

A comparative study of human IgE binding to proteins of a genetically modified (GM) soybean and six non-GM soybeans grown in multiple locations

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ABSTRACT

Prior to commercialization, genetically modified (GM) crops are evaluated to determine the allergenicity of the newly expressed protein. Some regulators require an evaluation of endogenous allergens in commonly allergenic crops including soybean to determine if genetic transformation increased endogenous allergen concentrations, even asking for IgE testing using sera from individual sensitized subjects. Little is known about the variability of the expression of endogenous allergens among non-GM varieties or under different environmental conditions. We tested IgE binding to endogenous allergenic proteins in an experimental non-commercial GM line, a non-GM near-isoline control, and five non-GM commercial soybean lines replicated at three geographically separated locations. One-dimensional (1D) and two-dimensional (2D) immunoblotting and ELISA were performed using serum or plasma from eleven soybean allergic patients. The results of immunoblots and ELISA showed no significant differences in IgE binding between the GM line and its non-GM near-isoline control. However, some distinct differences in IgE binding patterns were observed among the non-GM commercial soybean lines and between different locations, highlighting the inherent variability in endogenous allergenic proteins. Understanding the potential variability in the levels of endogenous allergens is necessary to establish a standard of acceptance for GM soybeans compared to non-GM soybean events and lines.

1. Introduction

Many genetically modified (GM) crops have been developed by the insertion of a gene, which encodes a protein that confers the traits of interest (NASEM, 2016). Common traits in GM crops include herbicide resistance, drought tolerance, delayed ripening, bacterial disease resistance, high oleic acid levels, and resistance to insect pests (Chrispeels, 2014; Delmer, 2014; Metcalfe et al., 1996; Newell-McGloughlin, 2014; Taylor and Hefle, 2001). Farmers in the United States (U.S.) have rapidly adopted the use of GM soybean with herbicide tolerance and GM maize with herbicide tolerance and insect resistance since they were first commercialized in 1996 (Fernandez-Cornejo et al., 2014; James, 2008). The use of new GM traits has grown remarkably since then. New GM crops intended for food and/or feed use undergo a rigorous safety assessment to ensure no biologically

relevant intended or unintended changes occurred in their nutrients, anti-nutrients, toxins, and allergens (Goodman, 2014; Goodman et al., 2013; König et al., 2004).

Soybean [*Glycine max* (L.) Merr.] ranks as one of the most common allergenic foods recognized by regulatory authorities worldwide. The use of soybean must be declared on labels of processed food in the U.S. and the European Union (EU) (EU, 2011; FDA, 2004). However, soybean allergy only affects approximately 0.1–0.2% of the general population although higher prevalence is found in infants and children under 10 years of age, although substantially lower than the incidence of allergy from other commonly allergenic foods (Gupta et al., 2011; Roehr et al., 2004; Sicherer and Sampson, 2010; Taylor et al., 2015). Because soybean is a common allergenic food, there is an additional regulatory requirement for developers of GM soybean compared to those developing GM-maize and other GM crops that are not considered

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; 2-DE, two-dimensional gel electrophoresis; CCD, cross-reactive carbohydrate determinants; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EU, European Union; EFSA, European Food Safety Authority; GM, genetically modified; HRP, horseradish peroxidase; IgE, immunoglobulin E; LS-Means, least squares means; NFDm, non-fat dry milk; OD, optical density; PBS, phosphate buffered saline; PBST, PBS plus 0.05% Tween 20; vdc, volts of direct current; w/v, weight to volume

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major sources of food allergy. Based on regulatory guidance, all GM crops are assessed for allergenicity by considering the source of the gene, comparison of the amino acid sequence of the newly expressed protein to known allergens, evaluation of the stability of the newly expressed protein to pepsin digestion at acidic pH, and determination of abundance of the protein in the plant tissues used as food. However, for soybean and other commonly allergenic crops, the additional measure is the relative level of known endogenous allergens to ensure that the genetic modification has not increased the level of endogenous allergens to an extent that would adversely impact human health. A number of human serum IgE binding studies have been performed with new GM soybeans (Burks and Fuchs, 1995; Panda et al., 2013). The European Food Safety Authority (EFSA) requested specific serum IgE screening, to evaluate whether the GM crop has approximately equivalent IgE binding to its closest non-GM counterpart, the near-isoline, and other non-GM commercial varieties of the crop (EFSA, 2006, 2011). Recently the European Union (EU) began requiring comparison of IgE binding using individual soy allergic subjects or analytical methods to measure certain endogenous allergens in the GM plant compared with its non-GM comparator(s) (EU, 2013).

Currently, there is a lack of understanding of the range of variation of endogenous allergens between different non-GM varieties with different genotypes and the natural variability of endogenous proteins in non-GM varieties of a crop. Yet, this information is required by some regulators to evaluate whether differences found between a GM crop and its non-GM counterpart(s) are biologically relevant (Doerr et al., 2010). When the EU mandated quantitative analysis of individual proteins identified as allergens in the comparative compositional analysis of soybean (EU, 2013), they included a number of proteins that had not been characterized or proven to be allergens. Additionally the natural variation of the expression of the individual allergens among the non-GM commercial soybean lines had not been determined. A recent proteomics study showed the variation for some soybean allergens can be large across soybean lines using proteomics methods (Houston et al., 2010).

The objectives of this study were to evaluate the differences in IgE binding between an experimental non-commercial GM line, its non-GM near-isoline control and five non-GM commercial soybean lines of different genetic backgrounds. The intent was to determine whether the transformation or the transgene might cause differences or whether the different genetic backgrounds or other factors were likely sources of variation in IgE binding. Since the environmental conditions, agronomic practices, soil conditions may change the expression of endogenous allergens (Fernandez et al., 2013), natural variability was also taken into account by including soybean lines replicated in multiple different geographical locations in this study.

2. Materials and methods

2.1. Soybean samples

Fifteen individual full-fat, ground samples of soybean representing multiple geographical replicates of the GM soybean line, the non-GM near-isoline control soybean, and five other non-GM commercial soybean lines from replicated field trials were tested (listed in Table 1). Non-soybean control samples including raw Spanish peanuts, raw navy bean and maize meal that were purchased at a grocery store in Lincoln, NE.

2.2. Human serum and plasma

A total of 11 human sera and plasma samples were acquired from two sources (Table 2). Human sera from seven soy-allergic patients were obtained from the University Hospital Zürich, Switzerland and plasma from four soy-sensitized subjects were purchased from PlasmaLab International, Everett, WA, U.S. Diagnosis of the seven soy-

Table 1

List of soybean samples with multiple geographical replicates.

Soybean lines	Test sites	Number of individual soybean lines
GM	Iowa (IA), Indiana (IN), Kansas (KS)	3
Non-GM near-isoline control	IA, IN, KS	3
Non-GM commercial line 1	IA, IN	2
Non-GM commercial line 2	KS	1
Non-GM commercial line 3	IA, IN, KS	3
Non-GM commercial line 4	IN	1
Non-GM commercial line 5	IA, KS	2

Table 2

Soybean allergic human serum and plasma samples.

Human Subject ID	ImmunoCAP® (kU/I)			Symptoms as reported by history ^a
	Soy	Gly m 4	Total IgE	
<i>Soy-allergic Human Sera from University Hospital Zürich</i>				
Neb-3	1.18	34.4	N/A ^b	OAS ^c
Neb-4	2.06	N/A	N/A	OAS, nausea, emesis
Neb-5	9.23	N/A	N/A	OAS, dyspnea, drop blood pressure
Neb-6	4.55	N/A	N/A	OAS, dyspnea, angioedema, conjunctivitis, tightness throat
Neb-7	< 0.35	26.2	N/A	OAS
Neb-8	6.42	1.58	N/A	OAS, nausea, emesis, diarrhea, gastrointestinal pain
Neb-9	2.10	9.12	N/A	OAS, urticaria, angioedema
<i>Soy-sensitized Human Plasma from PlasmaLab International</i>				
19392-CS	71.60	N/A	896	Angioedema, vomiting
20197-BH	3.00	N/A	N/A	Itchy throat with nuts and raw vegetables.
22329-JE	5.00	N/A	1953	N/A
9735-RE	5.00	N/A	> 5000	Anaphylaxis to peanut. Soybean causes sore throat, itchy mouth, and queasy stomach.

^a The reported symptoms from soy-allergic subjects were soy-allergic symptoms only. The reported symptoms from soy-sensitized subjects were not strict to soy-related symptoms.

^b N/A, not available.

^c OAS, oral allergy syndrome.

allergic patients from Switzerland was confirmed by double-blind placebo-control food challenge or very clear clinical histories and diagnostic tests. The four soy-sensitized subjects collected by PlasmaLab were identified based on self-reported allergies to soybean using a standard questionnaire and positive detectable ImmunoCAP® IgE binding to soybean. Human sera from five individuals without reported allergy to soybean who did not experience symptoms in an open food challenge with soy milk and with very low soybean-specific IgE (< 0.35 kU/L ImmunoCAP® value) were provided by the University Hospital of Zürich as negative controls (clinical data not shown). All serum and plasma samples were collected from subjects who provided voluntary consent following specific institutional review board approval (University Hospital in Zürich) or collections by a U.S. Food and Drug Administration licensed facility (PlasmaLab).

2.3. One-dimensional (1D) IgE immunoblotting

The 15 individual full-fat soybean flour samples, as well as ground peanut, navy bean and maize samples were extracted with phosphate buffered saline (PBS) buffer containing protease inhibitor cocktail (Thermo Fisher Scientific). The protein content of the clarified extracts were determined using a Lowry DC protein assay kit (BioRad). Equal

volumes of the geographical replicated soybean extracts of the same soybean lines were pooled to provide a single sample of each line for immunoblots. A total of seven pooled soybean PBS extracts, including GM soybean extract, non-GM near-isoline control extract and each non-GM commercial line were analyzed using SDS-PAGE. The SDS-PAGE was performed under both reducing (with β -mercaptoethanol and heating) and non-reducing conditions to check the staining pattern of the denatured proteins as well as for immunoblots as described by Panda et al. (2013). Proteins were separated by electrophoresis in 10–20% Tris-glycine gels (Invitrogen) along with the Precision Plus molecular weight marker (BioRad). A representative gel was stained with Colloidal Brilliant Blue G250 (Sigma). Proteins in identically treated gels were electro-transferred to polyvinylidene difluoride (PVDF) membranes and then blocked with 5% non-fat dry milk (NFDM) in PBS plus 0.05% Tween 20 (PBST) for 2 h. Individual soybean allergic or non-allergic control sera were diluted appropriately (1: 5, 1:10 or 1:20) in 2.5% NFDM before incubating with the blocked membrane overnight. Bound IgE was detected, after washing off unbound IgE, using monoclonal horseradish peroxidase (HRP) conjugated anti-human IgE (1:1000 dilution) with SuperSignal West Dura Extended Duration chemiluminescence substrate (Thermo Fisher Scientific). Emitted light images were captured using a Kodak Gel Logic 440 image station. A Nitrocellulose membrane spotted with dilutions of purified myeloma human IgE (Abcam) was used as an intensity control. Nitrocellulose membranes were blocked with 5% NFDM and incubated with secondary antibody and substrate similar to the treatment of PVDF immunoblots to evaluate relative signal strength.

2.4. Two-dimensional (2D) IgE immunoblotting

Due to the limited availability of soybean allergic sera, the two-dimensional gel electrophoresis (2-DE) and immunoblotting were designed for optimum relative comparison of four soybean extracts (the GM, non-GM near-isoline and one separate and one pool of two non-GM commercial lines) captured at a fixed time for each donor serum or plasma sample. Based on the results of 1D immunoblot, commercial line 3 appeared to have a minor band difference and was used separately. Commercial lines 1 and 2 appeared to be identical in IgE binding and equal amounts of ground samples were pooled prior to extraction. Proteins were extracted from each of the four pooled soybean samples using a method modified from the publication of Natarajan et al. (2005). Each 1 g of pooled soybean flour sample was mixed with 10 ml of cold acetone solution containing 10% trichloroacetic acid and 0.07% β -mercaptoethanol and stored at -20°C overnight. After centrifugation, the pellet was washed by addition of 10 ml of cold acetone followed by centrifugation and then removal of acetone. The washing step was repeated and the pellet was then dried for 20 min in a chemical hood to eliminate most of the acetone. The pellets were suspended in 10 ml of 8 M urea with 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) buffer by vortexing and shaken for 2 h. Samples were clarified by centrifugation and supernatants aliquoted into microfuge tubes and frozen at -80°C . One tube was thawed for each sample and the protein content was determined by Bradford assay (BioRad) to ensure equivalent sample application.

A PROTEAN[®] i12[™] isoelectric focusing system (BioRad) was used to focus the proteins at their isoelectric points. Samples representing 40 μg of protein were diluted to a final volume of 125 μl of rehydration buffer with volumes adjusted to represent 8 M urea, 2% CHAPS, 50 mM dithiothreitol and 0.5% ampholytes. The 125 μl sample was applied to non-linear 7 cm immobilized pH gradient strips (pH 3–10) in the focusing tray. Samples were covered with mineral oil. Active rehydration was performed at 50 V of direct current (vdc), at 20°C for 12 h, followed by separation with the program set to rapid ramping to 250 vdc for 15 min followed by gradual ramping up to 4000 vdc for 2 h for a total of 37,500 integrated volt-hours during this final step. Sample positions were maintained with a constant 500 vdc after isoelectric

focusing was complete. The next morning, the strips were removed and proteins were reduced with dithiothreitol and acetylated with iodoacetamide containing equilibration buffer. Separation in the second dimension of 2-DE was performed on NuPAGE[®] Novex 4–12% Bis-Tris ZOOM[®] gels (Invitrogen) by placing the immobilized pH gradient strip in the wide-well of the SDS-PAGE gel. Precision Plus molecular weight marker was added to the small well as a standard. The wells were sealed with low-temperature agarose. Samples were electrophoresed at 150 vdc for 60 min. Representative gels were stained overnight in EZBlue[™] Gel Staining Reagent (Sigma) and the images captured using the Kodak GL440 Image Station. Proteins from identically loaded and electrophoresed sample gels were transferred to PVDF membranes without fixing. The PVDF membranes were then immunoblotted with soybean allergic or soybean non-allergic serum and plasma samples with bound IgE detected using HRP conjugated anti-IgE as described for 1D immunoblots. Standard amounts of diluted IgE was applied to nitrocellulose and were detected along with the immunoblots to provide an indication of the relative exposure time and sensitivity.

2.5. ELISA analysis

The 15 individual soybean PBS extracts prepared in section 2.3 were tested as IgE targets in ELISA assays. The extracts were diluted to a protein concentration of 0.01 mg/ml in carbonate-bicarbonate buffer (pH 9.6) and applied to wells of a 96-well microtiter plate (1 $\mu\text{g}/\text{well}$, Nunc) in triplicate wells and incubated overnight at 4°C . The plate was then washed and blocked with 1% bovine serum albumin for 1 h at 37°C . Eleven individual soybean allergic sera or plasma samples and a pool of five non-soybean allergic sera were diluted (between 1:5 and 1:20) with the blocking buffer before applying 100 μl of diluted sera/plasma to designated wells. Plates were incubated for 2 h at 37°C . After washing to remove unbound IgE, 100 μl of 1:5000 diluted monoclonal anti-human IgE-HRP (SouthernBiotech) was added to each well and incubated for 1 h at 37°C . The plate was then washed and incubated with 100 $\mu\text{l}/\text{well}$ of tetramethylbenzidine substrate (Sigma) in the dark for 20 min at room temperature. Reactions were stopped by adding 100 $\mu\text{l}/\text{well}$ of 1 N sulfuric acid. Absorbance was measured immediately at 450 nm using a PowerWave HT 340 microplate reader (BioTek).

2.6. Statistical analysis

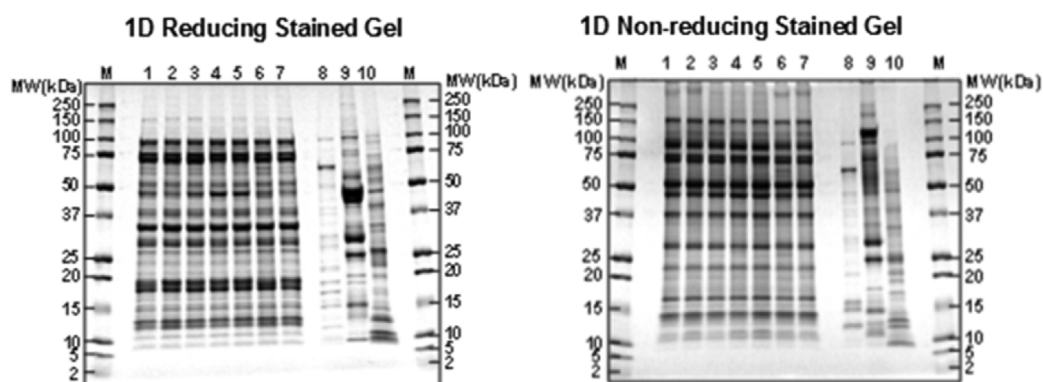
The raw optical density (OD) values were measured at a wavelength of 450 nm in the ELISA reader and values were statistically analyzed using SAS[®] Version 9.2 (SAS Institute Inc.). A linear mixed model analysis complemented by the residual maximum likelihood estimation method was used to estimate the variance components, as well as the means of the lines and the means of the locations. For convenience, those estimated means are referred to as least squares means (LS-Means). Ninety-five percent confidence intervals for LS-Means differences between lines or locations were estimated utilizing Dunnett's multiple comparison adjustment method with line of non-GM near-isoline control soybean and location of Iowa used as references for the comparisons.

3. Results

3.1. 1D SDS-PAGE and 2-DE

There were no obvious qualitative differences in protein expression between soybean lines as demonstrated by Coomassie stained 1D SDS-PAGE run under reducing or non-reducing conditions using samples loaded with equivalent total protein per well (Fig. 1). However, some minor differences were observed in specific band intensities between the soybean lines. For example, the protein bands at ~ 48 kDa on reduced gels were more intense in the non-GM near-isoline control (lane 4) and GM line (lane 5) than in commercial lines (lanes 1, 2, 3, 6, and

1D SDS-PAGE Stained Gels



2-DE Stained Gels

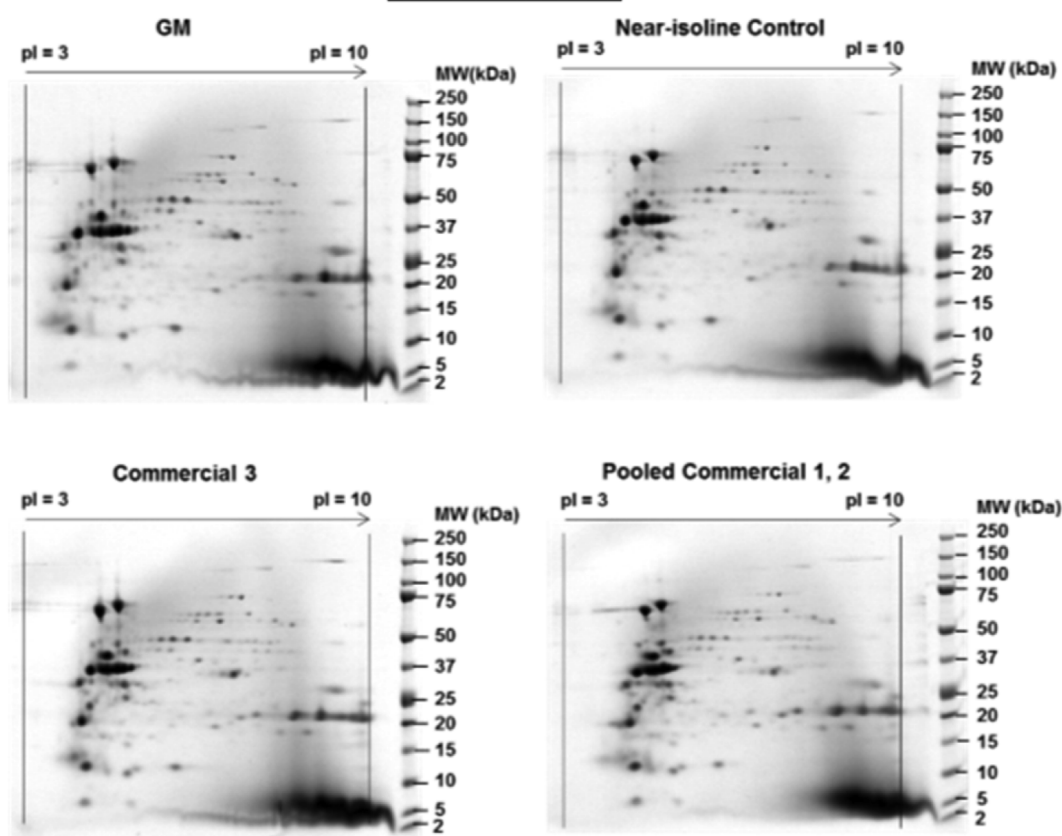


Fig. 1. 1D SDS-PAGE and 2-DE Stained Gels. The lane designations for 1D SDS-PAGE are commercial 1 (lane 1), commercial 2 (lane 2), commercial 3 (lane 3), non-GM near-isoline control (lane 4), GM (lane 5), commercial 4 (lane 6), commercial 5 (lane 7), peanut (lane 8), navy bean (lane 9), maize (lane 10), M (marker).

7). In addition, the 1D SDS-PAGE staining pattern for all soybean lines differed between the reduced and non-reduced conditions as reduction allows separation of subunits of proteins, such as Gly m 6 glycinins that are linked by disulfide bonds or separation of bound proteins containing either inter-peptide or intra-peptide disulfide bonds (Anfinson and Haber, 1961; Maruyama et al., 2003).

There were also no obvious qualitative differences in protein expression between soybean lines as demonstrated by 2-DE using samples loaded at equivalent total protein concentrations per gel (Fig. 1). As is typical for a soybean seed extract (Natarajan, 2014; Xu et al., 2007), most of the proteins have an acidic pI between 3 and 7, and most single proteins are localized between 10 and 90 kDa as shown on the 2-DE gels. Notably, some of the protein spots that separate as a series of spots

at close to the same MW are likely to be minor variants of the same protein that represent either minor isoforms or differences created by post-translational modifications or post-extraction chemical changes such as phosphorylation (Green-Church et al., 2008).

3.2. Qualitative comparison of IgE binding by 1D IgE immunoblotting

Fig. 2 shows the 1D IgE immunoblotting results from 4 representative soybean allergic subjects (Neb-3, Neb-9, 19392-CS, and 22329-JE). For all allergic subjects (data is only shown for four representative subjects), there was no clear qualitative differences detected in IgE binding between the GM soybean (lane 5) compared to the non-GM near-isoline control soybean (lane 4). The non-allergic control

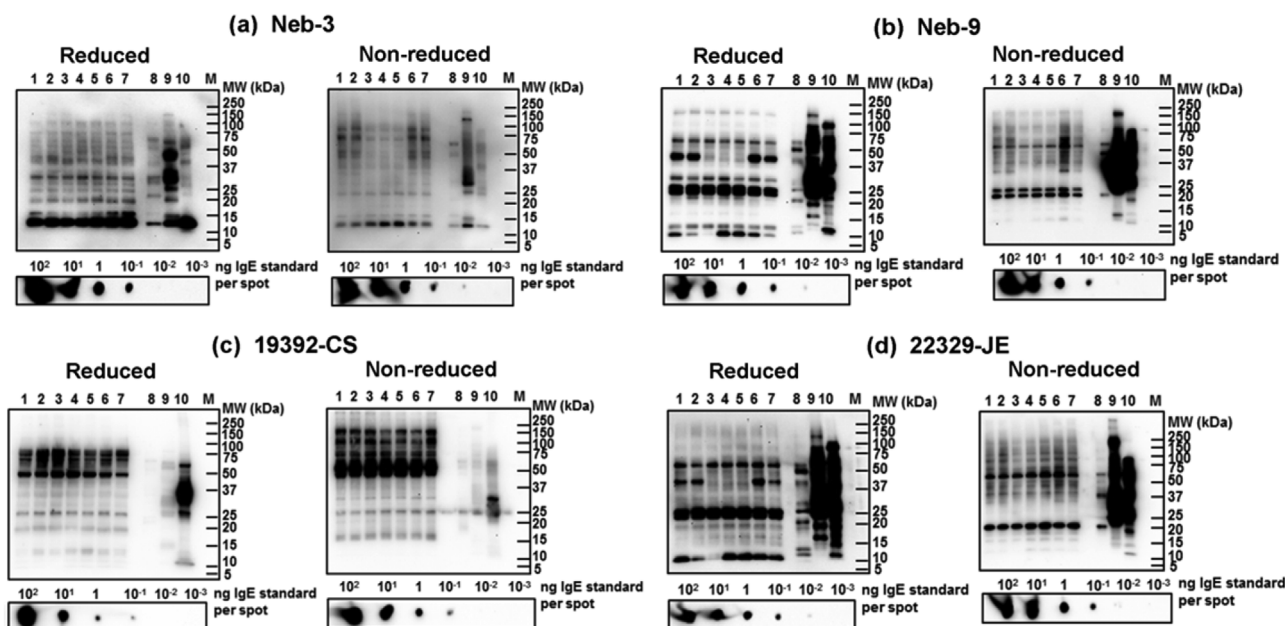


Fig. 2. Representative 1D IgE immunoblots with subjects Neb-3 (a), Neb-9 (b), 19392-CS (c) and 22329-JE (d). Lane designations are same as in Fig. 1.

subjects showed extremely faint IgE binding to a few proteins in the soybean lines at an intensity less than 100 pg of pure IgE on dot blots (not shown). In addition, for all allergic subjects, IgE binding patterns differed between reducing and non-reducing conditions. This was expected as it is known that some subjects have IgE binding to conformational epitopes that may not be present under reducing conditions and furthermore, some common allergenic proteins, such as Gly m 6 glycinins have subunits that separate upon reduction (Maruyama et al., 2003).

For subjects Neb-3, Neb-9 and 22329-JE (Fig. 2 a, b, d), IgE binding patterns to the non-GM commercial lines 1 (lane 1), 2 (lane 2), 4 (lane 6) and 5 (lane 7) were very similar to each other. Whereas, GM (lane 5), non-GM near-isoline control (lane 4) and non-GM commercial line 3 (lane 3) were more similar to each other compared to the other commercial soybean lines. Similar results were also observed in the 1D IgE immunoblots for subjects Neb-7 and 9735-RE (data not shown). In Fig. 2 b and d, a clear IgE binding band was visible at ~40 kDa in the commercial lines 1, 2, 4 and 5, but not in GM, non-GM near-isoline control, or non-GM commercial line 3; a minor IgE binding band was visible at ~10 kDa in all soybean lines except commercial line 3. Thus, minor variation of expression of endogenous allergens were noted across different non-GM commercial soybean lines with different genetic backgrounds.

Subject 22329-JE showed darker IgE binding bands from the GM (lane 5) compared to the non-GM near-isoline control soybean (lane 4) in the non-reduced blot, but the intensity of IgE binding to the GM line was similar to IgE binding to the five non-GM commercial soybean lines (Fig. 2 d). In contrast, IgE binding to the GM line was weaker than to the non-GM near-isoline control soybean in the reduced blot for subject 19392-CS (Fig. 2 c).

Many of the allergic subjects showed IgE binding to proteins in the non-soybean control extracts, indicating that the patients might have multiple food allergies or at least IgE to various natural plant proteins. Importantly, IgE binding to a protein *in vitro* does not accurately predict food allergy as the history of the patients and clinical reports are needed for accurate diagnosis (Goodman et al., 2013). For subjects Neb-3 and Neb-9 (Fig. 2 a and b), strong IgE binding to a protein of ~37 kDa in navy bean in reduced extracts (lane 9) and faint binding to many proteins in all the soybean reduced extracts suggests probable IgE binding to cross-reactive carbohydrate determinants (CCD) on glycoproteins. The 37 kDa protein in navy bean is likely

phytohemagglutinin, which has multiple sites for asparagine linked carbohydrate addition and those are usually CCD with alpha 1–3 fucose or beta 1–2 xylose substitutions. However, the CCD epitopes are considered not relevant for allergic reactivity (Malandain, 2005).

3.3. Qualitative comparison of IgE binding by 2D IgE immunoblotting

Fig. 3 shows the 2D IgE immunoblotting results from the same 4 human subjects of Neb-3, Neb-9, 19392-CS, and 22329-JE used in 1D blots. A pooled sample of five non-soybean allergic control sera did not show any IgE binding to the four soybean extracts in 2D IgE immunoblots (data not shown). For most of the subjects, GM and non-GM near-isoline control showed identical IgE binding patterns to the proteins separated by 2-DE. However, significant differences of IgE binding patterns were observed between the non-GM commercial line 3 and the pool of non-GM commercial lines 1 and 2.

Serum Neb-3 (Fig. 3a) showed moderately strong IgE binding to spot 1 in the GM and non-GM near-isoline control soybeans, but faint binding to spot 1 in the other two non-GM commercial extracts. Spot 2 was invisible in commercial line 3.

Serum Neb-9 (Fig. 3b) showed strong IgE binding to spot 5 in the GM soybean, but faint binding in the non-GM near-isoline control and the pooled commercial lines 1 and 2. Spot 8 was visible in both GM and non-GM near-isoline control soybeans, but not in either commercial extracts. Spot 9 was visible only in the pooled commercial lines 1 and 2 extract.

Plasma 19392-CS (Fig. 3c) showed only relatively minor differences in intensities of binding between soybean lines.

Plasma 22329-JE (Fig. 3d) showed faint IgE binding to spot 2 in the GM, non-GM near-isoline control and commercial line 3. Faint spots 5, 10 and 16 were visible in both the GM and non-GM near-isoline control extracts. Faint spot 6 was visible only in the GM and commercial 3 extracts. Spot 11 was only absent in the non-GM near-isoline control. Spot 18 was only visible in pooled commercial lines 1 and 2. Notably, the IgE binding to the pooled commercial lines 1 and 2 extract included binding to an acidic spot approximately 50–55 kDa for subjects Neb-9 (spot 9 in Fig. 3b) and 22329-JE (spot 18 in Fig. 3d) that was not present in the other three extracts. This extra IgE binding spot correlates with additional IgE binding bands that were observed at ~40 kDa in the reduced 1D SDS-PAGE immunoblots of the same two subjects.

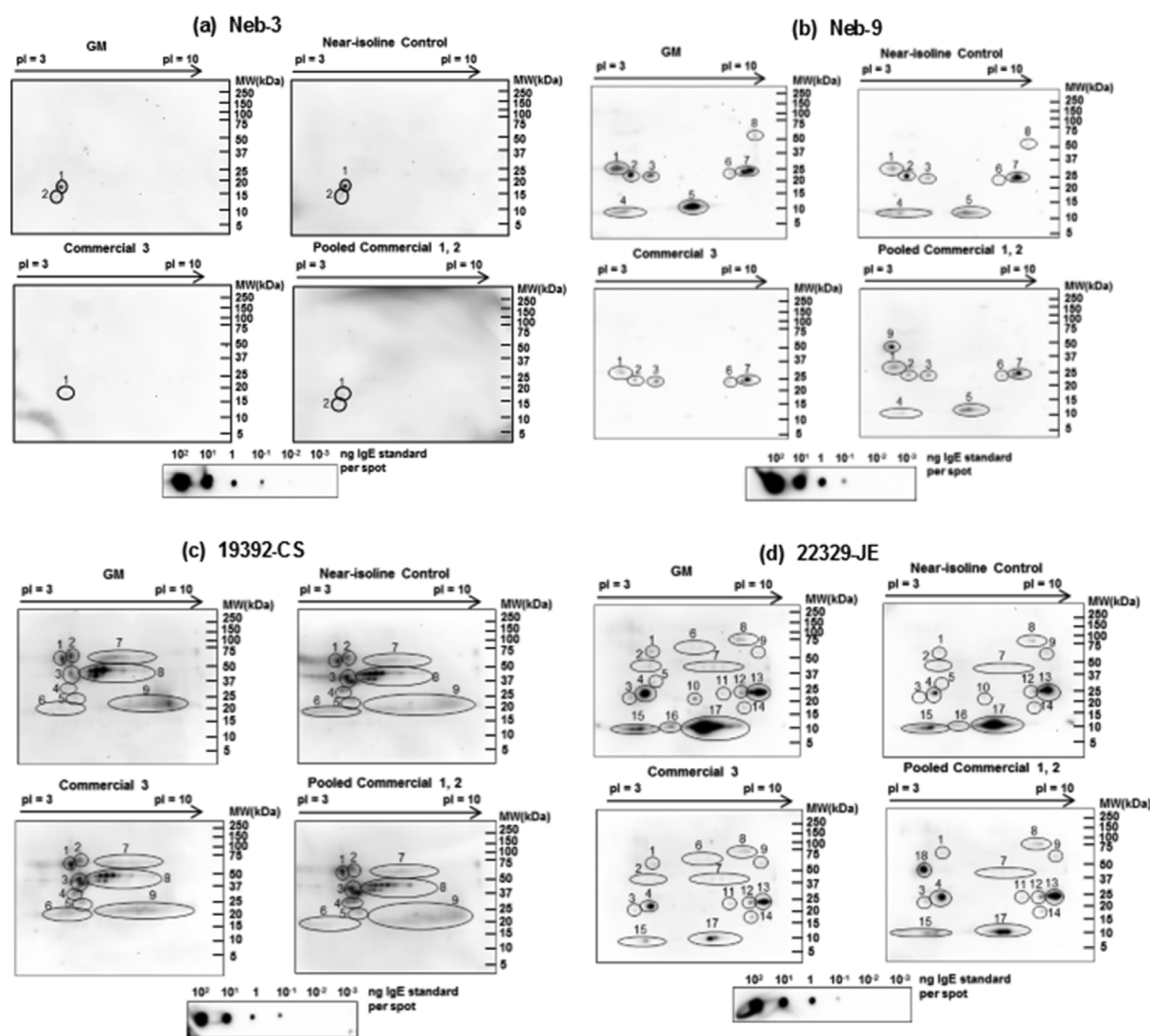


Fig. 3. Representative 2D IgE immunoblots with subjects Neb-3 (a), Neb-9 (b), 19392-CS (c) and 22329-JE (d).

3.4. ELISA analysis

The IgE binding of each serum to each individual soybean sample (soybean lines and geographical replicates) was measured by direct ELISA. The raw OD values were plotted in Fig. 4 without differentiating between the five non-GM commercial lines. Results from the geographical locations of the soybean lines were nested with each subject. Fig. 4 illustrates that there is a noticeable amount of variability between soybean lines and a mild level of variability between locations for each subject. It is noteworthy that IgE binding to the GM soybean line is at the lower end of binding to non-GM commercial lines for each location and for every subject. These data demonstrate that the level of IgE binding and the presumed level of allergenic reactivity of the GM soybean would not be higher than the population of other commercial soybeans including the non-GM near-isoline control soybean and other non-GM commercial lines that were included in this study.

Point estimates and the 95% confidence interval of the LS-mean differences between each soybean line (compared to non-GM near-isoline control) and between each geographical location (compared to Iowa) are shown in Fig. 5. A statistical difference was not identified in OD values between the GM and the non-GM near-isoline control soybeans (P value = .9972). The OD value difference between the GM and non-GM near-isoline control soybean is substantially less than the differences between the non-GM near-isoline control and other non-GM

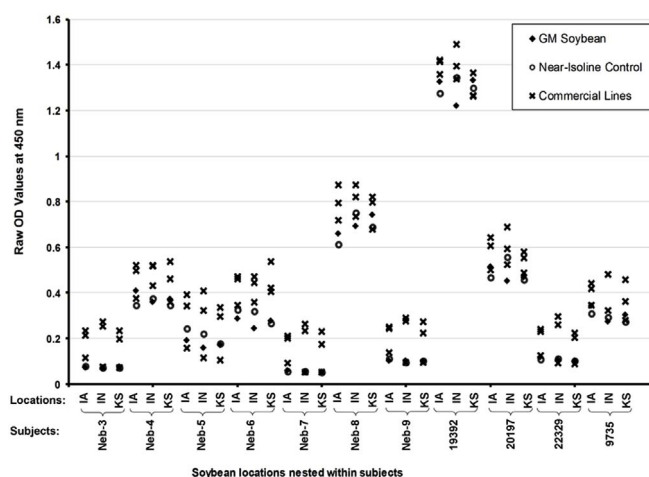


Fig. 4. Comparative analysis of IgE binding to GM, non-GM near-isoline control and non-GM commercial soybean lines using ELISA. The commercial soybean lines 1 to 5 were not differentiated. The locations of the commercial lines were nested within subjects. IA-Iowa, IN-Indiana, KS-Kansas.

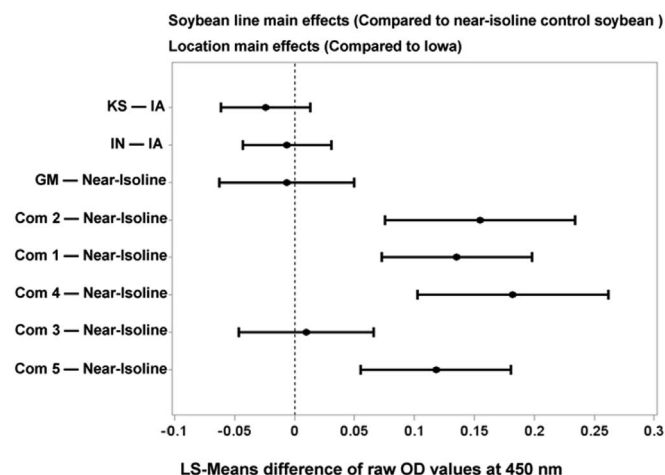


Fig. 5. LS-Means differences between lines or locations estimated with Dunnett's multiple comparison adjustment method. Black dots represent the point estimate of LS-Means differences between lines or locations with non-GM near-isoline control or IA as references, respectively. Horizontal lines with bars at the ends represent 95% confidence intervals for LS-Means differences. The dashed vertical line represents no difference in the estimate of LS-Means. IA-Iowa, IN-Indiana, KS-Kansas, Com-commercial.

commercial soybean lines with different genetic backgrounds (except commercial line 3). The statistical differences between locations (Kansas to Iowa and Indiana to Iowa) for all soybean lines were not significant and not as large as differences between commercial soybean lines and the non-GM near-isoline. The results in this study indicate that genetic differences between soybean lines accounted for more of the variation of IgE binding than the presence of the transgene or the environmental differences. Interestingly, Baker et al. (2006) reported results of a metabolomics study of GM wheat compared to non-GM wheat and Baudo et al. (2006) presented evaluation of the transcriptomes of transgenic and non-transgenic wheat lines. The studies were performed at the Rothamsted Research Center under the direction of Peter Shewry and both showed results demonstrating that the parental genes had a greater impact on the expression of genes than the differences between GM and near-isogenic wheat lines across environments.

4. Discussion

The results of the qualitative (1D & 2D IgE immunoblots) and comparative (ELISA) analyses indicate that the experimental non-commercial GM line was not significantly more potent in IgE binding than the non-GM near-isoline or non-GM commercial soybean lines evaluated in this study. Furthermore, this study demonstrates that normal variation of the expression levels of endogenous allergens in five non-GM commercial lines was larger than the variation between the GM and its non-GM near-isoline. Measurement of samples grown in three geographical locations demonstrates that environmental conditions can influence expression of some endogenous allergens. Thus, evaluation of the genotypic and environmental variation is necessary to put the minor differences observed between the GM and non-GM near-isoline in perspective and certainly to determine whether insertion of a gene is likely to significantly alter expression of endogenous allergens.

Similar results were obtained from other research studies that demonstrated the allergenic potential of soybeans were not affected by the presence of the transgene when compared multiple GM soybeans with wild-type soybeans (Sten et al., 2004; Panda et al., 2013). Analytical measurement of endogenous allergens in conventional soybean lines have also demonstrated a wide range of concentrations due to the differences in genetics in commercial varieties (Houston et al., 2010). These studies add to the weight of evidence that genetic background and environmental factors, such as temperature, moisture, insolation, nutrients, plant pathogens, insect loads, harvesting time, or storage

conditions have much bigger impact on protein (e.g. allergen) expression than insertion of a small number of genes (Goodman et al., 2013; Ladics et al., 2014; Panda et al., 2013).

Recently, novel analytical methods and molecular profiling techniques that do not require human sera have been used by some developers to evaluate concentrations of some endogenous soybean allergens. The EFSA requires quantitative measurement of individual allergens within the compositional analysis (EU, 2013). However, the list of allergens that EFSA lists does not represent clinically significant allergens of soybean as some proteins in their list have not been characterized or sequenced and there is not an accepted range of clinical importance for those proteins. The use of serum IgE tests from samples of soybean allergic subjects in this study helps to focus the evaluation on likely allergens. Regardless of the approach utilized to comparatively assess the endogenous allergens in the GM plants, whether via IgE binding assay or via analytical methods, it is important to place the data interpretation in the context of natural variation. This study has provided additional information to help understand inherent variability in endogenous allergenic proteins in soybean currently used for food. It highlights the importance of establishing adequate non-GM data to capture the range of allergen variability in the soybean, which incompletely known. Importantly, for risk management, soybean allergic individuals should always avoid foods containing soybean regardless of whether it is produced using GM or non-GM soybeans to ensure they are safe. Soybeans are a commodity traded in bulk with mixtures of multiple lines grown in many geographies. Based on this study, it is clear that there are differences that can be measured in the content of IgE binding proteins (presumed allergens) when soybeans are grown in different geographies. The most recent EFSA guidelines call for evaluating the characteristics of proteins grown in eight field trial sites. That requirement puts a large burden on developers and it is clear from this study that differences are likely to be random between soybean lines and locations. The safety evaluation of a new GM soybean should be based on an overall evaluation of whether the GM line is as safe as the range of soybeans currently in commerce. In reviewing our results and results of other studies including analytical measurements of individual proteins (e.g. Houston et al., 2010), the focus of the safety evaluation can be made based on the safety of the introduced protein and the overall agronomic and nutritional characteristics of the crop. A recent analytical study used mass spectrometry to quantitate 14 proteins that were termed endogenous allergens (Hill et al., 2017). They reported markedly different concentrations of the 14 proteins from 21 varieties, tested in seven laboratories. The proteins varied from five to fifty fold across measurements. Their assay did not evaluate IgE binding from allergic sera and some of the proteins are extremely low in abundance. Others have not been proven to be allergens. As we have discussed previously, no limits have been set based on science that differentiate between potential risk and increased or lack of risk for GM varieties compared to non-GM varieties (Goodman et al., 2013; Panda et al., 2013). When considering food safety, we should acknowledge that some foods have marginal amounts of soybean protein such as soy sauce, while others are dominated by native or processed proteins in soybean including texturized soy protein and soy protein isolate. Those products are safe for those who do not have allergies to soybean. A clear question to ask then is how much of an increase might be acceptable for specific endogenous allergens in a GM soybean compared to a non-GM soybean? If we do not have a science-based answer, it seems this question should not be part of the safety evaluation of GM soybeans. The logical answer for soybean allergic consumers is to avoid products containing any soybean. The rest of the population would not have a health risk from the consumption of soybean.

5. Conclusions

From this study, we conclude there is no increase in endogenous allergens in this GM soybean, compared to non-GM near-isoline or non-

GM commercial lines. Thus, the GM soybean line would not pose any additional risk of allergenicity to the consuming public compared to other soybean lines. The results of this study demonstrate that IgE binding is variable between different soybean varieties, between the same soybean varieties grown in different environmental conditions, as well as between different soybean allergic subjects. Variability in the expression of endogenous allergens in soybean is mainly affected by the genetic background of the soybean, not the transgene, or insertion point. Our ELISA results measured the overall IgE binding of each subject's blood sample with an extract from each soybean line. The results suggest environmental conditions and soybean line genotypes can influence expression of some allergens and the overall allergenic activity of the soybeans. Understanding the inherent variability of IgE binding and expression of endogenous allergens in soybeans are necessary for context of biological relevance regarding the safety of GM soybeans. As this study and others have shown, soybeans of different genetic backgrounds, grown under different conditions present a range of concentrations of allergens. It is not clear that highly refined measurement of each allergen reported from soybean using LC-MS/MS or protein-specific IgG ELISA assays will improve the safety evaluation of new GM events. It is also not clear that differences in the expression of any soybean protein alters risks of food allergy for those with or without allergy to soybeans. Importantly, people consume foods that contain diverse soybean lines, grown in different locations. Thus we suggest that requirements for such tests are not relevant to food safety evaluation.

Conflict of interest statement

Authors declare that there are no conflicts of interest. The costs for this study was supported by a grant from an unnamed biotechnology company.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.fct.2018.01.001>

References

- Anfinsen, C.B., Haber, E., 1961. Studies on the reduction and re-formation of protein disulfide bonds. *J. Biol. Chem.* 236, 1361–1363.
- Baker, J.M., Hawkins, N.D., Ward, J.L., Lovegrove, A., Napier, J.A., Shewry, P.R., Beale, M.H., 2006. A metabolomic study of substantial equivalence of field-grown genetically modified wheat. *Plant Biotechnol. J.* 4, 381–392.
- Baudo, M.M., Lyons, R., Powers, S., Pastori, G.M., Edwards, E.J., Holdsworth, M.J., Shewry, P., 2006. Transgenesis has less impact on the transcriptome of wheat grain than conventional breeding. *Plant Biotechnol. J.* 4, 369–380.
- Burks, A.W., Fuchs, R.L., 1995. Assessment of the endogenous allergens in glyphosate-tolerant and commercial soybean varieties. *J. Allergy Clin. Immunol.* 96 (6), 1008–1010.
- Chrispeels, M.J., 2014. Global production and consumption of genetically engineered crops. *J. Huazhong Agric. Univ.* 33, 120–132.
- Delmer, D., 2014. GM crops and food security. *J. Huazhong Agric. Univ.* 33, 1–9.
- Doerr, N., Ladics, G., McClain, S., Herouet-Guicheney, C., Poulsen, L.K., Privalle, L., Stagg, N., 2010. Evaluating biological variation in non-transgenic crops. In: Executive Summary from the ILSI Health and Environmental Sciences Institute Workshop, November 16–17, 2009, Paris, France. *Regulatory Toxicology and Pharmacology*, vol 58, pp. S2–S7.
- EFSA, 2006. Guidance document for the risk assessment of genetically modified plants and derived food and feed by the Scientific Panel on Genetically Modified Organisms (GMO) - including draft document updated in 2008. *EFSA J.* 9, 1–100.
- EFSA, 2011. Guidance for risk assessment of food and feed from genetically modified plants. *EFSA J.* 9, 1–50.
- EU, 2011. Food Allergen Labelling and Information Requirements under the EU Food Information for Consumers Regulation No. 1169/2011: Technical Guidance.
- EU, 2013. European commission implementing regulation (EU) No. 503/2013 of 3 April 2013 on applications for authorization of genetically modified food and feed in accordance with regulation (EC) No. 1829/2003 of the European Parliament and of the council and amending commission regulations (EC) No. 641/2004 and (EC) No. 1981/2006. *Offic. J. Eur. Union* 56, 1–48.
- FDA, 2004. Food Allergen Labeling and Consumer Protection Act of 2004.
- Fernandez-Cornejo, J., Wechsler, S., Livingston, M., Mitchell, L., 2014. Genetically Engineered Crops in the United States, Economic Research Report No. (ERR-162). 60 pp, February 2014. United States Department of Agriculture, U.S.
- Fernandez, A., Mills, E.N.C., Lovik, M., Spoek, A., Germini, A., Mikalsen, A., Wal, J.M., 2013. Endogenous allergens and compositional analysis in the allergenicity assessment of genetically modified plants. *Food Chem. Toxicol.* 62, 1–6.
- Goodman, R.E., 2014. Biosafety: evaluation and regulation of genetically modified (GM) crops in the United States. *J. Huazhong Agric. Univ.* 33, 85–114.
- Goodman, R.E., Panda, R., Ariyaratna, H., 2013. Evaluation of endogenous allergens for the safety evaluation of genetically engineered food crops: review of potential risks, test methods, examples and relevance. *J. Agric. Food Chem.* 61, 8317–8332.
- Green-Church, K.B., Nichols, K.K., Kleinholz, N.M., Zhang, L., Nichols, J.J., 2008. Investigation of the human tear film proteome using multiple proteomic approaches. *Mol. Vis.* 14, 456–470.
- Gupta, R.S., Springston, E.E., Warrier, M.R., Smith, B., Kumar, R., Pongracic, J., Holl, J.L., 2011. The prevalence, severity, and distribution of childhood food allergy in the United States. *Pediatrics* 128, e9–e17.
- Hill, R.C., Oman, T.J., Wang, X., Shan, G., Schafer, B., Herman, R.A., Tobias, R., Shippar, J., Malayappan, B., Sheng, L., Xu, A., Bradshaw, J., 2017. Development, validation and interlaboratory evaluation of a quantitative multiplexing method to assess levels of ten endogenous allergens in soybean seed and its application to field trials spanning three growing seasons. *J. Agric. Food Chem.* 65, 5531–5544.
- Houston, N.L., Lee, D.-G., Stevenson, S.E., Ladics, G.S., Bannon, G.A., McClain, S., Privalle, L., Stagg, N., Herouet-Guicheney, C., MacIntosh, S.C., Thelen, J.J., 2010. Quantitation of soybean allergens using tandem mass spectrometry. *J. Proteome Res.* 10, 763–773.
- James, C., 2008. Global status of commercialized biotech/GM crops: 2007. *J. Chin. Biotechnol.* 28, 1.
- König, A., Cockburn, A., Crevel, R.W.R., Debruyne, E., Grafstroem, R., Hammerling, U., Kimber, I., Knudsen, I., Kuiper, H.A., Peijnenburg, A.A.C.M., Penninks, A.H., Poulsen, M., Schauzu, M., Wal, J.M., 2004. Assessment of the safety of foods derived from genetically modified (GM) crops. *Food Chem. Toxicol.* 42, 1047–1088.
- Ladics, G.S., Budziszewski, G.J., Herman, R.A., Herouet-Guicheney, C., Joshi, S., Lipscomb, E.A., McClain, S., Ward, J.M., 2014. Measurement of endogenous allergens in genetically modified soybeans – short communication. *Regul. Toxicol. Pharmacol.* 70, 75–79.
- Malandain, H., 2005. IgE-reactive carbohydrate epitopes-classification, cross-reactivity, and clinical impact. *Eur. Ann. Allergy Clin. Immunol.* 37, 122–128.
- Maruyama, N., Fukuda, T., Saka, S., Inui, N., Kotoh, J., Miyagawa, M., Hayashi, M., Sawada, M., Moriyama, T., Utsumi, S., 2003. Molecular and structural analysis of electrophoretic variants of soybean seed storage proteins. *Phytochemistry* 64, 701–708.
- Metcalf, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L., Fuchs, R.L., 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit. Rev. Food Sci. Nutr.* 36, 165–186.
- NASEM, 2016. Genetically engineered crops: experiences and prospects. In: *Medicine* (Ed.), E. The National Academies of Science. The National Academies Press, Washington, DC.
- Natarajan, S., Xu, C., Caperna, T.J., Garrett, W.M., 2005. Comparison of protein solubilization methods suitable for proteomic analysis of soybean seed proteins. *Anal. Biochem.* 342, 214–220.
- Natarajan, S.S., 2014. Analysis of soybean seed proteins using proteomics. *J. Data Min. Genom. Proteonomics* 5, 1–3.
- Newell-McGloughlin, M., 2014. Future directions, development and application of GM crops in the USA. *J. Huazhong Agric. Univ.* 33, 31–39.
- Panda, R., Ariyaratna, H., Amnuaycheewa, P.T., Afua, Pramod, S.N., Taylor, S.L., Ballmer-Weber, B.K., Goodman, R.E., 2013. Challenges in testing genetically modified crops for potential increases in endogenous allergen expression for safety. *Allergy* 68, 142–151.
- Roehr, C.C., Edenharter, G., Reimann, S., Ehlers, I., Worm, M., Zuberbier, T., Niggemann, B., 2004. Food allergy and non-allergic food hypersensitivity in children and adolescents. *Clin. Exp. Allergy* 34, 1534–1541.
- Sicherer, S.H., Sampson, H.A., 2010. Food allergy. *J. Allergy Clin. Immunol.* 125, S116–S125.
- Sten, E., Skov, P.S., Andersen, S.B., Torp, A.M., Olesen, A., Bindslev-Jensen, U., Poulsen, L.K., Bindslev-Jensen, C., 2004. A comparative study of the allergenic potency of wild-type and glyphosate-tolerant gene-modified soybean cultivars. *APMIS Acta Pathol. Microbiol. Immunol. Scand.* 112, 21–28.
- Taylor, S.L., Hefle, S.L., 2001. Will genetically modified foods be allergenic? *J. Allergy Clin. Immunol.* 107, 765–771.
- Taylor, S.L., Remington, B.C., Panda, R., Goodman, R.E., Baumert, J.L., 2015. Detection and Control of Soybeans as a Food Allergen, Handbook of Food Allergen Detection and Control. Woodhead Publishing, pp. 341–366.
- Xu, C., Caperna, T.J., Garrett, W.M., Cregan, P., Bae, H., Luthria, D.L., Natarajan, S., 2007. Proteomic analysis of the distribution of the major seed allergens in wild, landrace, ancestral, and modern soybean genotypes. *J. Sci. Food Agric.* 87, 2511–2518.