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Challenges in testing genetically modified crops for potential increases in endogenous allergen expression for safety

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Abstract
Premarket, genetically modified (GM) plants are assessed for potential risks of food allergy. The major risk would be transfer of a gene encoding an allergen or protein nearly identical to an allergen into a different food source, which can be assessed by specific serum testing. The potential that a newly expressed protein might become an allergen is evaluated based on resistance to digestion in pepsin and abundance in food fractions. If the modified plant is a common allergenic source (e.g. soybean), regulatory guidelines suggest testing for increases in the expression of endogenous allergens. Some regulators request evaluating endogenous allergens for rarely allergenic plants (e.g. maize and rice). Since allergic individuals must avoid foods containing their allergen (e.g. peanut, soybean, maize, or rice), the relevance of the tests is unclear. Furthermore, no acceptance criteria are established and little is known about the natural variation in allergen concentrations in these crops. Our results demonstrate a 15-fold difference in the major maize allergen, lipid transfer protein between nine varieties, and complex variation in IgE binding to various soybean varieties. We question the value of evaluating endogenous allergens in GM plants unless the intent of the modification was production of a hypoallergenic crop.

Keywords: endogenous allergen, genetically modified, IgE, maize, rice, soybean

Abbreviations: ANOVA, analysis of variance; GM, genetically modified; HRP, horse radish peroxidase; kU/L, kilounits of IgE per liter; mLTP, maize lipid transfer protein; MAb, monoclonal antibody; NFDM, nonfat dry milk; PBST PBS, containing 0.05% Tween 20; PVDF, polyvinylidene difluoride; TCA, trichloroacetic acid; TMB, 3,3´,5,5´-tetramethylbenzidine.

Genetic improvement in agriculturally important plants has contributed to increased food, fiber, and energy production for centuries and increasingly during the past 40 years. Until the 1990s, genetic changes were introduced by relatively uncontrolled methods including out-crossing with wild relatives, radiation or chemical mutagenesis followed by back crossing and selection (1, 2). Since 1985, genetically modified (GM) plants have been developed using *Agrobacterium tumefaciens* transformation vectors (3, 4) or DNA-coupled particle bombardment (5) to introduce genes from unrelated sources (e.g. bacteria into soybeans). The GM trait is then introduced into diverse genetic varieties by classical breeding for use in various geographical regions and climatic conditions. These methods will help to meet growing food demand as the population climbs toward 9 billion by 2050 (6–8). Today <200 GM events have been introduced in 25 crops (primarily soybean, maize, and cotton) and approved for production in at least one country (9).

Approval of GM crops for commercial production and for importation as food or feed follows extensive testing and is the responsibility of individual countries or cooperating countries (e.g. the European Union). Ideally, regulations follow internationally vetted guidelines of the Codex Alimentarius Commission for the assessment of food safety of GM organisms (10), to facilitate international trade (11). The allergenicity assessment

is an important focus because food crops in common use are generally recognized as safe except for individuals with specific food allergies. The primary concern is the potential transfer of a major allergen from a different species into a food crop as was the case when a Brazil nut 2S albumin was transferred into soybean to improve nutritional quality (12). The Codex (10) calls for serum IgE testing if the source of the gene is allergenic. Potential cross-reactivity is also evaluated based upon amino acid (protein) sequence identity comparisons to known allergens. If a significant sequence match is identified (e.g. >35% identity over 80 or more amino acids) using a well-curated allergen database such as the peer-reviewed FARRP database (www.allergenonline.org), similar IgE testing would be required (11). The assessment also evaluates the likelihood that the novel protein might sensitize susceptible individuals. Factors considered by Codex (10) and the European Food Safety Authority (EFSA) (13) include stability of the protein in pepsin and abundance in food materials. However, the risks of de novo sensitization and subsequent food allergy are difficult to predict and health risks of most allergens are relatively low (14).

The possibility of significant increases in the expression of endogenous allergens due to insertion of the transgene is in Codex (10) as part of a "compositional analysis of key components" including nutrients and anti-nutrients or toxicants and is addressed by EFSA (13, 15). Most countries have required evaluating endogenous allergens only for GM host plants (gene recipients) that are common causes of allergy (e.g. soybean), but not maize. The Codex guideline did not specify testing methods or criteria for acceptance or rejection, and the EFSA guidance has changed markedly from 2006 to 2011. Testing methods accepted by specific regulatory agencies have varied between submissions of applications even under the same guidelines from 1994 until 2012. Tests of herbicide-tolerant soybean event 40-3-2 (Monsanto Co., St. Louis, MO, USA) showed no differences in IgE binding to extracts of the parental line and two other commercial soybean varieties using 1D gel immunoblotting with a pool of five soybean food challenge–positive allergic subjects (16). The 40-3-2 trait has been bred into genetically diverse commercial varieties and is the dominant GM crop globally. Similar studies were not required for insect-resistant maize (MON810), herbicide-tolerant maize, or GM cotton as those crops present little risk of food allergy. Recently, the use of complex testing methods (e.g. proteomics) has been suggested by some regulatory agencies along with requests for testing of crops that rarely cause food allergy (e.g. corn). Scientists in the Japanese regulatory agency have performed tests to evaluate possible changes in endogenous allergens expressed in a GM rice event (17). Yet food allergy to rice is extremely rare and rice is used as a "safe" weaning food in many countries (18). While the theoretical possibility of increased expression of endogenous allergens is a scientifically interesting question (19), individuals allergic to a specific food (e.g. soybean or maize) must avoid consuming conventional and GM varieties of the crop to remain symptom free. Furthermore, the amount and variety of individual commodity crop materials in processed foods is highly variable and the commodity is often processed in ways that markedly alter allergen content or form (e.g. soybean protein isolate, lecithin or fermentation), which may alter allergen content and exposure. Yet, risks of food allergy are rarely considered for food products containing non-GM commodity materials. This study presents results of tests evaluating the endogenous allergen content in a small number of commercially available maize and soybean varieties as well as a specific GM soybean event to provide some examples of variation in the expression of allergens. Based on our previous experiences performing studies to evaluate endogenous allergen content of four GM soybean events, appropriate tests are difficult to perform and expensive. Submitted dossiers often stimulate additional technical questions from regulators that have caused delays of approvals in some countries, potentially causing additional indirect costs for food and animal feed without improving safety.

Materials and methods

Maize major allergen, lipid transfer protein

Pastorello et al. (20) characterized maize LTP (mLTP) as a food allergen for Italian subjects that also experience allergic reactions to peaches due to a nearly identical LTP. Previously, a peptide (AARTTADRRA) corresponding to amino acids 67–76 of mLTP was synthesized and used to immunize rabbits to produce antiserum used in this study (RE Goodman unpublished at Monsanto, 2002). Natural mLTP was purified from maize following Pastorello's procedure (20) for use as a standard for immunoassays. The identity of the purified mLTP was confirmed by LC-MSMS analysis, with 84% coverage of the sequence of Tchang et al. (21). Extracts of corn and purified mLTP were separated in SDS-PAGE, immunoblotted, and detected using the rabbit anti-mLTP-peptide serum followed by goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) and SuperSignal West-DURA chemiluminescent substrate (Thermo Scientific, Rochester, IL, USA). Emitted light from blots was captured by a Kodak Gel Logic 440 image station (Carestream Health, Rochester, NY, USA). A semiquantitative dot-blot immunoassay was developed and validated using equal volume spots of a dilution series of reduced and denatured mLTP on nitrocellulose. Samples of nine distinct non-GM maize hybrids (DKC-50-20, DKC60-19, DKC61-73, DKC63-46, Mo17xB73, N60-B6, N69-P9, N70- F1, and N76-D3) grown without irrigation in replicate plots (n = 2) at the University of Nebraska research station at Mead, NE, were extracted. Equal volumes (2 µl) of reduced and denatured sodium acetate extracts of grain samples (10 µg total protein) containing 2% of β-mercaptoethanol (Sigma, St. Louis, MO, USA) were heated to 95°C prior to spotting (in triplicate) on nitrocellulose. The mLTP was detected and the concentration of mLTP estimated from image densities using 1D software (Kodak, Carestream Health, Rochester, NY, USA) compared with diluted, purified mLTP. Previous tests (unpublished) demonstrated that spotting of equal concentrations of pure mLTP and pure LTP spiked into extracts of unrelated plant protein produced equivalent spots.

Soybean endogenous allergen evaluation

BASF Plant Science (Research Triangle Park, NC, USA), collaborated with EMBRAPA (Empresa Brasileira de Pesquisa Agropecuaria, Brazilia, Brazil) to develop a GM soybean, BPS-CV127-9 that is tolerant to imidazolinone herbicides due to expression of a transgene encoding imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) from *Arabidopsis thaliana*. The expressed protein was previously characterized as presenting a low potential risk of allergenicity based on Codex guidelines (10) based on nonallergenic source, lack of sequence similarity to allergens, low abundance, and rapid digestion in pepsin using an assay similar to Ofori-Anti et al. (22). Here, we report a summary of the study to evaluate potential changes in the expression of endogenous allergens following Codex (2003) and EFSA (2011) guidelines.

BASF provided full-fat flour samples of the GM event BPS-CV127 (no. 3410-T), near-isoline (no.3410-I), parental variety Conquista (EMBRAPA), two nontransgenic commercial soybean lines: MON8001 (no. 3415-M) and Coodetec 217 (no. 3416-C). Soybean flour samples and samples of ground and defatted peanuts (*Arachis hypogaea*), ground navy beans (*Phaseolus vulgaris*) and maize grain were extracted at room temperature in PBS with protease inhibitor cocktail (Thermo Scientific). Samples were clarified by centrifugation and filtration. Protein concentrations were measured by Lowry DC protein assay (BioRad, Hercules, CA, USA). Additional extracts of soybeans were prepared for 2D gel electrophoresis using a trichloroacetic acid (TCA)/acetone precipitation method modified from Natarajan et al. (23). Briefly, samples of full-fat soybean flour were mixed with a 10% TCA solution (Sigma) containing 2% of 2-mercaptoethanol (BioRad), in cold acetone (Thermo Scientific) and then precipitated at –20°C overnight before centrifugation at 10,000 × g for 30 min at 4°C. Pellets were washed twice with cold (–20°C) acetone, air-dried, and dissolved in a solution of 8 M urea (Invitrogen, Life Technologies, Grand Island, NY, USA) with 2% CHAPS (Invitrogen). Samples were clarified by centrifugation, and protein concentrations were determined by Bradford assay (BioRad).

Historical serum or plasma samples from nine soybean allergic and six non-soybean allergic controls (Table 1) that were collected under consent and ethical approval at a clinic, or from US Food and Drug Administration (FDA) licensed facilities (SeraCare Life Sciences, Millford, MA, USA; PlasmaLab International, Everett, WA, USA) were tested for IgE binding. Soybean-specific IgE levels in soybean allergic donors ranged from 0.8 to 47 kU/l as measured by ImmunoCAP® (Phadia AB, Uppsala, Sweden) or IMMULITE® (Siemens Healthcare Diagnostics, Inc., Tarrytown, NY, USA). The soybean allergic subjects also bound IgE to peanuts ranging from 5 to 100 kU/l. Three of the six control subjects reported allergies to either lupine or pea, but not soybean.

One-dimensional IgE immunoblotting was performed under denaturing conditions (in Laemmli buffer), both with and without reducing agent using individual serum and plasma samples as described previously (24–26). Samples with β-mercaptoethanol were heated to 95°C prior to electrophoresis. Those without reducing agent were not heated. Proteins were separated by electrophoresis in Novex 10–20% tris-glycine minigels (Invitrogen). Representative gels were fixed and stained with Colloidal Bril-

Table 1. Human serum and plasma samples

Serum No.	Reported food allergies	Total IgE kU/L	Soy-specific IgE (ImmunoCAP* or IMMULITE#) kU/l
Soybean allergic subjects			
297	Soybean and peanut: anaphylaxis	nd	Soy: 0.8#; peanut: 70#
714	Soybean: symptoms not specified	nd	Soy: 15.9#; peanut: 22#
715	Soybean: oral, dermal, respiratory; peanut: anaphylaxis	644	Soybean: 17.8*; peanut: 100*
716	Soybean: symptoms not specified; peanut, no information	nd	Soybean: 7.2#; peanut: 18#
719	Soybean and wheat: asthma	1406	Soybean: 22*; peanut: 23*
721	Soybean: no information; peanut: reported but symptoms not specified	14725	Soybean: 47*; peanut 44*
RG-LEG-103	Soybean: no information; peanut: hives, throat swelling	1032	Soybean: 12.2*, 1.7#; peanut: 100#
RG-LEG-105	Soybean: oral itch, facial edema, breathing difficulty	1023	Soybean: 2.3*; peanut: 5*
RG-LEG-118	Soybean and peanut: hives and edema of face, throat and tongue	915	Soybean: 6.6*; peanut: 100*
19392-CS	Soybean: angioedema, vomit, EOS G; milk, egg, meat, fruit, peaches, pears	nd	Soy: 68#; peanut: 15#
Non-soybean allergic controls			
RG-71	Allergic to lupin, no symptoms to soybean or peanut	nd	Soybean: 1.5*; peanut: 1.5*
RG-73	Allergic to pea (no claim of allergy, but weak skin test positive to peanut and soy)	nd	Soybean: 0.7*; peanut: 15*
RG-74	Allergic to lupin, oral symptoms to peanut, no symptoms to soybean	nd	Soybean: nd; peanut <0.35*
SNP	No known allergies	nd	nd
RS-ID-1	Asthma, uncertain cause	nd	Soybean: nd; German cockroach 31*
RS-ID-3	Asthma, uncertain cause	nd	Soybean: nd; German cockroach 42*

Nd, not done.

* ImmunoCAP assay (Phadia, Uppsala, Sweden)

IMMULITE assay (Siemens Healthcare, Erlangen, Germany)

liant blue G250 (Sigma). Proteins from gels for immunoblotting were electro-transferred to PVDF membranes without fixation and then blocked with 5% NFDM in PBST. Soybean allergic or control human serum or plasma samples were diluted 1 : 10 or 1 : 20 (v:v) in 2.5% NFDM in PBST 1 h before addition to membranes and then incubated overnight at 22°C. Membranes were washed four times with PBST followed by addition of 1 : 1000 diluted monoclonal (MAb) anti-human IgE (SouthernBiotech, Birmingham, AL, USA) conjugated with HRP. Bound antibodies were detected with chemiluminescence as described above. A nitrocellulose membrane (Invitrogen) spotted with diluted purified human MAb IgE (ABCAM Inc., Cambridge, MA, USA) was blocked and detected simultaneously with soybean blots to gauge the relatively intensity of IgE signals.

Two-dimensional immunoblotting was performed using individual human samples to detect IgE binding to four soybean samples (GM 3411-T, near-isogenic line 3410-I, parental line Conquista, and commercial line 3416-C). Samples representing 25 µg of TCA-/acetone-precipitated protein were diluted to 125 µl in isoelectric focusing (IEF) sample buffer [8 M urea, 2% CHAPS, 50 mM DTT (Thermo Scientific) containing 0.5% ampholyte, pH 3–10 (BioRad)] and applied to 7 cm, pH 3–10 nonlinear IPG strips (BioRad) in a BioRad PROTEAN IEF system. Active rehydration was performed at 50 vdc for 12 h. Separation was performed using 250 vdc for 15 min; 4000 vdc ramping for 2 h and 4000 vdc limit-step for 30 000 integrated vhr; followed by 500 vdc to maintain focusing. Strips were reduced in a solution of 6 M urea, 2% SDS, 0.375 M Tris–HCl, pH 8.8, 20% glycerol, and 130 mM DTT and then acetylated with 135 mM iodoacetamide (BioRad). Second dimension (SDS-PAGE) separation was performed in NuPAGE® Novex 4–12% Bis-Tris ZOOM® Gels (Invitrogen). Representative gels were stained with Coomassie blue. Proteins of unstained gels were transferred to PVDF membranes, blocked with NFDM in PBST, incubated with diluted human samples, and detected as described for 1D immunoblots.

ELISA inhibition was performed using a pool of serum from clinically characterized, soybean allergic subjects using a study design recommended by European allergen extract regulators for testing the potency of allergenic extracts used for diagnosis (27). Assays were replicated (n = 3) with fresh extracts of each sample. An equal protein pool of all five soybean lines (diluted in pH 9.6 carbonate–bicarbonate buffer (Sigma) at 10 µg protein per ml) was used to coat Maxisorp ELISA plates (Nunc-Thermo Scientific). Nonspecific binding was blocked with 1% BSA fraction V (Sigma) in PBST. A pool of eight soybean IgE-positive serum and plasma samples (297, 714, 715, 716, 719, 721, RE-LEG-103, and RG-LEG- 118) was generated with volumes adjusted (715 and RG-LEG 103 used at half-volumes) to provide balanced IgE binding to a variety of soybean proteins based on prior direct ELISA and immunoblotting results. A pool of six nonsoybean allergic human samples (RG-71, RG-73, RG-74, SNP, RS-ID1, and RS-ID3) was used as the negative control. Triplicate inhibition dilution series were produced for the standard (pooled soybean) curve and for each individual soybean sample by serially diluting the extracts to a final concentration of 125, 25, 5, 1, 0.2, or 0.04 µg in 100 µl in a fixed concentration of the soybean allergic pool. Individual tubes were mixed and held at room temperature for 2 h to allow IgE binding to soluble soybean proteins before adding the mixtures to the soybean-coated ELISA plates. The plates were incubated for 2 h at 37°C before washing four times with PBST. Monoclonal HRP-labeled anti-hIgE (SouthernBiotech) was diluted 1:5,000 with 1% BSA in PBST before addition to the plate, and after 1 h of incubation, excess anti-IgE was removed by washing prior to the addition of substrate (3,3′,5,5′-tetramethylbenzidine [TMB], Sigma). Reactions were stopped after 20 min by the addition of 100 µl of 1N sulfuric acid (Thermo Scientific). The absorbance at 450 nm was measured using a Biotek® Powerwave XS2 reader (BioTek Instruments, Winooski, VT, USA). The average absorbance of uninhibited binding with the soybean nonallergic control pool was subtracted from test well readings as background and represented 100% inhibition of IgE binding (minimum absorbance). The average absorbance readings from direct serum binding to each soybean sample without inhibitor represented 0% inhibition (maximum absorbance). Inhibition lines were calculated for the standard soybean extract pool and each individual soybean extract. The EC50 values (inhibitor concentration yielding 50% inhibition of binding) were calculated from a logistic response model that was fit to inhibition values. The EC50 values of individual soybean lines were compared using an unbalanced one-way ANOVA (analysis of variance). The GLM Procedure of SAS (version 9.1, SAS Institute, Cary, NC, USA) was used for the analysis, and a 95% level (P < 0.05) was chosen for significance.

Results

Maize LTP

Validation testing showed the rabbit anti-mLTP-peptide IgG is highly specific and the mLTP standard highly pure (data not shown). The mLTP dot-blot standard curve (Fig. 1A) was reproducible (replicates not shown) and allowed the determination of LTP concentration in grain extracts. Measurement of mLTP from the nine non-GM commercial hybrids showed good reproducibility. The means of the nine hybrids differed by 15-fold across hybrids under rain-fed conditions (Fig. 1B).

Soybean IgE binding

The total protein concentrations of the five soybean samples extracted with PBS were similar (15.4–17.8 mg/ml), and the same samples extracted following TCA/acetone precipitation were similar (8.1–10.2 mg/ml). There were no obvious qualitative differences of Coomassie stainable protein bands among the five soybean varieties in 1D SDS-PAGE under reducing or nonreducing conditions although minor differences in intensity were observed for Conquista in high MW bands compared with the other lines (Fig. 2). Similarly, the 2D PAGE stained gel patterns of the four tested soybean varieties showed only minor qualitative differences (not shown).

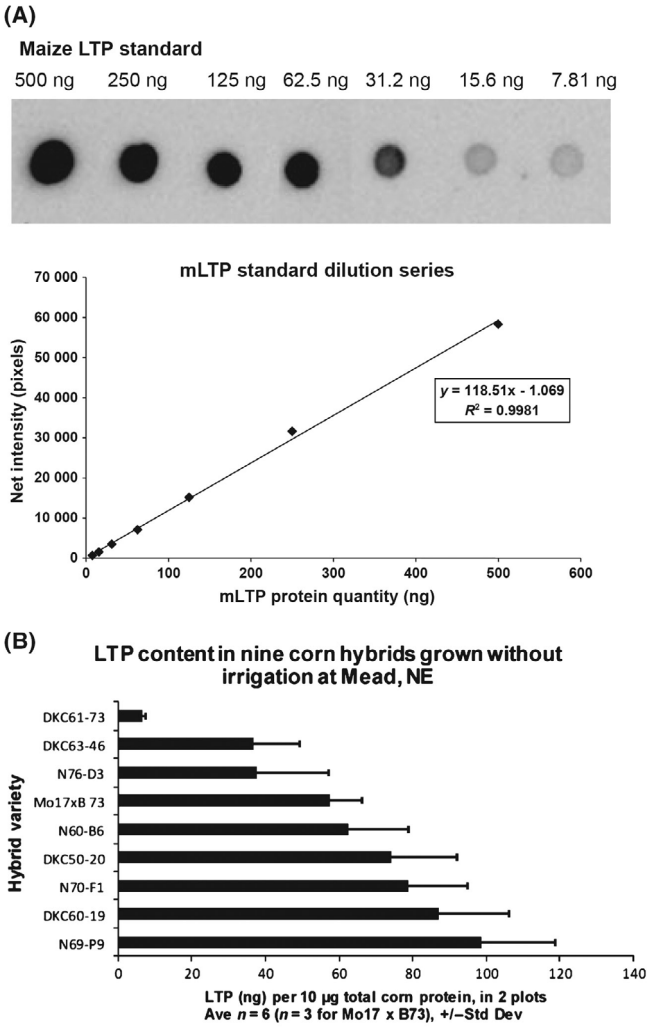

Figure 1. (A) Maize LTP SDS-PAGE–stained gel and immunoblotting with mLTP-specific rabbit IgG. SDS-PAGE reducing gel, lane (A) 10 µg protein from maize extract, (B) 0.5 µg purified maize LTP, (C) 10 µg protein from maize embryo extract, (M) MW marker. Immunoblot of a PVDF membrane blot of an identical gel using rabbit mLTP peptide–specific IgG, followed by goat, anti-rabbit-HRP, and chemiluminescent substrate. (B) Maize LTP accumulation in nine commercial non-GM varieties of maize grown under nonirrigated (dry land) conditions at Mead, Nebraska (USA). Grain samples of each maize field plot were extracted triplicate and 10 µg of each protein extract was spotted on nitrocellulose membranes, then incubated with rabbit anti-mLTP and then detection antibody and chemiluminscent substrate. Images were captured using a Gel Logic 440, and pixel densities were plotted against the mLTP diluted standard curve. (B) Mean and standard deviations are plotted showing a 15-fold difference in hybrids.

One-dimensional immunoblotting patterns of the five soybean lines were similar for soybean allergic sera except for subject RG-LEG 118 (lanes 1, 2, and 5 compared with lanes 3 and 4). However, patterns differed markedly between subjects as demonstrated by blots of four representative soybean allergic sera (Fig. 2). Binding to control extracts demonstrate that some individuals (e.g. 716) have IgE to other sources of allergens, although IgE binding to navy bean at 34 kDa (lane 7) is likely due to binding to cross-reactive carbohydrate determinants (CCD) on phytohemagglutinin and is unlikely to represent a significant risk of allergy based on our unpublished tests with basophils and sera from those with similar binding patterns. IgE binding to peanut proteins (lane 9) are prominent for some subjects (RG-LEG 118 and 715), while binding to maize (lane 11) is generally less intense and less common. Differences between reducing and nonreducing blots are obvious for some subjects, as might be expected due to sensitization to multiple subunits of glycinins, which separate into acidic and basic subunits under reducing conditions (28). There were few notable differences across soybean lines except a prominent band that is missing at 60 kDa for soybeans MON8001 and CD217 (lanes 3 and 4) for serum RG-LEG 118 and apparent differences in intensity in high molecular weight proteins under nonreducing conditions for plasma 715. Furthermore, 4 of 9 sera showed faint binding to a minor band at approximately 12 kDa that was only visible in commercial variety CD217 (lane 4) under reduced conditions with serum 716 (Fig. 2). The results of 1D gel immunoblots showed no specific differences in IgE binding to proteins of the transgenic soybean line (lane 2) compared with the near-isoline (lane 1). The results demonstrated that qualitative and apparent quantitative differences in IgE binding occur between non-GM commercial soybean lines for some allergic subjects. No IgE binding was observed in 1D immunoblots with non-soybean allergic sera (not shown). The 2D immunoblot IgE binding patterns (Fig. 3A,B) are diverse and difficult to analyze in part due to minor migration differences between gels and probable technical difficulties in blotting. Replication of blots of each sample and serum was not possible due to limited availability of serum samples. Thus, no attempt was made to quantitatively estimate spot intensities by densitometry. The IgE binding spots were visually compared between four soybean lines (transgenic 3411-T, near-isoline 3410-I, commercial line CD217 and parental line Conquista). Spot patterns are very similar for individual subjects with exceptions noted below, but differed markedly between subjects as expected from 1D immunoblotting. Images of immunoblots to all four soybean varieties are shown for two representative subjects (Fig. 3A), with no obvious differences in binding between the transgenic and near-isoline soybean lines. However, two additional IgE binding spots (no. 12 and no. 14) are visible in commercial variety 3416-C (CD217) with plasma sample 19392-CS that are not visible in the other three soybean lines. Spots no. 18 and no. 19 are visible in sample 3416-C (CD 217) for plasma 297 (Fig. 3A), but those spots are not visible in the other extracts. Immunoblots of the transgenic (3411-T) and near-isoline (3410-I) are also shown for four other soybean allergic subject's samples (Fig. 3B), demonstrating remarkably different patterns between subjects, but without obvious qualitative differences (and only minor quantitative differences) between the transgenic and near-isogenic lines.

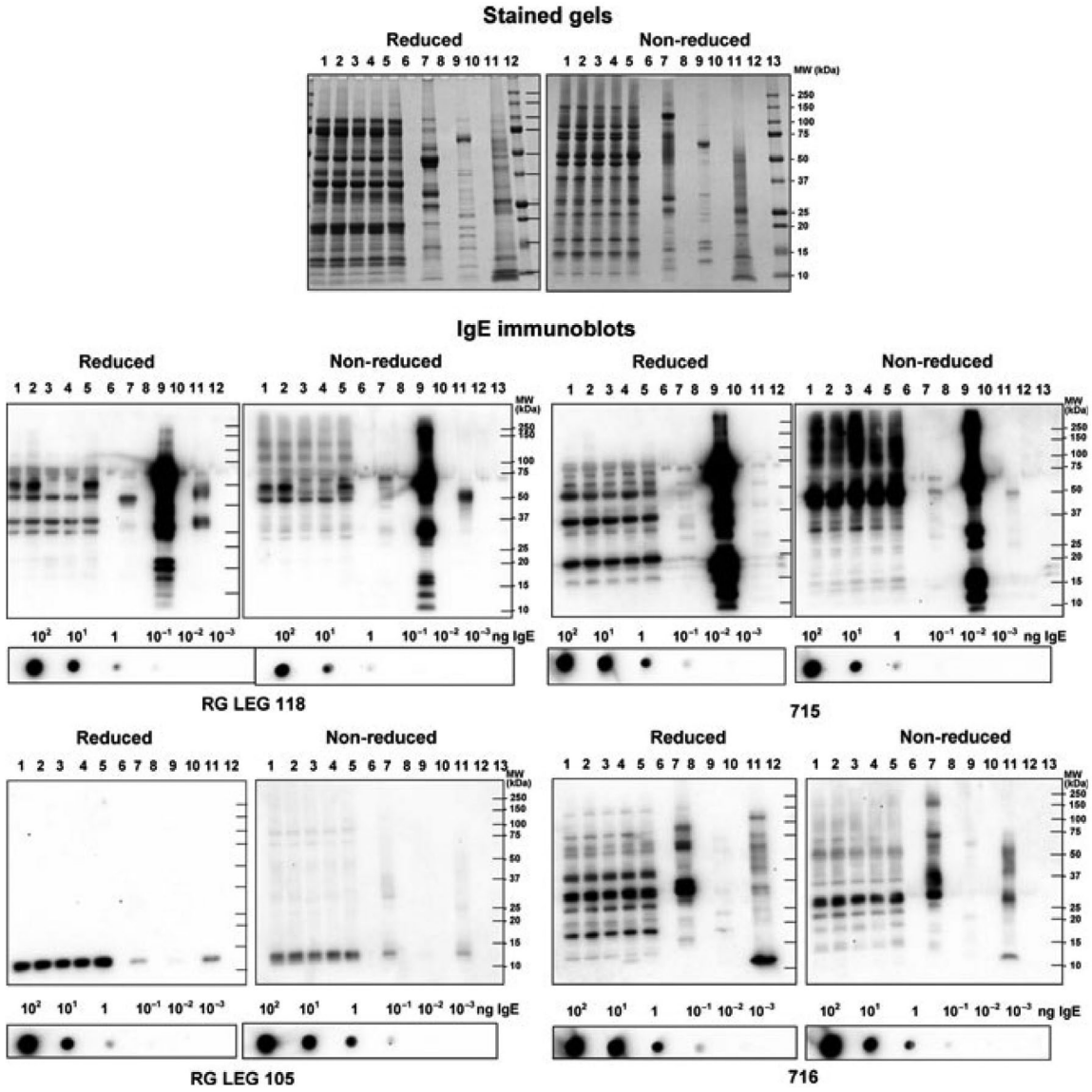

Figure 2. One-dimensional SDS-PAGE of soybean and control samples and IgE Immunoblots. Extracts were separated under reducing and denaturing conditions with SDS, mercaptoethanol and heat (Reduced), or under denaturing conditions with SDS, but without mercaptoethanol or heat (nonreduced). Representative Coomassie blue stained gels are shown (top). Immunoblots of four representative soybean allergic subject IgE binding patterns are shown under reduced and nonreduced conditions with donor samples listed below each set of blots.. Samples were as follows: Lane 1, near-isoline 3410-I (10 µg); Lane 2, transgenic line 3411-T (10 µg); Lane 3, commercial variety 3415-M/MON8001 (10 µg); Lane 4, commercial variety 3416-C/CD217 (10 µg); Lane 5, commercial variety Conquista (10 µg); Lane 6, empty; Lane 7, navy bean (10 µg); Lane 8, empty; Lane 9, peanut (2 µg); Lane 10, empty; Lane 11, corn (10 µg); Lane 12, molecular weight marker (reduced) or empty (nonreduced); Lane 13, molecular weight marker nonreduced). Nitrocellulose strips shown below each image represent dilutions (100 ng to 1 pg) of human IgE spotted and detected along with the immunoblots to gauge development intensity.

IgE inhibition ELISA assay

Individual soybean allergic (n = 9) and a pool of non-soybean allergic (n = 6) serum and plasma samples were tested for IgE binding to a pool of all five soybean extracts by direct binding ELISA to select subjects to pool for the inhibition ELISA (Fig. 4A). The mean absorbance at 450 nm varied remarkably between subjects. Soybean allergic sample RG-LEG 105 binding hardly differed from the non-allergic control pool, while sample 715 binding was more than double the next highest binding sample (RG-LEG 103) and ten-fold higher than sample 719. This ELISA data and 1D immunoblotting patterns were used to select subjects and concentrations of human samples for the standard inhibition ELISA pool. For ELISA inhibition, the mean absorbance values across replicate assays are plotted in Fig. 4B. The fit of the regression lines was high, $r^2 \geq 0.99$ (data not shown). Inhibition values between soybean lines were obviously similar, although parental Conquista was statistically more potent

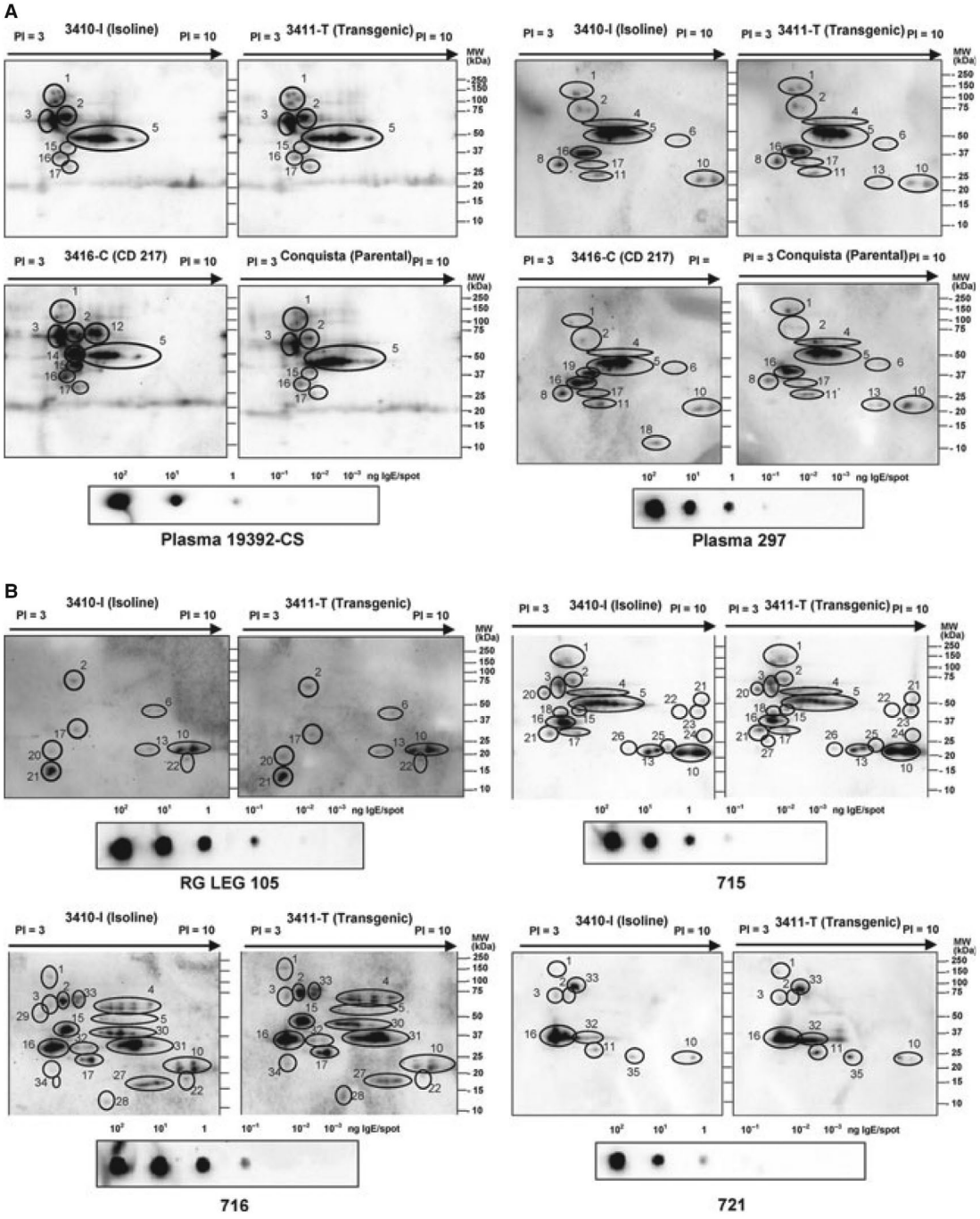

Figure 3. Representative 2D immunoblots with human serum or plasma samples (listed in figure) of soybean extracts: 3410-I, 3411- T, 3416-C, and Conquista. Twenty-five micrograms of protein was separated first in nonlinear pH 3–10 IPG strips and then Bis–Tris 4–12% PAGE in. Spots circled and labeled with the same numbers represent the proteins with the same pI and MW between blots. (A) Immunoblots of all four soybean samples with two representative human samples demonstrating the additional spots (12, 14, 18, and 19) that were only visible in nontransgenic sample 3416-C. (B) Immunoblots of four human samples showing results for only isoline 3410-I and transgenic 3411-T soybeans as the other nontransgenic soybean blots did not differ.

and commercial non-GM, MON8001 was statistically less potent than other soybean lines (Table 2). However, the differences of all lines compared to the pooled standard were within a normal tolerance range (50–200%) for judging the potency of allergenic extracts (29, 30). There is less than a two-fold difference in the EC50 values across the soybean lines, with observed potency being greatest for Conquista (EC50 = 3.11 µg/well) and least for MON8001 (3415-M, EC50 = 5.39 µg/well). The EC50 values of the transgenic, BPS-CV127-9 (3411-T), and near-isoline (3410-I) were similar (4.31 *vs* 4.68 µg/well, respectively; $P = 0.846$).

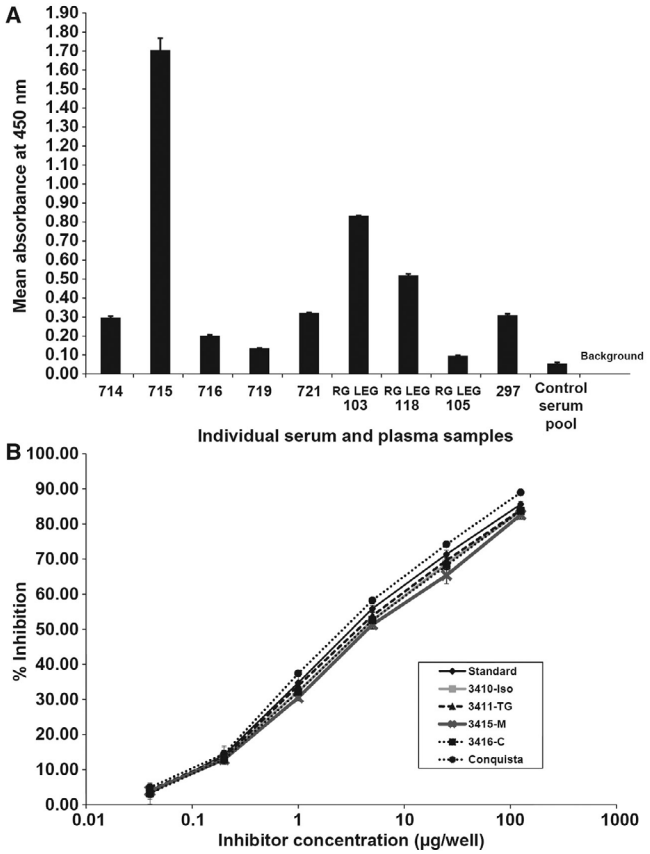

Figure 4. Direct and Inhibition ELISA IgE binding to soybean extracts. Individual serum and plasma samples were tested for total soybean binding using direct binding to a pool of soybean extracts coated on the plate (A). The data were used to adjust serum concentrations in the pool of sera used for inhibition. Inhibition ELISA (B) plots the percent inhibition of binding resulting from pre-incubating the soybean allergic serum pool (n = 8) with soluble extracts of a standard pool or individual extracts of soybean lines at specific protein concentrations. The concentrations of specific soybeans required to achieve fifty percent inhibition (EC50) are shown (table insert).

Table 2. IgE Inhibition ELISA EC50 values: averages from 3 assays

Sample	EC50 (mg/well)	SD	Reciprocal% of pooled standard EC50†
Pooled standard	3.86	0.45	–
3410-I	4.68	0.7	0.82%
3411-T	4.31	0.11	0.90%
3415-M	5.39*	0.27	0.72%
3416-C	4.76	0.35	0.81%
Conquista	3.11**	0.19	1.24%

ANOVA comparison of all five soybean lines compared with the standard pool and each other.
* Significantly different from the pooled Standard, Dunnett, $P < 0.05$.
** Significantly different than all other soybean lines, Tukey, $P < 0.05$.
† Reciprocal% EC50: sample EC50/3.86 (Standard Pool) × 100%.
Acceptance range for a diagnostic extract would be 50–200% (Lorenz et al. 2008).

Discussion

The studies reported here are presented as examples that might be expected in evaluating potential differences in endogenous allergen accumulation in maize and soybean varieties used in food production today. The intent was to demonstrate that natural variation exists between varieties of commodity crops that are being transformed for future products as well as show the complexity of the analysis. Regulatory agencies in various countries are asking for similar studies to be performed on every new GM event without the benefit of data regarding natural variation, and without guidance regarding the variation in available commercial crops.

Maize LTP varied up to 15-fold in concentration in mature grain across commercial hybrids grown under typical field conditions in Nebraska, a major maize producing state in the United States. This suggests a wide variation in the major allergen (LTP) content is likely in commercial food and feed as well as in extracts used to diagnose allergy by skin prick testing.

We report representative results from a regulatory study performed to evaluate a GM soybean event, BPS-CV127-9 following requests for data from EFSA. Those tests were performed using serum and plasma samples from individuals with clinically diagnosed soybean allergy or with suspected allergy and clear *in vitro* IgE binding to soybean proteins. The soybean study included 1D immunoblots with individual serum samples testing under reducing and nonreducing conditions, individual serum samples in 2D immunoblots, and ELISA inhibition using a well-characterized serum pool. This study went beyond the requirements of US FDA and EU agencies for the regulation of allergenic extracts that are used as diagnostic allergen products. Variation in qualitative IgE binding to proteins was evident between non-GM commercial varieties that were greater than differences between the GM and isogenic soybeans. There were also statistically significant quantitative differences between two of the non-GM commercial lines as measured by ELISA inhibition. Notably, the differences were within the likely tolerance limits (50–200%) for diagnostic extracts (29, 30). However, current regulatory guidelines do not set limits of acceptable variation of allergens in GM crops. The inference based on compositional analysis of a GM variety compared to a near-isogenic variety is that statistically significant differences would be given intense review. Based on previous decisions evaluating statistically significant differences in nutrient composition of GM crops, if the differences fall within statistical tolerance interval of known commercially available lines, the product is likely to be accepted by regulators. Yet, formal guidelines for endogenous allergens are lacking.

We believe it is important to reconsider the relevance of the question of food safety of GM crops regarding endogenous allergen expression. First, even though soybean is con-

sidered a commonly allergenic food, the majority of soy-allergic individuals are infants and young children who outgrow their soy allergy rather quickly (31), and relatively few subjects have severe reactions to soybeans compared with peanut and tree nuts. Second, allergenic foods only pose a risk of allergy for those who are allergic. There are no data to demonstrate that specific doses of allergens are responsible for sensitization, while lower doses are tolerogenic. The allergic individuals must avoid consumption of foods containing their allergenic source to avoid adverse reactions. Food allergy is highly specific to the individual, both in the specific allergenic proteins that bind IgE and in the dose of allergen that elicits a reaction. Severely allergic subjects may only tolerate a few milligrams of a whole food before reacting, while others tolerate gram quantities. Thus, food allergic subjects are told to avoid consumption of the foods that elicit their reactions. There are no mechanisms to evaluate and segregate hypoand hyper-allergenic varieties (GM or not) in the food supply.

If tests are required, it is important to consider alternative testing methods, costs, and possible conclusions. Acquisition of a sufficient number of well-characterized soy-allergic adult serum donors is difficult. For a food crop with a relatively simple allergen profile such as maize, with one major allergen (LTP), and very rare occurrence of allergy, analytical methods may be the only practical way to evaluate endogenous allergen levels. But, natural variation in maize LTP levels is high. Thus, an extremely large increase in the level of maize LTP should necessary to raise concerns for GM maize. In the case of soybeans, IgE binding is very complex with many different proteins bound by sera from different allergic subjects. A large number of soy proteins have been identified as potential soy allergens on the basis of IgE binding in various studies (28, 32) and at least 3 soy proteins (Gly m 4, 5, and 6) qualify as major soy allergens. Other investigators have previously shown wide variation in the content of allergens in some fruits and commodity crops (11, 33), illustrating similar difficulties may be encountered with other foods. Some regulators and investigators suggest using analytic proteomics methods to measure changes in endogenous allergens (34). However, differences in isoform expression and expression of previously unknown allergens could be missed by a proteomic analysis.

These results and consideration of risks of allergy posed by non-GM food crops should raise questions about the relevance of such testing as a general safety requirement for GM plants. Previously, such evaluations were focused on GM varieties of commonly allergenic foods, principally soybean. But, regulators in some Asian countries are asking for similar evaluations for GM rice and some European countries have asked for similar evaluations of GM maize. Do these tests protect consumers? Those allergic to soybean should avoid soybean containing foods. Those allergic to maize or rice should avoid maize or rice. If regulators are going to continue to ask for similar studies of new GM crops, there are many issues to address. What tests provide sufficient information to allow confident conclusions of safety or of unacceptable risk? Should similar tests be required for all new genetic varieties of new crops regardless of the source of the genetic variation? And finally, if only low allergen expressing varieties are selected for food production, will there be a negative impact on production of food and feed across diverse environments? Some allergenic proteins are pathogenesis related proteins and presumably act to protect the plant when attacked by insects or pathogenic microbes or when under environmental stress. We strongly recommend removing this requirement from safety testing of new GM crop varieties unless there is a very specific question to answer. For example, if a developer claimed to have developed a hypoallergenic food crop, then the claim should be verified and the product marketed under a different name.

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Contributions — R. Panda performed the serum IgE binding study on soybean varieties and statistical analysis and drafted a significant portion of the manuscript; H. Ariyarathna purified the maize LTP, developed and performed the dot-blot assays for maize LTP quantitation, and developed the figures on LTP; P. Amnuaycheewa helped develop and refine the 2D gel separation procedure for soybean extracts and figure preparation; A. Tetteh performed serum screening of IgE binding of some of the subjects and helped develop protocols for IgE binding assays; SN Pramod advised on the purification of mLTP and development of the quantitative LTP assay; S. Taylor provided funding for some experiments and helped draft the manuscript; B. Ballmer-Weber provided guidance and criteria for allergic subject selection for soybean food allergy blood donors provided some serum samples and ImmunoCAP data; R. Goodman guided the overall study design, conduct, and manuscript revision.

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