped by adding H₂SO₄ (0.04 M). The histamine released into the supernatant was measured with a fluorescence spectrophotometer²³. The percentage of histamine release was calculated as follows: (stimulated released histamine – spontaneous released histamine) / total released histamine x 100%.

203

204 **Statistical analyses**

205 Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA,
206 USA). Data are presented as mean $\pm$ standard error of the mean (SEM). P-values ≤ 0.05 were
207 considered statistically significant. All the results of Non-GM and GM soybeans were compared
208 between groups and analyzed by paired Student's t test.

Results

GM soybean Confirmation by PCR of 35S gene

GM soybean was confirmed using PCR at the 35S region (35S-promoter; originated from cauliflower mosaic virus) with primer 35S-Forward and 35S-Reverse. All GM soybeans showed positive results at the 35S 192 bp fragment (104GM lane 01-05 and 104GM lane 06-10) , compared to negative results for Non-GM soybeans (104TNGM lane 01-05 and 104TNGM06-10). The internal control of rbCL gene 599 bp fragment appeared in both GM and non-GM soybeans (Fig. 1).

Gene expression of Soybean Allergens compared between GM and Non-GM

Gene expression was determined using the “Next Generation Sequencing Analysis System” which involved Transcriptome sequencing and Illumina Solexa[®] sequencing. RNA-Seq was used to identify on a genome-wide scale of soybean the potential candidates within the National Center for Biotechnology Information (NCBI) database. After whole-genome profiling, a total of 58,885 transcript gene expressions were compared between GM and Non-GM soybeans. Among these, 1,947 transcript genes with Fragments Per Kilobase of transcript per Million mapped reads (FPKM) were >0.3 and with Two-Fold change (FC) >2. Findings indicated that many genes were altered after genetic modifications. In this study, we focus only on the transcript genes of allergenic components. Three transcript genes of soybean allergens have been characterized: Gly m Bd 30K, Gly m 4 and Gly m Bd 28K. Transcript gene expressions of these allergens were compared. Results showed that Gly m 4 (stress-induced protein SAM22) and Gly m Bd 28K (soybean allergen precursor) increased (ratio: 22.32 of Gly m 4; 29.62 of Gly m Bd 28K) after genetic modifications (Table 1).

Identification of IgE binding proteins in the 3 different soybeans

Three different soybeans were studied here: Organic soybean in No. 1, Non-GM soybean in No. 2, and GM soybean in No. 3 (Fig. 2A). Protein profiles on SDS-PAGE revealed several bands in the soybean crude extracts, a pattern that was similar across different sources of soybeans (Fig 2B). IgE binding proteins were analyzed with immunoblotting. Ten serum samples derived from soybean-allergic patients were used for IgE binding protein identification. Soybean crude extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Serum was added to the membrane strip for overnight in 4 °C. IgE binding proteins were identified with anti-IgE antibody using immunoblotting. Results showed that 9 out of 10 serum samples (except serum A) reacted positively with the protein of MW around 30 kDa, (Fig. 2C). The sequence information of the 30kDa protein was further obtained with the LC/MS/MS analyses of peptides. Twenty-four interpretable mass spectra were obtained from trypsin-digested peptides, and the sequences of these tryptic fragments matched the amino acid sequence of ID (GI: 84371704). (http://140.116.98.49/mascot/cgi/master_results.pl?file=.%2Fdata%2F20160521%2FF001887.dat&REPTYPE=peptide&sigthreshold=0.05&REPORT=AUTO&_server_mudpit_switch=0.000000001&_ignoreionsscorebelow=0&_showsubsets=0&_showpopups=TRUE&_sortunassigned=scoredown&_requireboldred=0#Hit1) That corresponded to Glycine max Gly m Bd 30K according to the NCBI database (shown in the supplementary data Figure-S1). This finding showed that Gly m Bd 30K was the major allergen of soybean for the studied patients.

Purification of rGly m 4 and preparation of the rabbit anti-Gly m 4 antibodies

To identify contents of allergenic component-Gly m 4 in the crude extracts of various soybeans, recombinant Gly m 4 and anti-Gly m 4 antibodies were prepared. Chromatography with a Ni-NTA sepharose affinity column was used to purify His-tagged Gly m 4 from *E. coli* induction lysate. Protein purification profiles on SDS-PAGE showed a high expression at approximately 15 kDa in the lysate. After binding with affinity column and elution, the recombinant Gly m 4 with

molecular weight of 15kDa was found (Fig 3A). Protein profiles on SDS-PAGE showed several bands with no apparent disparity across different sources of soybeans (Fig 3B). Polyclonal antibodies anti-Gly m 4 were prepared from a New Zealand rabbit, which received a subcutaneous injection of r Gly m 4 emulsified with Freund's adjuvant. The soybean proteins were identified with Western Blotting, and crude extracts were detected with anti-Gly m 4 antibodies at the molecular weight of 15kDa (Fig 3C). In the case when rabbit pre-immune serum was used as controls, soybean crude extracts could not be detected immunologically (Fig 3D). The sequence of the protein of 15kDa molecular weight was confirmed with the LC/MS/MS analyses. Fifty interpretable mass spectra were obtained, and 51% of protein sequence was covered. The sequence of these tryptic fragments matched the amino acid sequence of ID (GI:351724911). This corresponded to the stress-induced protein SAM22 of Glycine max (Gly m 4) according to the NCBI database (shown in the supplementary data Figure-S2) with 72% protein identity to allergen Bet v 1.

Preparation of the anti- Gly m Bd 30K antibodies in the mouse

Soybeans, including 10 non-GM (No. 1-5 & No. 11-15) and 10 GM (No. 6-10 & No. 16-20) were studied here. In order to evaluate major allergenic component from different sources of soybeans, recombinant Gly m Bd 30K and anti- Gly m Bd 30K antibodies were prepared first. Protein profiles on SDS-PAGE revealed several bands in the soybean crude extracts, a pattern that was similar across different sources of soybeans (Fig 4A). The proteins were identified by Western Blotting by the binding to anti- Gly m Bd 30K antibodies at the molecular weight of 30 kDa (Fig 4B). Mouse pre-immune serum acted as a control confirmed no similar binding (Fig 4B).

Total IgE binding proteins compared between GM and Non-GM soybeans

In order to compare allergenic components of soybeans, we analyzed the ten lines of GM and ten

lines of non-GM soybeans based on protein profiles and Western blot analysis. IgE binding proteins were analyzed by immunoblotting assay with pooled human serum. These serum samples from soybean allergic subjects (as determined by ImmunoCAP) and healthy subjects (negative to soybean) were used to analyze specific IgE-binding allergenic components in the ten lines of soybeans. The total IgE binding proteins in these crude extracts were compared after immunoblotting with pooled human serum and anti-IgE antibody (Fig. 5A). Results showed some differences in IgE-binding activity among the ten soybean lines. In contrast no response of IgE-binding was found with healthy subjects. The Western blots of IgE-binding activity were quantified using a digital imaging system “ImageQuant[™] LAS 4000”. The mean binding strength to Gly m Bd 30K IgE was 219.02 signal intensity(SI) for GM soybeans and 206.35 SI for non-GM soybeans (Fig. 5B) with no statistical differences.

Comparisons of Gly m Bd 30K and Gly m 4 Compositions in GM and Non-GM Soybean by ELISA

Gly m Bd 30k and Gly m 4 in the extracts of Non-GM and GM soybeans were compared by binding to specific antibodies. A total of 20 soybeans seeds (10 Non-GM and 10 GM) were analyzed using the ELISA method. ELISA plates were coated with different concentrations of Gly m Bd 30K (from 781 to 50,000 pg/ml) and Gly m 4 (from 156 to 10,000 pg/ml) as the standards. The soybean crude extracts were coated in duplicate at protein concentrations of 0.4 µg/ml to determine their allergen concentrations. The concentrations of Gly m 4 in Non-GM soybeans were 224±78.4 pg/µg and in GM soybeans 250.4±68.73 pg/µg (Fig 6A). The concentrations of Gly m Bd 30K in Non-GM soybeans were 3,564±1,696 and in GM soybeans 4,573±783.2 pg/µg (Fig 6B). However no statistically significant differences were found with respect to these concentrations of Gly m 4 and Gly m Bd 30K between Non-GM and GM soybeans. Results indicated that the intrinsic allergens of Gly m Bd 30K or Gly m 4 in these two soybeans had little effects on their

protein levels.

Comparison of Allergenicity by Histamine Release after Stimulation by Soybean from GM or Non-GM Lines

Serum samples from soybean-allergic patients and healthy individuals were tested with an in vitro basophil histamine release assay following stimulation with soybean crude extracts. Passive sensitization of basophils was performed with washed polymorphonuclear cells obtained from nonatopic donors. After sensitization, basophils were stimulated with different lines of soybeans. A total of six GM and six non-GM soybeans were tested after incubation with soybean allergic serum. The mean histamine releases were 0.77 ng/ml with GM-soybeans and 0.71 ng/ml with non-GM soybeans. On analyzing histamine release triggered by different lines of soybeans, there were no statistically significant differences found between GM and non-GM soybeans (Figure 7). This meant that there were no apparent differences of allergenic components in GM soybean or non-GM soybean by this evaluation method.

Discussion:

In this study, we found that 9 out of 10 of soybean-allergic patients (who had specific IgE to soybean crude extract in their sera) were sensitive to the allergenic component of Gly m Bd 30K. Gly m Bd30K could there be considered a major allergen of soybeans in the Taiwan population. Gly m Bd 30K allergen was also reported by others to be the major allergen of soybean⁷. Gly m Bd 30K is a protease and has IgE binding ability^{8, 15}. It is localized in storage vacuoles of soybean seeds. It has also been identified as major allergen of Gly m 1 (also known as Gly m Bd30K or P34) (The GenBank accession numbers, AB013289)⁷.

Polyclonal antibody-based ELISA was developed in this study to quantify Gly m Bd 30K and Gly m 4. Using the standard of recombinant Gly m Bd 30K/ Gly m 4 as internal standard, we found a good correlation between the content of Gly m Bd 30K/Gly m 4 in the crude extract and recombinant Bd 30K/Gly m 4. Allergic reactions caused by allergens are related to the levels of allergen concentrations. Soybean proteins are potential allergens for food allergy and the allergic symptoms closely related to the level of allergen concentration, therefore measurement of allergen levels in the soybeans will offer adequate information to lay down rules to help regulating the commercialization of soybeans. The use of this method of quantification allows rapid assessment of soybean samples, enabling the determination of allergenic components.

We provided here the first report on the intrinsic allergens (Gly m Bd 30K/ Gly m 4) on GM soybeans. The two allergenic components (Gly m Bd30K/ Gly m 4) only slightly increased their concentrations after GM and with no statistical significance. It has been reported that the nutrient composition of transgenic soybean (event DAS-81419-2) grown in the United States and Brazil is equivalent to nontransgenic soybean²⁴. Consistent with our findings, the allergic risks of GM soybeans has been investigated to be unaltered in terms of the IgE sensitization ratios. Similar finding had been reported that the IgE sensitization rates to wild-type and GM soybeans were identical (3.8% of allergic adults), and circulating IgE antibodies specific for the two extracts were

comparable²⁵. The ELISA inhibition test, SDS-PAGE, and IgE immunoblotting showed a similar composition of IgE-binding components within the wild-type and GM extracts, which was confirmed using two-dimensional gel electrophoresis, IgE immunoblotting, and amino acid sequencing²⁵. It has been investigated the variability of five allergen levels (Gly m 4, Gly m 5, Gly m 6, Gly m Bd 28k, and Gly m Bd 30k) in conventional soybean by ELISAs, the multivariate statistical analyses and pairwise comparisons shows that environmental factors have a larger effect on allergen levels than genetic factors²⁶.

Gly m 4 can induce birch pollen-related soy allergy is well known¹⁹. Severe allergic reaction after consuming soy products in patients with birch pollinosis has been reported¹⁸. The mechanism was considered to be related to IgE antibodies against rGly m 4¹⁸⁻¹⁹. Our study showed that anti-Gly m4 antibody bound to a protein band at 17kDa in both GM and non-GM soybeans. The quantitative analysis of Gly m4 levels slightly increased in the GM soybeans but the increase was not statistically significant. Cross-reaction between Bet v 1 and Gly m 4 has been reported before, and that is consistent with our finding in supplementary data (Figure-S2). Patients with anaphylaxis to soybean also reported to have IgE binding to Gly m 4 in the soy extract. Results indicated that GM soybeans did not aggravate the allergic reactions for patients with pre-existing soybean allergy. Therefore, we believe that it is unlikely that GM-soybeans augment the allergic reaction in patients with Bet v 1 allergy.

In this study, transcript gene expressions of soybean allergens (Gly m Bd 30K, Gly m 4 and Gly m Bd 28K) were compared using the “Next Generation Sequencing Analysis System” which involved Transcriptome sequencing and Illumina Solexa[®] sequencing. Results showed Gly m 4 (stress-induced protein SAM22) and Gly m Bd 28K (soybean allergen precursor) fold increased (ratio: 22.32 of Gly m 4; 29.62 of Gly m Bd 28K) after genetic modifications. But there were no significant differences between non-GM and GM soybean in the protein level. We speculated the detection sensitivity of gene expressions by Transcriptome sequencing is higher than protein

quantification by antibodies we used. There is another possible reason that the gene expression is controlled at different stages and many different ways, including transcriptional regulation and post-transcriptional regulation. Therefore, in most of the cases, change in mRNA levels and protein levels do not correlate that well mainly due to the regulation control at different levels.

Our study also demonstrated a slight increase of Gly m Bd 30K protein levels in the GM soybean, but with no statistical significance in terms of IgE binding and histamine release. Results are suggestive of no additional allergic reactions caused by GM in patients with soybean allergy. Gly m Bd 30k was slightly increased (insignificant), but IgE binding and histamine release were unchanged. It could be explained by the GM which did not affect the functional epitope of Gly Bd 30k. The exact underlying mechanisms remain to be further explored on epitope mapping.

Reactions to soybean have been reported in some patients with IgE-mediated cow milk allergy¹⁷. Gly m Bd30K, as recognized by IgE antibodies of serum from cow milk allergic patients, was reported to be involved in the cross-reactivity between soybean and bovine caseins¹⁶. In our study, we did not find differences in GM and non-GM soybeans on histamine release and IgE-binding. Therefore, there could be no augmentation of allergic reaction in cow-milk (caseins) allergic patients. In conclusion, Gly m Bd 30k is likely the major allergen in soybean and it is present in both GM and non-GM soybeans, as recognized by anti-Gly m Bd 30K and soybean allergic serum. Anti-Gly m Bd30k can be used for the major allergen identification in soybeans. The concentrations of Gly m Bd 30k were similar in GM and non-GM soybeans. Similar finding was also observed for Gly m 4. Given that the intrinsic allergens of soybean including Gly m Bd 30K and Gly m 4 were similar after GM. GM soybean will not augment the allergic reaction in soybean-sensitive patient. The limitations of this study included small samples analyzed, and that the allergenic components in the soybeans were not comprehensively investigated. Although the allergenicity of soybean in GM and non-GM detected by current experimental techniques are similar, it cannot be concluded that the allergen in GM-soybean is not upregulated until all

398 soybean allergens been identified. In this study, only two major allergens in soybean had been
399 evaluated after the GM. We cannot therefore rule out the possibility that other allergens in
400 GM-soybeans could be upregulated. And more samples of soybeans will be needed to clarify the
401 altered gene expressions focusing in the allergens caused by GM.
402

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Table:

Table 1: The altered gene expressions in soybean allergens between GM and Non-GM

Gene name	Protein product	Functional Description (Allergen)	GM	Non-GM	Ratio	
			Soybean	soybean	(GM/Non-GM)	p_value
LOC548062	NP_001238219.1	P34 probable thiol protease precursor (Gly m Bd 30k)	0.19	0.18	1.07	1
LOC547915	NP_001236038.1	stress-induced protein SAM22 (Gly m 4)	12.43	0.56	22.32	0.0104
LOC547942	NP_001267501.1	Soybean allergen precursor (Gly m Bd 28K)	17.23	0.58	29.62	0.0058

Figure legends:**Figure 1. GM soybean Confirmation by PCR of 35S gene.**

PCR products were amplified with 35S-Forward and 35S-Reverse primers, and analyzed with 1% agarose gel electrophoresis; M: DNA marker; rbCL gene 599 bp fragment as internal control of soybean; GM: genetic modified; NGM: non- genetic modified.

Figure 2. Comparison of IgE-binding allergenic components in organic, non-GM and GM soybean.

(A) Soybean seed from three different sources showing its size and appearance. (B) Electrophoresis of crude extracts (20 µg) from organic, non-GM and GM soybeans were performed on 12 % SDS-PAGE. M: Protein molecular marker; lane 1: organic soybean; lane 2: non-GM soybean; lane 3: GM-soybean. (C) Western blot analysis performed by soybean crude extracts reacted with serum from soybean-allergic individual. Lane 1: organic soybean, Lane 2: non-GM soybean, Lane 3: GM soybean. Healthy: serum from nonallergic individuals.

Figure 3. Protein profiles and Western blot of different soybean crude extracts.

(A) The recombinant Gly m 4 was purified from *E. coli* induction lysate using His-tagged affinity chromatography with Ni-NTA-Sepharose. Lane 1: supernatant collected from lysate after sonication and centrifugation; lane 2: flowthrough after resin binding; lane 3: rGly m 4 elution tube 1; lane 4: rGly m 4 elution tube 2. (B) Electrophoresis of crude extracts (20 µg) from organic, non-GM and GM soybeans were performed on 12 % SDS-PAGE. M: Protein molecular marker; lane 1: organic soybean; lane 2: non-GM soybean; lane 3: GM-soybean. (C) Western blot analysis after reaction with rabbit anti-Gly m 4 polyclonal antibodies. (D) Rabbit pre-immune serum acting as the control for immunization.

Figure 4. Protein profiles and Western blot analysis of different soybean crude extracts

based on anti- Gly m Bd 30K. (A) Electrophoresis of crude extracts (20 µg) from non-GM (lines

1-5,11-15) and GM soybean (lines 6-10, 6-10 and 16-20) were performed on 12 % SDS-PAGE. M: Protein molecular marker. (B) Left panels: Western blot reacted with mouse anti- Gly m Bd 30K polyclonal antibodies. (B) Right panels: Mouse pre-immune serum acted as a control for immunization.

Figure 5. Comparison of allergic component-Gly m Bd30k in non-GM and GM soybeans.

(A) Electrophoresis of crude extracts from non-GM (lines 1-5 & lines 11-15) and GM soybean (lines 6-10, 6-10 and 16-20) were performed on 12 % SDS-PAGE. Western blot analysis was reacted with pooled serum samples from soybean-allergic patients or healthy subjects. (B) The Western blots of IgE-binding intensities were quantified with a digital imaging system “ImageQuant[™] LAS 4000”. Data were presented as mean $\pm$ standard deviation.

Figure 6. Quantitative results on Gly m 4 and Gly m Bd 30K in GM and Non-GM soybeans.

(A) Allergen levels of Gly m 4. (B) Allergen levels of Gly m Bd 30K. A total of 20 soybeans seeds (10 Non-GM and 10 GM) were used for the allergen determination using enzyme-linked immunosorbent assay (ELISA). The contents of Gly m 4 and Gly m Bd 30 K in soybeans were evaluated by different concentration of Gly m Bd 30 K (range between 781 pg/ml to 50,000 pg/ml) and Gly m 4 (range between 156 pg/ml to 10,000 pg/ml) were coated on ELISA plates as standard. The soybean crude extracts were coated in duplicate at the protein concentration of 0.4 ug/ml to determine its allergen concentration.

Figure 7. Histamine release assay of GM and non-GM soybeans. Basophils were presensitized with sera from soybean-allergic individuals and were then stimulated with different lines of soybean at 10 μ g/mL. GM: Genetic Modified; non-GM: non genetic modified. A23187: calcium ionophore (Calcimycin); as a positive control for histamine release.

537 **Abbreviations:**

538 **GM**, genetic modification; **PR**, pathogenesis-related; **Bet v 1**, the major white birch (*Betula*
539 *verrucosa*) pollen allergen; **PCR**, polymerase chain reaction; **qPCR**, real-time quantitative PCR;
540 **ELISA**, Enzyme-linked immunosorbent assay; **OPA**, O-phthalaldehyde; **NGM**, non- genetic
541 modified; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis; **PBS**,
542 phosphate buffered saline; **Gly m 4**, *Glycine max* allergen; **Gly m Bd 30 K**, 30kDa soybean
543 allergen; **SAM22**, Stress-induced protein of *Glycine max*; **CFA**, Complete Freund's adjuvant; **OD**,
544 optical density; **FPKM**, Fragments Per Kilobase of transcript per Million mapped reads.

Figures

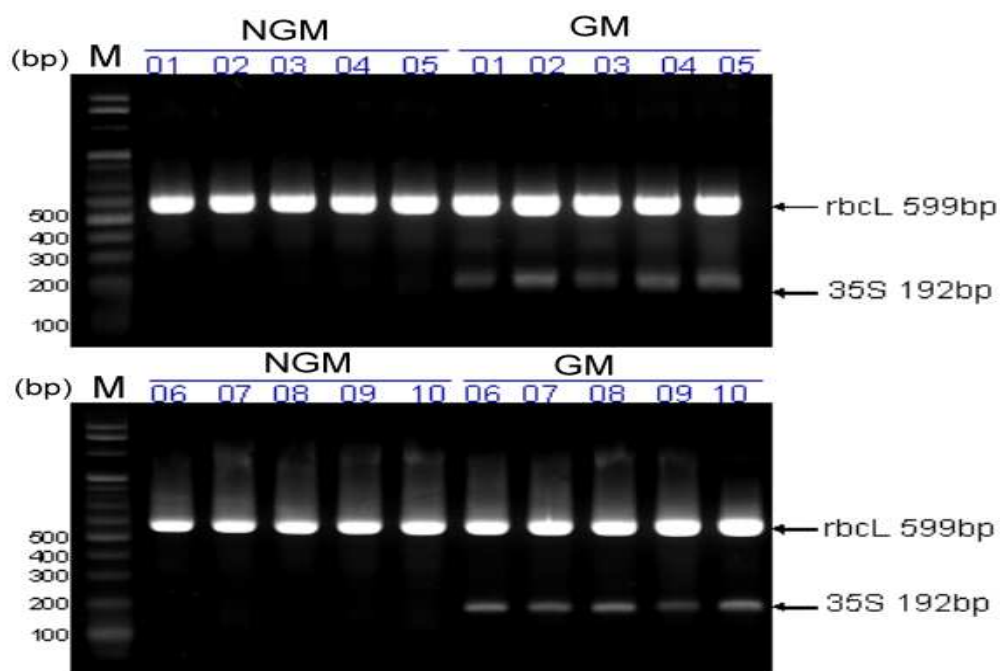


Figure 1. GM soybean Confirmation by PCR of 35S gene.

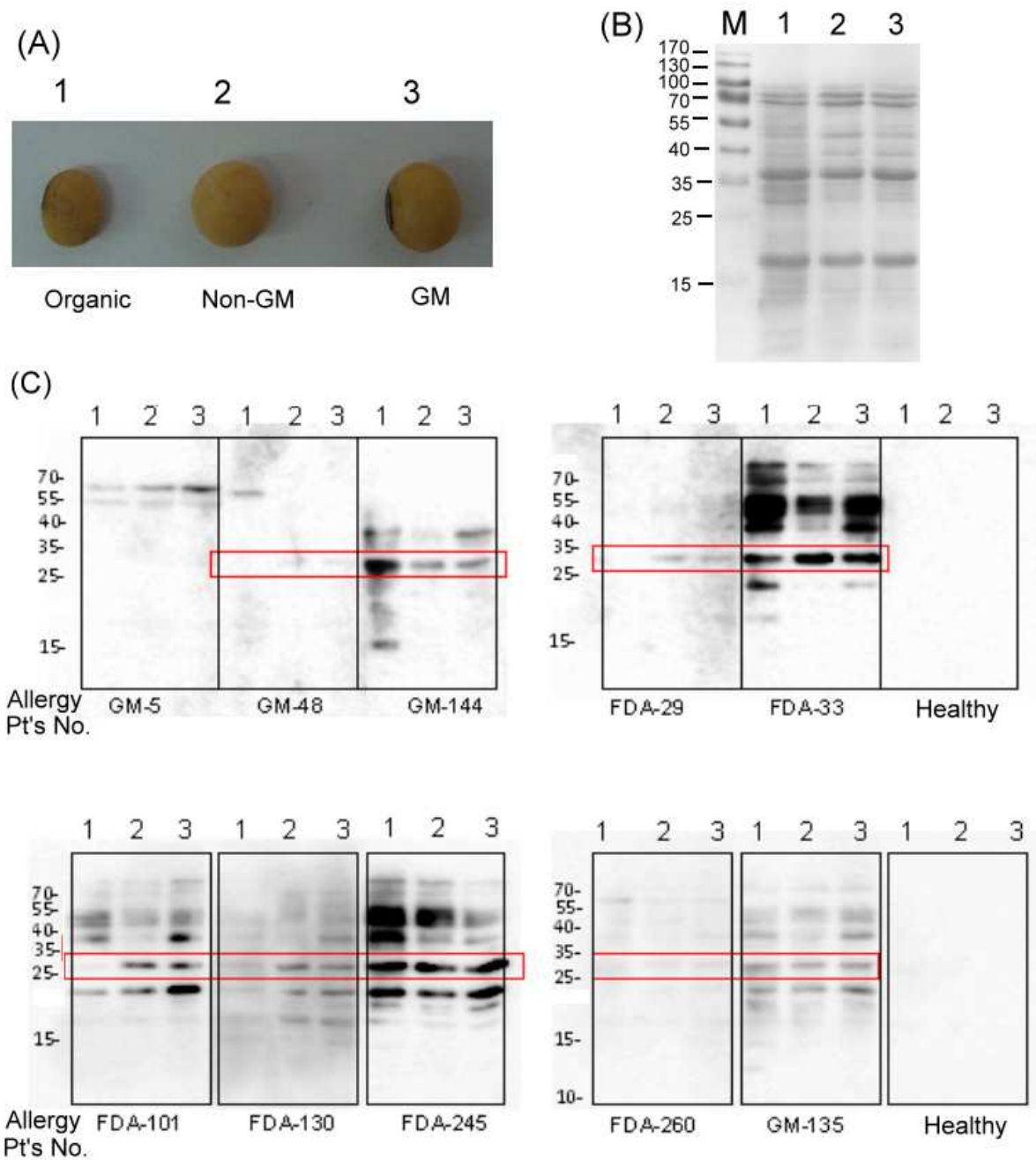


Figure 2. Comparison of IgE-binding allergenic components in organic, non-GM and GM soybean.

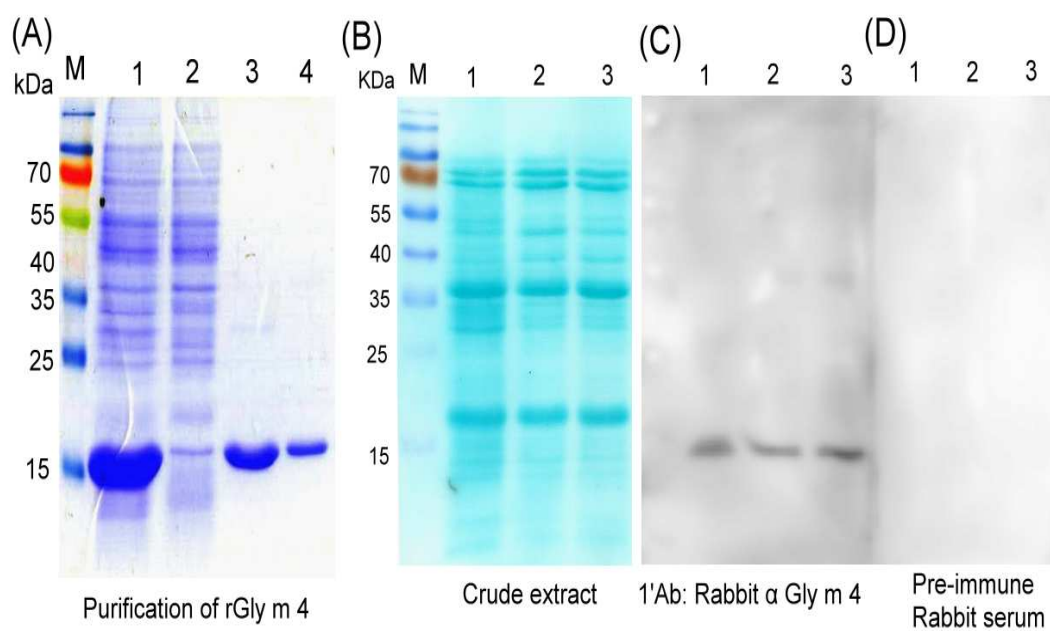


Figure 3. Protein profiles and Western blot of different soybean crude extracts.

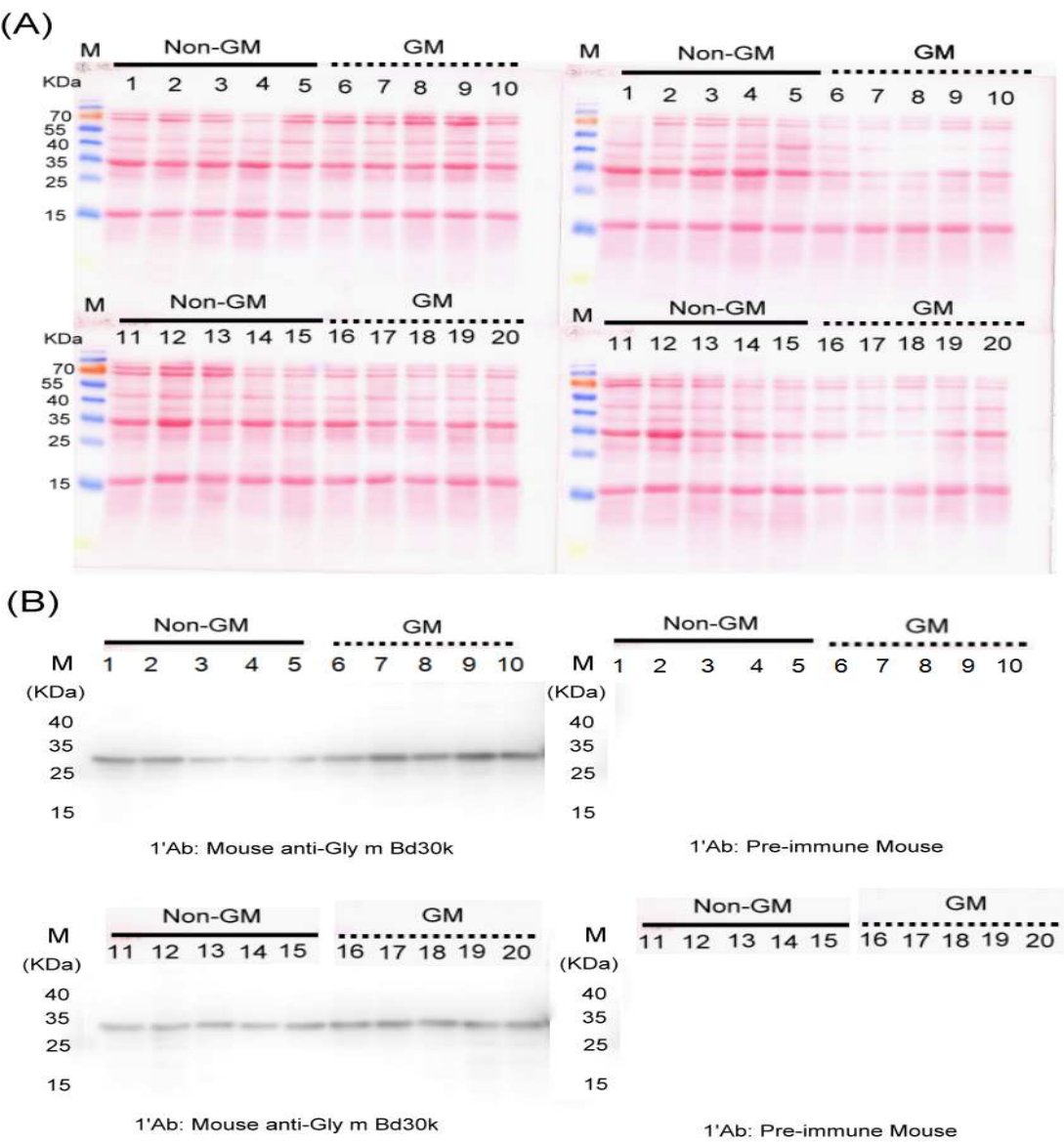


Figure 4. Protein profiles and Western blot analysis of different soybean crude extracts based on anti- Gly m Bd 30K.

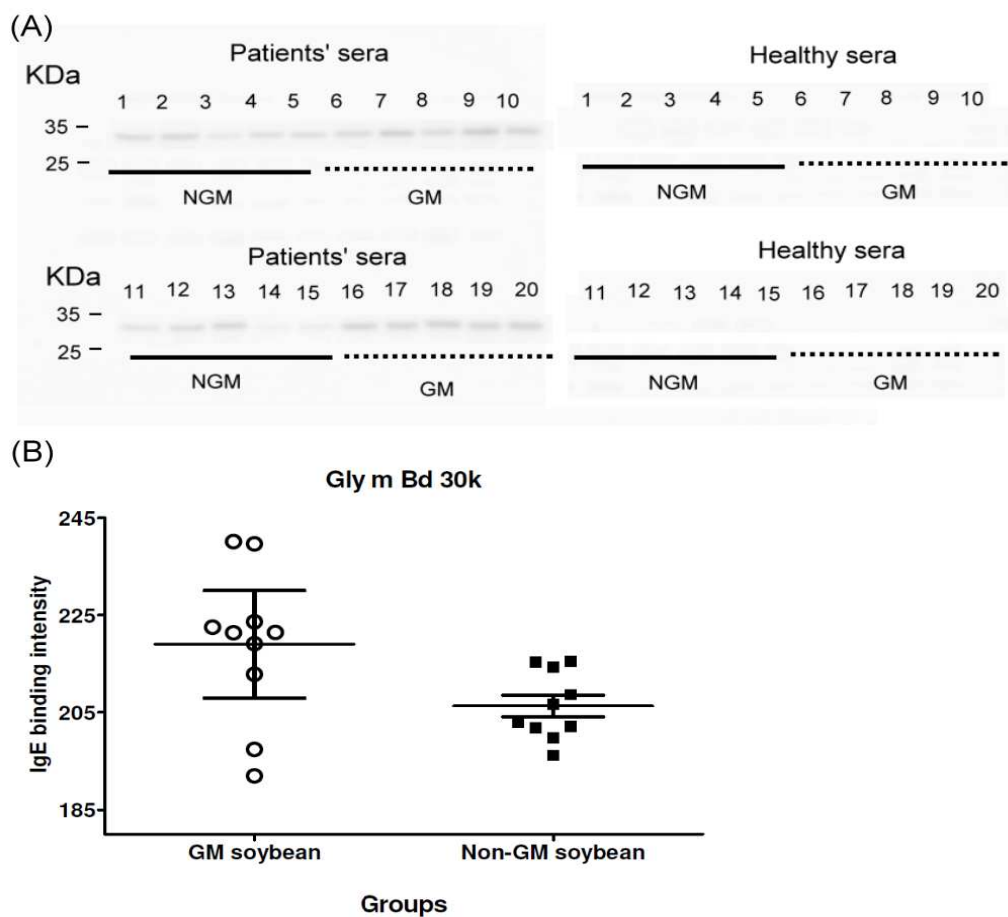


Figure 5. Comparison of allergic component-Gly m Bd30k in non-GM and GM soybeans.

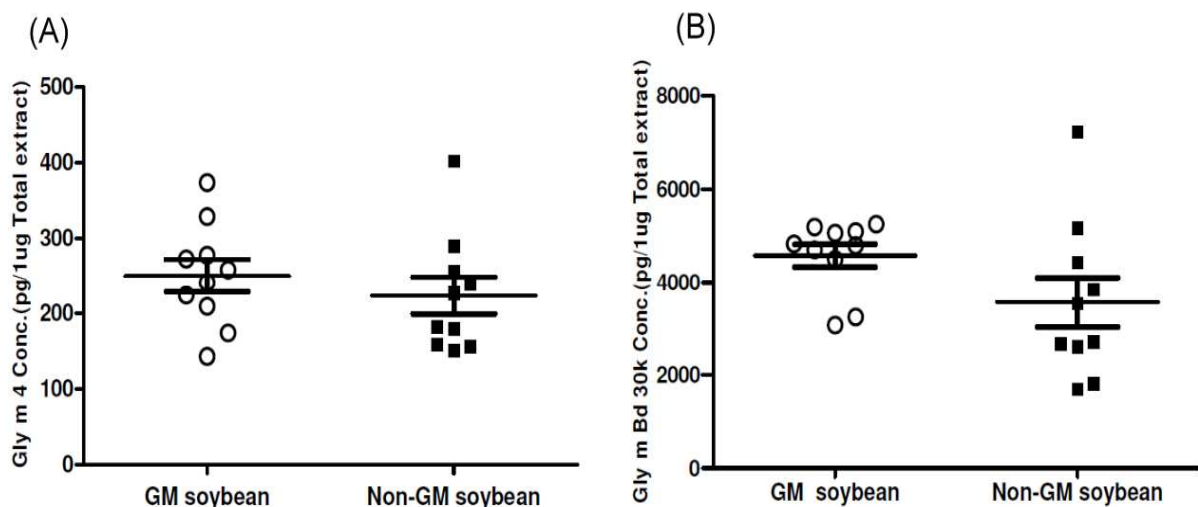


Figure 6. Quantitative results on Gly m 4 and Gly m Bd 30K in GM and Non-GM soybeans.

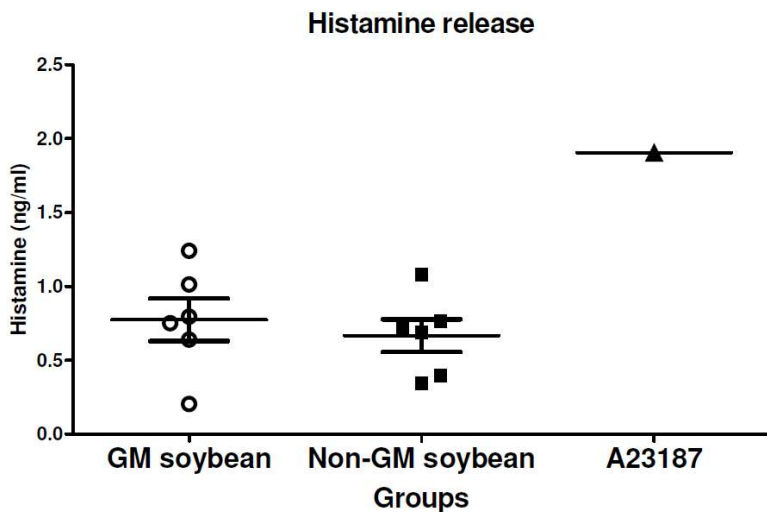
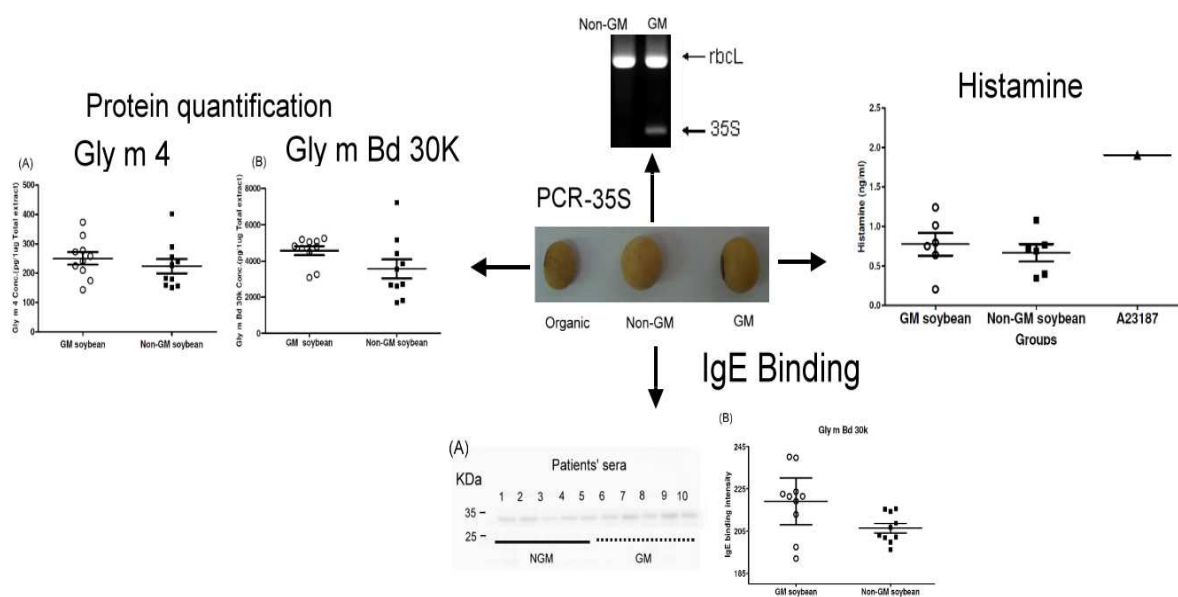


Figure 7. Histamine release assay of GM and non-GM soybeans.



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