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Evaluation of Endogenous Allergens for the Safety Evaluation of Genetically Engineered Food Crops: Review of Potential Risks, Test Methods, Examples and Relevance

Richard E. Goodman*

Food Allergy Research and Resource Program, University of Nebraska—Lincoln, 143 Food Industry Complex, Lincoln, Nebraska 68583-0955, United States

Rakhi Panda

Food Allergy Research and Resource Program, University of Nebraska—Lincoln, 143 Food Industry Complex, Lincoln, Nebraska 68583-0955, United States

Harsha Ariyarathna

Department of Veterinary Pathobiology, University of Peradeniya, Peradeniya, Sri Lanka

ABSTRACT: The safety of food produced from genetically engineered (GE) crops is assessed for potential risks of food allergy on the basis of an international consensus guideline outlined by the Codex Alimentarius Commission (2003). The assessment focuses on evaluation of the potential allergenicity of the newly expressed protein(s) as the primary potential risk using a process that markedly limits risks to allergic consumers. However, Codex also recommended evaluating a second concern, potential increases in endogenous allergens of commonly allergenic food crops that might occur due to insertion of the gene. Unfortunately, potential risks and natural variation of endogenous allergens in non-GE varieties are not understood, and risks from increases have not been demonstrated. Because regulatory approvals in some countries are delayed due to increasing demands for measuring endogenous allergens, we present a review of the potential risks of food allergy, risk management for food allergy, and test methods that may be used in these evaluations. We also present new data from our laboratory studies on the variation of the allergenic lipid transfer protein in non-GE maize hybrids as well as data from two studies of endogenous allergen comparisons for three GE soybean lines, their nearest genetic soy lines, and other commercial lines. We conclude that scientifically based limits of acceptable variation cannot been established without an understanding of natural variation in non-GE crops. Furthermore, the risks from increased allergen expression are minimal as the risk management strategy for food allergy is for allergic individuals to avoid consuming any food containing their allergenic source, regardless of the crop variety.

KEYWORDS: *genetically modified, IgE, food allergy, soybean, maize, risk management*

INTRODUCTION

Genetically Engineered (Genetically Modified) Crops. We have used the term genetically modified (GM) here as the more commonly recognized term, but are restricting the definition to mean those engineered by insertion of a specific sequence of DNA, rather than the broader genetic modifications that occur through wide-genetic crosses or chemical and radiation mutagenesis. Development of a strategy and regulations for ensuring that foods from GM crops are as safe as their conventional counterparts occurred before the first plants were allowed into commercial production. The foundation of the assessment is the recognition that there is a long history of safe consumption of commonly consumed plant species; there is natural variation in the composition of foods produced from these organisms; and there are specific nutrients, antinutrients, toxins, and allergens in the commonly consumed species.[1] The safety assessment therefore focuses on characterizing the intended modification with specific tests for allergenicity or toxicity required on the basis of scientifically defensible hypotheses related to the introduced gene, gene products, and, if generated, metabolites. In addition, potential unintended effects are to be evaluated on the basis of agronomic characteristics, compositional analysis, and evaluation of key (species specific) nutrients, antinutrients, allergens, and toxins.[1] Approvals of some specific GM crops for importation or cultivation have been delayed for years in the European Union (EU) and some other countries due to questions related to possible changes in endogenous allergen levels, even though these same varieties have been approved in the United States, Canada, and many other countries (unpublished comparison of approvals recorded at www.cera-

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gmc/org for global approvals vs www.GMO-Compass.org for EU approvals). We are not aware of any adverse human health events that have occurred due to the consumption of foods produced from any approved GM event. Yet the search for even minor differences between every new GM and its nearest genetic relative continues. Therefore, we have provided a brief review of the genetic and compositional complexity of the host plants (gene recipient), modification methods, review risks of food allergy, and present new data from three of our studies evaluating natural variation in endogenous allergens in maize and in soybean to consider the relevance for evaluating endogenous allergen levels in GM crops as part of regulatory requirements.

Overview of GM Crop Development. The first commercial production of GM crops developed and approved for use in food, feed, and fiber was in 1996, primarily in the United States and in Canada, Argentina, and Australia.[2] These GM plants were developed by transformation of commonly used food crop varieties by insertion of relatively short segments of DNA encoding from one to a few proteins from naturally occurring bacteria or plant sources. Expression of the introduced genes has provided increased resistance to specific insects, tolerance to commonly used herbicides, resistance to specific viruses, or delayed ripening of soft fruits.[3] Although hundreds of different GM plants have been partially or fully developed, a few traits dominate the commodity market, have been bred into genetically diverse varieties, and have been widely adopted in many additional countries. Approved GM crops were grown on approximately 160 million hectares in 29 countries in 2011.[4]

Understanding the source of genetic diversity in GM plants requires an appreciation of inherent genetic diversity in non-GM plant species as well as a comparison of the methods of genetic engineering to other methods used to introduce new traits. Biolistic methods or more commonly a genetically modified infectious *Agrobacterium tumefacians* transformation binary vector system has been used to introduce the desired gene (DNA) into the host plant (www.cera-gmc/org). Transformed organisms such as soybean and maize have genomes that include between 10000 and 40000 genes, organized on multiple chromosomes that include more noncoding DNA than coding DNA. Thus, randomly inserted DNA has a very small chance of altering the expression of any single endogenous gene as altered expression is only likely if the insertion is in proximity to the coding region of a gene or gene promoter or if the inserted gene is a very strong transcriptional factor with plausible regulatory impact on genes encoding specific allergens. The developer will know whether the gene encodes a transcriptional factor through characterization of donor DNA and products. In the process of producing effective new GM crop varieties, thousands of transformed cells and plants fail to develop or are discarded as they do not grow normally or produce viable seeds because biologically important genes have been interrupted. Thus, the majority of genetically and phenotypically perturbed transgenic plants are removed as candidates for further development very early in the process. Developers also select plants that contain only a single DNA insert if at all possible during molecular characterization of the transformed plants. Plants are usually backcrossed to achieve true breeding, nearly homozygous breeding stock that is functionally similar to the original pretransformed parental line, with the exception of the inserted transgene(s). However, often a small percentage of genes will vary between the parental line, the transformed plant, and progeny as the parental line is rarely completely homozygous at all loci. The selected GM plants are tested for gene stability, performance under various field conditions, and production of nutrients that are typical of the specific crop to ensure performance. Following the proof of a specific GM trait is useful; it will likely be bred into plants with diverse genetic backgrounds to provide optimum production when grown in very different environments in North and South America, Asia, Australia, Africa, and Europe. Plant geneticists have only relatively recently begun to be able to document and fully understand the genetic differences that exist in similar-appearing varieties of the same species. The maize genome is thought to include roughly 20500 genes.[5] There are few data comparing genomes of varieties of crops. However, a genome and breeding study of two inbred maize lines, B73 and Mo17, estimated a complete comparison would show up to 10000 short, nonshared genetic fragments or chromosomal rearrangements between the inbred lines. However, following cross-pollination, the hybrids produce higher yields, with larger ears, and the plants are adapted to more diverse growing conditions.[5] Grain from these hybrids is used for food and feed purposes similar to uses of grain from the individual inbred parents. The soybean genome includes more than 10000 protein coding genes.[6] Although *Glycine max* (cultivated soybean) and *Glycine soja* (wild soybean) have many divergent genes and are generally self-pollinated, the plants can be manually cross-pollinated and produce viable progeny.[6] Wide genetic crosses with wild relatives, such as *G. max* and *G. soja*, provide genetic diversity that may be useful to introduce disease and stress resistance, but their introduction requires many rounds of backcrossing and selection to regain high-yielding agronomic properties of elite lines. The introduction of such genetic variants is highly likely to introduce minor phenotypic variation in the expression of many proteins including allergens and antinutrients, but those are rarely measured and not regulated. In addition, variable environmental factors including water availability, temperature, insolation, wind, soil nutrients, and the presence or absence of symbionts or pathogens can modulate gene expression and accumulation of proteins and metabolites.[7] An important consideration for breeders is whether the major nutritional and agronomic traits of new varieties provide acceptable yields and characteristics under the conditions for a given agricultural production area. The "natural" genetic and phenotypic variation that exists in agricultural production should be considered in the evaluation of new GM varieties for acceptability according to Codex (2003).[1]

Food Safety Paradigm. The goal of the food safety assessment is a determination that the GM crop food material is either substantially equivalent to the non-GM counterpart in terms of food safety or that it is unacceptably more hazardous or "risky" under common growth and processing conditions. It has long been recognized that no food can be judged 100% safe or without risk for all humans under any conceivable use. Gluten proteins in wheat, barley, and rye cause celiac disease (CD), which affects nearly 1% of the global population.[8] Production of most conceivable GM wheat varieties would not be expected to alter the risk of eliciting symptoms of CD unless the GM trait introduced a new gliadin or glutenin or knocked out one or more of the CD-eliciting genes. Similarly, peanuts, soybeans, wheat, and maize cause food allergy for a number of consumers. Collectively, approximately 4% of consumers (2–4% adults, 6–8% of children) in the United States, EU, and Japan experience food allergy due to the development of

specific IgE antibodies to one or more proteins in the major sources of food allergy, including these commodity crops. The prevalence of food allergy to the major food allergens in the United States is estimated primarily by carefully structured, detailed telephone surveys. Results of a validated and widely cited survey indicate that peanut allergy affects approximately 1% of young children and 0.6% of adults; tree nut allergy affects 0.5% of children and 0.6% of adults; cow's milk allergy affects 2.5% of young children and 0.3% of adults; chicken egg allergy affects 1.5% of children and 0.2% of adults; fish allergy affects 0.1% of children and 0.4% of adults, crustacean shellfish allergy affects 0.1% of children and 2% of adults; and soybean and wheat allergies affect ~0.4% of children and 0.3% of adults in the United States.[9] Mustard, celeriac, and lupin are also cited as common allergenic food sources in some countries of the EU as reported by Ventor and Arshad.[10] Additionally, buckwheat is cited as a major allergenic food crop for Japan.[11]

Any GM variety would be expected to cause similar food allergies (same people; similar severity symptoms) as the non-GM counterpart and pose the same risks for the same consumers. Those with celiac or food allergy must avoid the foods that cause their disease whether the food is produced from a GM or traditionally bred variety (Codex, 2003).[1] Commodities produced and sold around the world for food production are not segregated on the basis of high or low allergy elicitation. Rather, they are produced from harvests that include many varieties which change in availability and are often comingled. Importantly, the amount of allergenic food components may also vary markedly in processed foods, altering exposure, but in most countries foods must be labeled listing the contents (e.g., peanut, wheat, eggs), but not the quantities of these materials that are present.

New GM crops are tested more rigorously under regulatory scrutiny than crops modified by intentional or natural mutagenesis and those produced by crossing with wild relatives to introduce new plant traits such as disease resistance, drought tolerance, or other improved characteristics. The allergenicity assessment process recommended by Codex (2003)[1] was reviewed by Goodman et al.[12] The risk is for those with existing allergies with the focus on preventing the transfer of an allergenic protein into a new source that would not be recognized by the allergic consumer. Additionally, the transfer of a protein that is nearly identical to an allergen would present a risk of allergic cross-reactions that require testing if the sequence identity match of the introduced protein is high using computer alignment comparison to known allergens.[12] If needed, specific serum tests are used to evaluate IgE binding. If the introduced protein in the new GM crop shows evidence that it is an allergen or is highly likely to be cross-reactive, it is unlikely to be acceptable for regulators as consumers would not expect to find their allergen in foods made with a plant that should not contain their allergen.

In addition, the stability of the protein in pepsin and abundance are also considered in the risk evaluation as a number of common food allergens are stable and abundant. Regulators are hesitant to approve GM crops with new abundant, pepsin-stable proteins due to the suspicion of a higher risk of sensitization or elicitation by such proteins. Finally, there is a hypothetical concern that transformation may induce increases in the expression of endogenous allergens, increasing risks for those with allergies or increasing the chance of sensitization, yet this has turned into the major focus of the allergenicity assessment in some countries.

Compositional "Substantial Equivalence" Including Allergens. In addition to evaluating the safety of the new trait, food and feed materials from the new GM plant varieties are evaluated for potential changes in the composition of key nutrients as well as antinutrients, toxins, allergens, and celiac-eliciting proteins (if the GM plant is wheat or a near-wheat relative), which are specifically associated with the host plant (gene recipient). Tests performed to evaluate substantial equivalence are specific to plants derived from the individual transformational event. Unfortunately, there are few published data regarding the concentration of specific allergens or glutens found in different genetic backgrounds (varieties). Even fewer data are available from studies designed to evaluate environmental variation found in plants of the same variety grown in different locations or years. A few published studies suggest that certain allergenic food proteins are highly variable in abundance in some crops as reviewed by Goodman et al.[12] The concentration of lipid transfer protein (LTP), also referred to as Mal d 3, an allergen in apples that is reported to cause severe reactions in Spain, was found to vary in concentration by approximately 100-fold across 88 apple cultivars.[13] We recently published preliminary data demonstrating up to 15-fold variation in LTP concentration in grain of nine non-GM maize hybrids grown without irrigation in a field trial in Nebraska.[14] The publication also included some results of a study evaluating potential differences in IgE binding to proteins of a GM soybean line (BPS-CV127, from BASF Plant Science), a near-isogenic line, and three other commercial soybean lines. Minor qualitative differences were identified between soybean lines based on serum IgE immunoblots (one-dimensional reducing and nonreducing blots using individual sera and by two-dimensional immunoblots) using individual soybean allergic sera. However, no significant differences were measured on the basis of inhibition ELISA tests between extracts using pooled soybean allergic sera.[14]

Although the general guidance of Codex (2003)[1] for safety including evaluating potential risks of food allergy is followed by most countries that approve GM crops, some countries and the European Food Safety Authority (EFSA) have recently asked for more detailed studies of potential changes in endogenous allergens than were performed previously. The new requests include proteomic analysis or two-dimensional gel blotting with individual allergic subject's sera for each new submission and for food crops that rarely cause allergy.[15] Yet in terms of food safety, there is no scientific basis to demonstrate what levels of change would pose an unacceptable risk to allergic consumers.[16,17] The senior investigator (Goodman) has performed serum IgE binding studies on five different GM soybean events and one GM wheat event since 1999. In each case it has proven very difficult to obtain sufficient sera from subjects diagnosed with clear food allergies to soybean or wheat. Development of appropriate methods, interpretation of results, and evaluation of minor differences in IgE binding between the GM event and near-isogenic and representative commodity food grade materials have proven challenging. Because there are no standards of acceptable differences or optimal testing protocols, regulatory evaluation of scientific studies and data is hard to predict and may vary across jurisdictions. Therefore, it is important to have a broader discussion of the variation in prevalence of allergies for GM crop varieties under consideration, natural variation in allergen content for material in commerce today, and appropriate interpretation and risk management strategies.

Table 1. mLTP Content in Nine Maize Hybrid Grain Samples from Mead and Clay Center, NE, with or without Irrigation

hybrid maize	Mead, NE irrigated (av mg/g, SD)[a]	Mead, NE rain-fed (av mg/g, SD)[a]	Clay Center, NE irrigated (av mg/g, SD)[a]	ratio: high/low
DKC50-20	4.36, 0.67[b]	7.42, 1.77[c]	3.65, 0.29[c]	2.03
DKC60-19	5.23, 0.42[c]	8.70, 1.91[c]	4.27, 0.52[c]	2.04
DKC61-73	2.43, 0.36[c]	0.66, 0.01[c]	1.70, 0.11[b]	3.68
DKC63-46	1.82, 0.46[c]	3.66, 1.27[c]	1.47, 0.16[c]	2.49
Mo17xB73	3.32, 0.58[c]	5.74, 0.88[b]	2.03, 0.18[c]	2.83
N60-B6	4.68, 0.41[c]	6.24, 1.65[c]	3.43, 0.82[c]	1.82
N69-P9	3.40, 0.44[c]	9.86, 2.01[c]	2.72, 0.15[c]	3.63
N70-F1	1.15, 0.10[c]	7.90, 1.60[c]	1.50, 01.5[c]	6.87
N76-D3	2.80, 0.77[c]	3.76, 1.95[c]	2.55, 0.40[c]	1.47
ratio: high/low	4.55	14.94	2.90	

[a]Average content is reported in mg mLTP per g total protein, standard deviation (SD, rounded). [b]Sample size is 3 (1 plot, 3 replicates). [c]Sample size is 6 (2 plots, 3 replicates).

As noted, those with food allergy must avoid the source of their allergies. Most of the foods that are consumed today are processed in some form and contain highly varied amounts of a given component (e.g., soybean protein isolate). The protein isolates may be processed by different methods and are made from market-available varieties of plants that are grown under diverse environmental conditions. There is no practical way to measure and control the allergen dose in each food. Furthermore, the risk of consuming an allergen is for those who are sensitized, as other consumers can consume high amounts of allergenic foods with no adverse effect. The only practical risk management strategy at this time for those with a specific allergy is to avoid any food that contains any protein from the source of their allergic disease.

The prevalence of soybean allergy may be as high as 0.4% in children and 0.3% in adults. Reactions to soybeans are rarely life-threatening. There are fewer than five documented cases of fatal food allergic reactions to soybean from a Swedish allergy group.[18] No other reports of fatalities were found in the literature, although some other severe systemic allergic reactions are documented.[19] There are three dominant soybean allergenic protein classes, glycinins, β-conglycinins, and a pathogenesis-related protein that cross-reacts with a birch pollen allergen,[20−22] but little is known about the variability of expression of these proteins between varieties, environmental growing conditions, or variation due to food processing.

New Studies in Maize and Soybean. Food allergy to maize is relatively rare, and the only significant reported food allergen is a nonspecific LTP.[23] This paper includes the complete data summary of a study of maize LTP accumulation from irrigated and rain-fed conditions and representative results from three different serum IgE binding studies with multiple lines of soybeans. The results are discussed in the context of risk assessment of GM crops.

MATERIALS AND METHODS

Maize Lipid Transfer Protein Analysis. Nine commercial non-GM maize (corn) hybrids were grown at two University of Nebraska field-trial sites in eastern Nebraska with irrigation (Clay Center) or with and without irrigation (Mead). Commercial hybrid samples DKC61-73, DKC60-19, N70-F1, N60-B6, N76-D3, N69-P9, DKC50-20, Mo17-B73, and DKC63-46 were grown at both sites and conditions, with duplicate plots for most samples (Table 1). Evaluation of the LTP content of grain was performed by Harsha Ariyarathna as part of her master's thesis (unpublished results). Three independent 10 g samples of grain from each plot were individually ground to a fine powder under liquid nitrogen, and proteins were extracted at 4 °C in 25 mM sodium acetate (pH 4.0) buffer and clarified by filtration, similar to the method of Pastorello et al.[23] The total protein content of extracts was determined by Lowry assay, and samples were adjusted to 5 mg/mL protein and 1% β-mercaptoethanol and then heated to ∼90 °C. Samples of serially diluted, 95% pure natural maize LTP (mLTP) characterized by LC-MSMS with 95% sequence coverage were prepared in a similar manner, but diluted in 0.1% bovine serum albumin (BSA) as carrier protein and β-mercaptoethanol added to 1% by volume. Two microliters of diluted mLTP standard and maize extract samples were spotted in triplicate on 0.45 μm pore size nitrocellulose membranes immediately after heating (95 °C) and cooling to room temperature. Membranes were blocked with 5% nonfat dry milk (NFDM) in phosphate-buffered saline with 0.2% Tween 20 (PBST) prior to incubation with mLTP peptide-specific (AARTTADRRA, corresponding to amino acids 67−76 of mLTP) diluted rabbit polyclonal IgG.[14] Excess IgG was removed by washing in PBST. Bound IgG was detected using diluted goat anti-rabbit IgG−horseradish peroxidase (Pierce Chemical, Rockford, IL, USA) in NFDM blocker. Following removal of unbound IgG, ECL Pico-west substrate solution (Pierce Chemical) was added to membranes, and approximately 2 min later emitted light was captured using a Kodak Gel Logic 440 imaging system. The pixel densities of sample and standard spots of images captured at the same time were used to compare sample extract immunospot densities to those of known concentrations of the diluted mLTP standard. Standard curves and correlation coefficients were calculated using Microsoft Office Excel 2007 software for each standard curve (in triplicate). Mean sample values (n = 6 for most or n = 3 for three samples) for each hybrid, growing condition, and location were compared using one-way analysis of variance (ANOVA). Fisher's protected least significance difference method (LSD) was used to determine which hybrids were statistically different using Statistical Analysis Software (SAS) version 9.1 (SAS Institute, Cary, NC, USA).

Soybean Samples for Endogenous Allergenicity Comparisons. Samples. Soybean seed samples were supplied from study sponsors (BASF Plant Science and Bayer CropScience) to compare IgE binding to endogenous soybean allergens for three different GM soybean lines, their non-GM, near-isogenic line (null-segregant sibling or parental lines), and multiple non-GM commercial lines, in separate studies (Table 2). Representative data from the BASF study (data not previously reported by Panda et al.)[14] and the two Bayer studies are presented to demonstrate the complexity, variation, and challenges of performing and interpreting such tests. Samples of soybeans were supplied as dehulled, defatted flour samples, certified as to identity by the study sponsors. Control samples of uncooked navy bean (*Phaseolus vulgaris*) and peanut (*Arachis hypogaea*) were purchased at local markets, ground to a fine powder, and stored frozen prior to extraction. Samples of dried corn grain (*Zea mays*) were obtained from a previously described field trial.[14]

Table 2. Soybean Samples Provided by Study Sponsors To Evaluate Potential Changes in Endogenous Allergen Accumulation

soybean type	soybean supplier		
	BASF Plant Science	Bayer CropScience 1	Bayer CropScience 2
GM	BPS-CV127-9[a]	LL55[b]	FG72[c]
(designation)	(3411-T)	(A5547-A127)	
near isogenic	null-segregant	near isogenic	parental
(designation)	(3410-I)	(A5547)	(Jack)
commercial	MON8001	Stine 2686-6	Stine 2686-6
(designation)	(3415-M)	(D)	(D)
commercial	CD217	Stine 2788	Stine 2788
(designation)	(3416-C)	(E)	(E)
commercial	Conquesta	Stine 3000-0	Stine 3000-0
(designation)	(parental line)	(F)	(F)

[a]Event BPS-CV127-9 is tolerant to imidazolinone herbicides due to insertion of a gene encoding a modified acetohydroxy acid synthase protein from *Arabidopsis thaliana*. [b]LL55 is a GE soybean that is tolerant to glufosinate ammonium herbicides due to insertion of a gene encoding a modified phosphinothricin acetyltransferase protein from *Streptomyces viridochromogenes*. [c]Event FG72 is tolerant to glyphosate and isoxaflutole herbicides due to insertion of genes encoding a modified maize (*Zea mays*) 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein and a modified *p*-hydroxyphenylpyruvate dioxygenase (HPPD W336) protein from *Pseudomonas fluorescens*.

Human Serum and Plasma Samples. Human donor samples were provided from clinical sources collected following informed consent and under ethical review at allergy clinics associated with the University of Zurich (Zurich, Switzerland) or Charity Hospital (Berlin, Germany) with summaries of clinical diagnosis or from FDA-licensed blood product collection centers (PlasmaLab, International, Everett, WA, USA) or SeraCare Life Sciences (Milford, MA, USA) with self-reported allergies and in vitro IgE ImmunoCAP values determined for whole-soybean antigen mixture. Donor samples were coded by suppliers and were never revealed to us. Descriptions of clinical histories of allergies and available clinical allergen-specific IgE test results (if available) were provided with samples (Table 3). We further screened serum and plasma samples by in vitro IgE immunoblotting to a non-GM soybean extract to select appropriate, informative donors for use in the studies. In some cases, food challenge positive subjects had no detectable IgE binding in our tests, whereas some with less certain allergic status showed strong in vitro IgE binding that may indicate sensitization without allergy or cross-reactivity with or without clinical symptoms.

One-Dimensional Immunoblots. Proteins of soybean and control samples were extracted (1:10 w/v) in phosphate-buffered saline (pH 7.2) with protease inhibitors as described previously.[14] Protein concentrations were determined using the Lowry method, and equal total protein concentration samples were separated using one-dimensional SDS-PAGE Tris−glycine 7 cm minigels in either reducing or nonreducing Laemmli buffer, with or without heating (respectively). Running buffer was 25 mM Tris, 0.192 M glycine, pH 8.3, 0.1% SDS. Proteins were then electrophoretically transferred to PVDF membranes prior to blocking in 2% nonfat dry milk (NFDM) and incubation with serum or plasma samples diluted in PBST with 2% NFDM (1:10 or 1:20, v/v) as described previously.[24,25] Bound IgE was detected by electrochemical luminescence (ECL) using monoclonal anti-human IgE conjugated with horseradish peroxidase (SouthernBiotech, Birmingham, AL, USA) diluted 1:1000, followed by ECL substrate (SuperSignal West-Dura, Thermo Scientific) as described previously.[14] Images of blots incubated with the same serum or plasma sample and separation procedure (reducing or nonreducing) were captured simultaneously along with a standard dilution series of purified human IgE, using a Kodak Gel Logic Image Station 440 (CareStream Health, Rochester, NY, USA). The specificity of the SouthernBiotech monoclonal anti-hIgE-HRP (clone B3102E8;

Table 3. Serum and Plasma Samples Used To Evaluate IgE Binding in Three Studies (Donor No.; Clinician- or Self-Proclaimed History of Food Allergies; *Soybean-Specific IgE by ImmunoCAP If Known, kU/L*)

BASF Plant Science BPS-CV127-9 (3411T)	Bayer CropScience 1 LL55	Bayer CropScience 2 FG 72
714; soybean, wheat, corn, milk, pollen, HDM; *16 kU/L*	**9735-RE**; soybean, peanut; *5 kU/L*	**9735-RE**; soybean, peanut; *5 kU/L*
715; soybean, peanut, lentils, hazelnut, apple, carrots, pollen, HDM; *17 kU/L*	**715**; soybean, peanut, lentils, hazelnut, apple, carrots, pollen, HDM; *17 kU/L*	**CC 03**; soybean, peanut, fish, walnut, peppers; *>1 kU/L*
716; soybean, shrimp, clam, pollen; *7 kU/L*	**716**; soybean, shrimp, clam, pollen; *7 kU/L*	**CC 04**; soybean, peanut, walnut, celeriac, pollen; *0.6 kU/L*
719; soybean, wheat, peanut; *21 kU/L*	**17006-RM**; soybean, peanut; *7.8 k/L*	**CC 08**; soybean, peanut, almond, walnut, celeriac; *>1 kU/L*
721; peanut, corn, wheat; *47 kU/L*	**18534-LN**; nuts, beans, seeds; *17.3 k/L*	**CC 10**; soybean, peanut, milk, almond, walnut, celeriac; *7 kU/L*
RGLEG103; peanut, cashew, pecan, walnut, Brazil nut, almond, mold, pollen; *12 kU/L*	**19392-CS**; nuts, beans, seeds; *72 kU/L*	**19392-CS**; nuts, beans, seeds; *72 kU/L*
RGLEG118; soybean, peanut, cashew, Brazil nut, pecan, almond, crustaceans; *7 kU/L*	**20197-BH**; nuts and raw vegetables; *3 kU/L*	**20197-BH**; nuts and raw vegetables; *3 kU/L*
RELEG105; peanuts, walnut, celery, cashew, pollen; *2 kU/L*	**20770-MH**; nuts, beans, seeds; soybean; peanut, throat swelling; *16.4 k/L*	**CC 15**; soybean, lentil, peanut, almond; *1 kU/L*
LPSCH102; soybean, peanut, walnut, hazelnut, celeriac, apple, kiwi, pollen; *2 kU/L*	**22206-DL**; nuts, beans, seeds; *7.85 k/L*	**CC 11**; peas, carrot, almond, walnut, cherry, celeriac; *0.1 kU/L*
RG68; soybean; *4 kU/L*	**22329-JE**; no recorded history of allergy; *8.58 kU/L*	**CC 12**; peanut, carrot, almond, celeriac, fruits; *0.1 kU/L*
RG77; soybean; *8 kU/L*		**CC 16**; carrot, hazelnut, apple, food challenges pollen; *0.04 kU/L*
	Not Soybean Allergic (Controls)	
CTL 712; NA; *NA*	**CTL SNP**; no known allergies	**CTL SNP**; no known allergies
CTL RG71; lupine; *1.5 kU/L*	**CTL RG71**; lupine; *1.5 kU/L*	**CTL RG71**; lupine; *1.5 kU/L*
CTL RG73; pea, *0.7 kU/L*	**CTL RG73**; pea; *0.7 kU/L*	**CTL RG73**; pea; *0.7 kU/L*
CTL RG 74; lupine, peanut; *<0.35 kU/L*	**CTL RG74**; lupine, oral allergy syndrome to peanut; *<0.35 kU/L*	**CTL RG74**; lupine, oral allergy syndrome to peanut; *<0.35 kU/L*
CTL 287; soybean, peanut; *<1 kU/L*		

9160-05) was verified by reciprocal binding to diluted human myeloma IgE (ABCAM Laboratories, Cambridge, MA, USA; AB65866-1), and purified IgG (Sigma 12511) as compared to binding with a monoclonal hIgG-HRP (SouthernBiotech clone 9042-05).

Two-Dimensional Immunoblots. Ground raw soybean seed samples were first extracted using the trichloroacetic acid/cold acetone precipitation method of Natarajan et al.,[26] modified as described previously[14] using 8 M urea and 2% CHAPS to extract protein. The protein content of clarified extracts was determined by using the Bradford assay (Bio-Rad, Hercules, CA, USA). Samples representing 25 μg of extracted protein were diluted in 125 μL of isoelectric focusing buffer [8 M urea, 2% CHAPS, 50 mM DTT (Thermo Scientific, Rockford, IL, USA), containing 0.5% ampholyte pH 3−10 (Bio-Rad)] and applied to 7 cm pH 3−10 nonlinear IPG strips from Bio-Rad for isoelectric focusing as described previously.[14] Proteins in focused strips were reduced and acetylated and then separated on the basis of apparent molecular mass in the second dimension in NuPAGE Novex 4−12% Bis-Tris ZOOM gels (Invitrogen). Following electrophoresis, either the gels were stained using Coomassie blue stain or proteins were electro-transferred to PVDF membranes for immunoblotting and detection of bound IgE using ECL substrate sera as described for 1D immunoblotting.

Direct ELISA. Direct binding enzyme-linked immunosorbent assays (ELISA) to soybean proteins were performed to qualify human blood samples (serum or plasma) for all studies using individual sera with pooled soybean samples as the antigen target. Ground soybean seeds (either defatted or full-fat) were extracted 1:10 w/v in pH 7.4 phosphate-buffered saline (PBS), and protein content was determined according to the Lowry method and then diluted to 10 μg protein/mL in pH 9.6 carbonate−bicarbonate buffer to coat Maxisorp ELISA plates (Nunc-Thermo Scientific) overnight at 4 °C. Similar extracts of peanut, wheat, or maize were used as controls in some assays along with empty wells to estimate background binding. Wells were washed with PBS containing 0.05% Tween 20 (polyoxyethylene sorbitan nonionic detergent, PBST) and then blocked with 1% bovine serum albumin (BSA) in PBS. Serum and plasma samples were diluted to between 1:5 and 1:20 v/v in PBS containing 1% BSA for 1 h at 37 °C. Human samples were diluted on the basis of previous determinations of soybean-specific IgE levels if data were available. Unbound IgE was removed by four sequential washes using PBST. Bound IgE was detected using 1:5000 diluted monoclonal anti-hIgE conjugated with horseradish peroxidase (SouthernBiotech) followed by four washes with PBST and then application of TMB substrate (tetramethylbenzidine from Sigma, St. Louis, MO, USA). Absorbance values were measured in a BioTek instrument Powerwave XS2 (Winooski, VT, USA).

Inhibition ELISA. Independent competitive serum IgE binding assays were performed for two of the GM soybean lines (BPS-CV127-9 from BASF and FG72 from Bayer) to compare binding to their near-isogenic or parental line counterparts as well as three other commercial soybean lines. The assay design is similar to that demanded by pharmaceutical regulators in the EU for testing the equivalence of potency of allergenic extracts used in diagnosis and immunotherapy for allergy.[27] ELISA plates were coated with pooled soybean extract pools of the GM, near-isogenic, and commercial lines using bicarbonate buffer as described for direct ELISA tests. Individual human blood samples (serum or plasma) for each study were chosen from subjects with described clinical symptoms to ingestion of soybean and marked serum IgE binding to soybean proteins demonstrated during immunoblotting and preliminary direct ELISA assays. The volumes of individual human samples were chosen on the basis of diversity of protein binding and strength of binding to ensure representative IgE specificity and balanced binding strength to minimize dominance by any individual subject.

RESULTS AND DISCUSSION

Maize Lipid Transfer Protein. LTP is produced and stored in the pericarp of the grain just under the waxy seed coat as well as throughout the embryo of mature grain (results from immunohistochemistry in Goodman laboratory, data not shown). The concentration of mLTP in finely ground whole grain (approximately 10% moisture) was determined for each of the nine hybrids from two locations, grown with irrigation or in one location without irrigation. The average total protein yield from each 10 g sample of grain ground extracted with sodium acetate ranged from 8 to 9.5% protein based on the Lowry protein assay. The mLTP was determined using a semiquantitative immunodot-blot assay, using equal total protein (10 μg) replicate samples. The mLTP was detected using peptide-specific mLTP rabbit IgG that was detected using HRP-labeled goat anti-rabbit IgG followed by ECL. The mLTP concentration in grain samples was estimated by comparing densitometry values of samples to dot blots of standard diluted purified natural mLTP using regression analysis. The lowest level of accumulated mLTP was approximately 0.66 mg mLTP/g total protein and the highest was 9.86 mg mLTP/g total protein (Table 1). The greatest differences in mLTP accumulation between all hybrids across sites and treatments varied over 2.9-fold for Clay Center (irrigated), 4.5-fold for Mead Center (irrigated), and 14.9-fold for Mead (nonirrigated) samples. The greatest difference across locations and treatments for any one hybrid was 5.27-fold. These widely divergent concentrations of mLTP across locations and growing conditions suggest that normal variation in exposure to the major maize allergenic protein in foods could easily be expected to be up to 15-fold difference across genetic and environmental differences. Because mLTP is considered a pathogenesis-related protein that may be induced by stress,[28] the differences are not surprising. Food allergy to maize is rarely noted. There are no published data that demonstrate this wide difference in the content of mLTP contributes to the risk of allergy to maize despite the fact that mLTP is the only maize protein known to be responsible for severe (anaphylactic) reactions following consumption by corn-allergic subjects.[23] On the basis of our data, one might expect that differences of 5-fold or more in LTP concentration between a GM corn compared to its nearest isogenic comparator might occur due to environmental conditions. Risk management for corn-allergic people is to avoid consuming any product with corn proteins, regardless of variety (GM or non-GM) or commercial lot of corn.

Soybean IgE Comparisons. Three independent studies were performed over a two year period to test relative IgE binding to the GM soybean lines and nearest genetic relative (null-segregant or parental lines) as well as two to three more distantly related soybean lines. Representative results of all three studies are shown. Results from some tests performed on BASF soybean samples were previously presented;[14] however, none of the results shown here were previously published. For each study we attempted to obtain human blood samples (plasma or serum) from subjects with objective, clinically confirmed allergies and verify the soybean-specific in vitro IgE binding prior to study initiation. However, few clinical allergists specializing in food allergy that were contacted had subjects with confirmed food allergy to soybeans. One center in Berlin, Germany, and one center in Zurich, Switzerland, provided a number of clinical samples from subjects with documented allergy to foods containing soybean products and with soybean-specific IgE clearly above background levels based on the standardized laboratory ImmunoCAP for soybean (f14) and/or Gly m 4 (f353) from Phadia (now ThermoScientific, USA). Some human samples had very low levels of soybean protein-specific IgE based on preliminary direct binding using

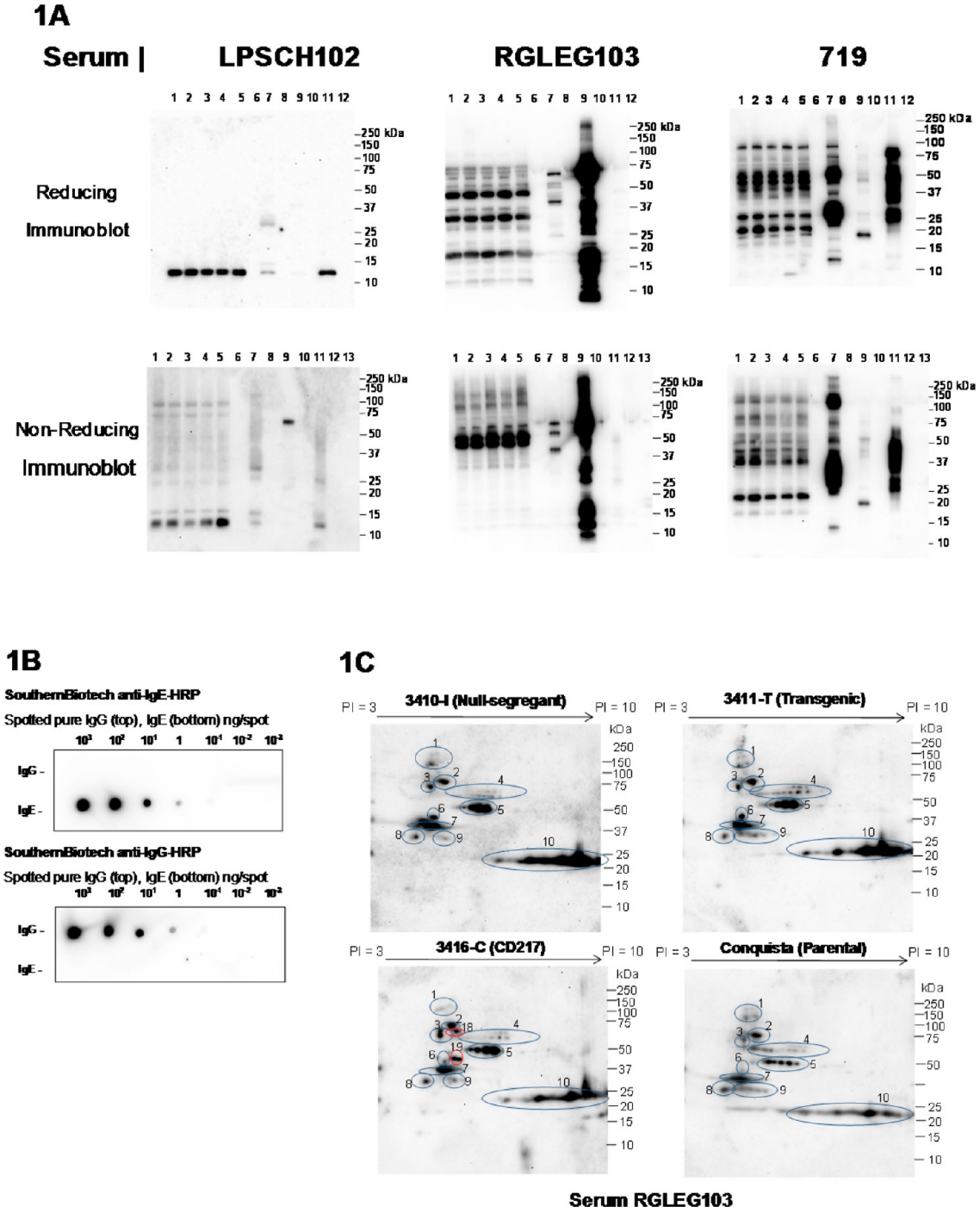

Figure 1. Soybean-allergic IgE binding to reduced and nonreduced proteins in 1D SDS-PAGE; BPS-CV127-9 (BASF). (A) Proteins in extracts of soybean (10 μg), navy bean (10 μg), peanut (2 μg), and maize (10 μg) were separated by SDS-PAGE under (a) reducing and (b) nonreducing conditions and immunoblotted with sera from three soybean-allergic subjects LPSCH102, RGLEG103, or 719 and detected using hIgE-specific mAb conjugated with HRP followed by ECL detection. Sample lanes: (1) 3410-I, null-segregant; (2) 3411-T, GM line BPS-CV127-9; (3) 3415-M, MON8001; (4) 3416-C, CD217; (5) Conquista, parental line; (6) none; (7) navy bean; (8) none; (9) peanut; (10) none; (11) maize; (12 MW marker. (B) Samples of purified human IgE and IgG were diluted and spotted onto nitrocellulose, representing from 1 pg−1 μg of immunoblobulin and then detected using monoclonal anti-hIgE or anti-hIgG to demonstrate immunoglobulin specificity. (C) Protein samples of null-segregant 3410-I GM, 3411-T, CD217, 3416-C, and Conquista (parental) soybean lines were separated by IEF in p*I* 3−10 nonlinear IPG strips then by size in SDS-PAGE, blot incubated, with serum RGLEG103.

immunoblots from reducing and nonreducing SDS-PAGE or direct binding to native protein by ELISA. Therefore, additional samples of plasma were obtained from FDA-licensed blood product suppliers from subjects that were screened by questionnaire and indicated probable food allergy to soybean, peanut, or other legumes and positive soybean-specific IgE testing using the ImmunoCAP system (Table 3). The EU values in Table 3 are for ImmunoCAP f14.

In all three studies diverse patterns of IgE binding were seen between subjects as demonstrated in 1D immunoblots (Figure

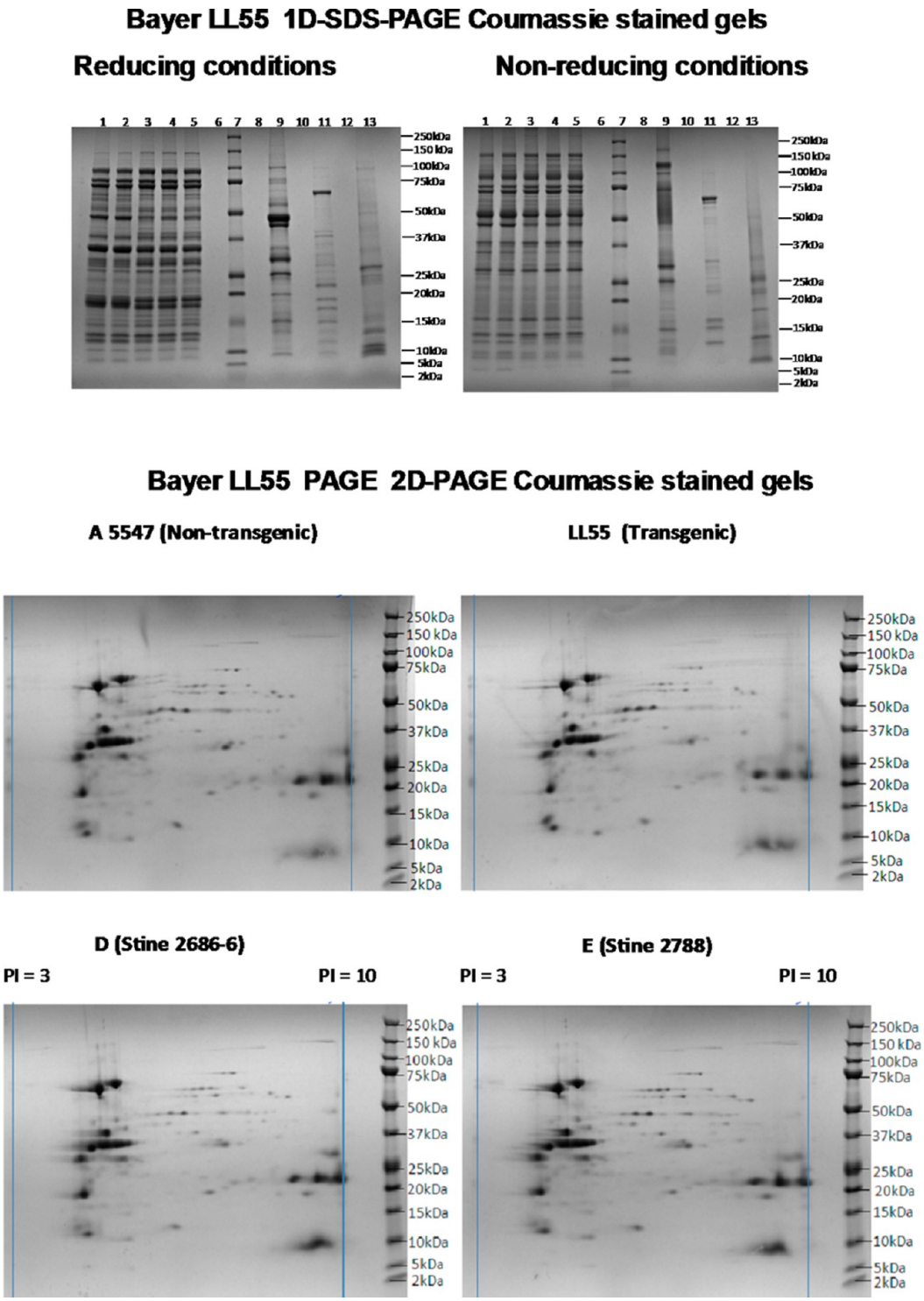

Figure 2. Extracts of soybean samples from the Bayer LL55 study were separated by 1D gel SDS-PAGE under reducing and nonreducing conditions as well as 2D gels (IEF first direction, p*I* 3−10 nonlinear gradient, then SDS-PAGE). Proteins in gels were stained with Coomassie blue. Proteins from identically run gels were transferred to PVDF membranes for immunoblotting. Sample lanes (1D): (1) A5547 (near-isogenic); (2) LL55 (transgenic); (3) Stine 2686-6; (4) Stine 2788; (5) Stine 3000-0; (6) empty; (7) MW marker; (8) empty; (9) navy bean; (10) empty; (11) peanut; (12) empty; (13) maize (corn). 2D gel samples: A5547 (isogenic); LL55 (transgenic); Stine 2686-6; Stine 2788.

1A). A few subjects exhibited binding to a single protein that is most visible under reducing conditions (e.g., subject LPSCH102), which may be a homologue of the LTP based on similar binding to a corn protein at ∼12 kDa using this subject's sera. For many subjects moderately complex binding was observed to at least three proteins including two that are linked by disulfide bonds (e.g., subject RGLEG103), and occasionally more complex patterns with binding to six or more bands (e.g., subject 719) was seen. The last case likely includes IgE binding to cross-reactive carbohydrate determinants (CCD) on some of the soybean proteins as demonstrated by strong binding to a protein of about 34 kDa (probably PHA,

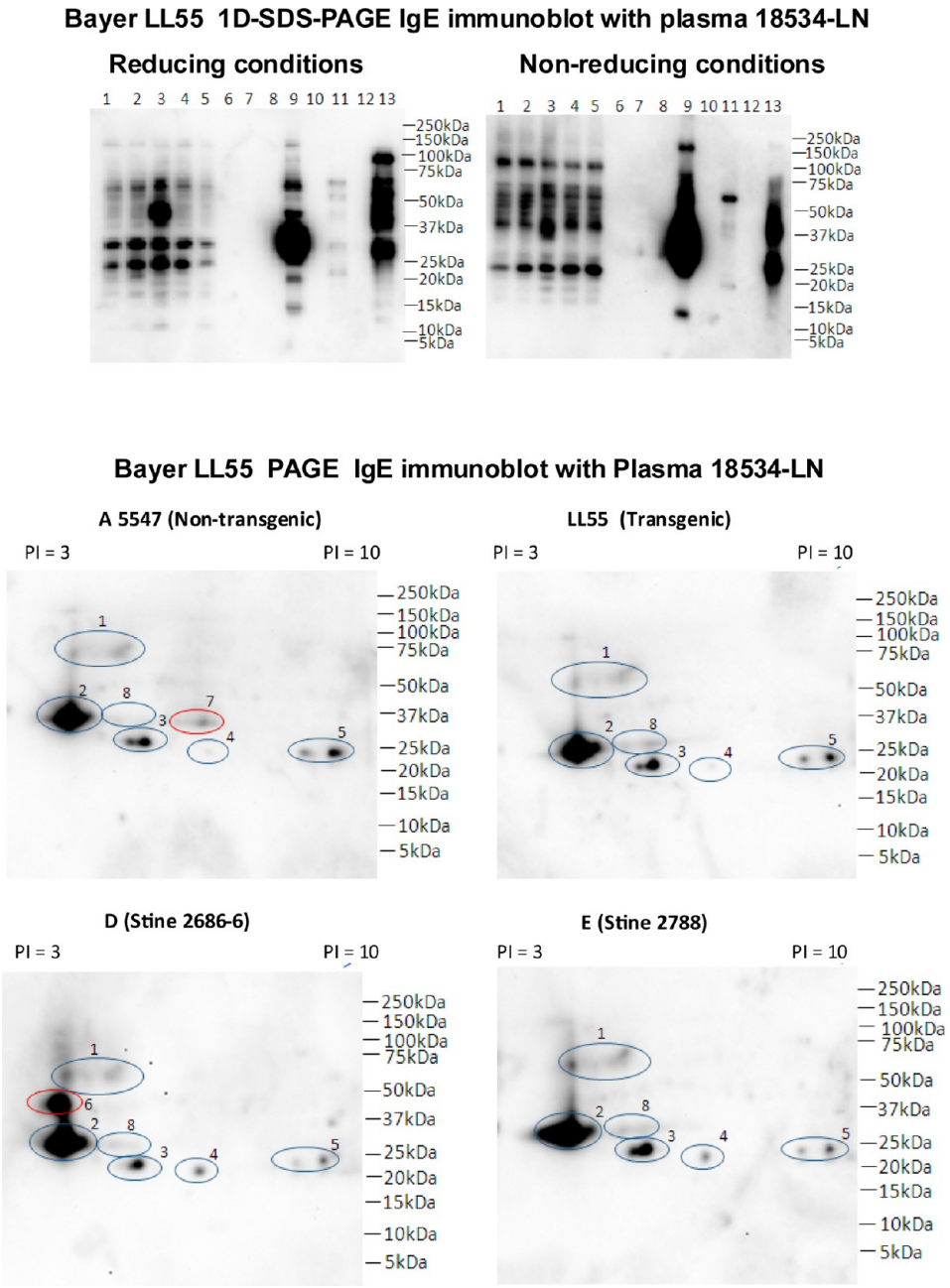

Figure 3. Serum IgE immunoblots of Bayer LL55 soybeans with soybean allergic plasma 18534-LN using 1D SDS-PAGE samples under reducing and nonreducing conditions and 2D PAGE. Sample lanes (1D): (1) A5547 (near-isogenic); (2) LL55 (transgenic); (3) Stine 2686-6; (4) Stine 2788; (5) Stine 3000-0; (6) empty; (7) MW marker; (8) empty; (9) navy bean; (10) empty; (11) peanut; (12) empty; (13) maize (corn). The notable difference in binding between soybean samples is at 50 kDa in nontransgenic sample lane 3 in reducing conditions, or lower MW in nonreducing conditions of 1D gel blots, and spot 6 of sample Stine 2686-6 in 2D gel blots. The only visible difference between near-isogenic line A5547 is the presence of light spot 7 in A5547 and the absence of the spot in LL55 (transgenic).

based on unpublished data) in navy bean extract and multiple proteins in maize extract (Figure 1, subject 719). IgE binding to CCD is rarely clinically relevant.[29] Many serum and plasma samples also showed IgE binding to at least one protein in the control plant extracts of navy bean, peanut, or maize, and the histories of most subjects (Table 3) indicated multiple food and airway allergies. However, it is important to stress that IgE binding alone does not prove that a given protein causes allergic symptoms, only that IgE antibodies are able to bind to the protein in vitro. Clinical laboratory results with positive soybean-specific IgE binding (>1 kUA/L specific IgE) often correlated with clear IgE binding detected in our study by immunoblot and/or ELISA, but in a few cases low levels or no detectable IgE binding was demonstrated even for subjects with reported positive food challenges, although some of those subjects (CC-3, CC-8, CC-11, CC-12, CC-15, CC-16) exhibited binding to high concentrations of native total soybean protein by dot-blot assay (data not shown). Whereas binding to peanut proteins typically correlated with allergy to peanut, binding to navy bean or maize was rarely matched with reports of clinical allergy to those foods. The 1D IgE blots for the BASF study of BPS-CV127-9 (3411-T) did not show obvious qualitative or quantitative differences compared to the null-segregant (3410-I) or the Conquista (parental line), but there

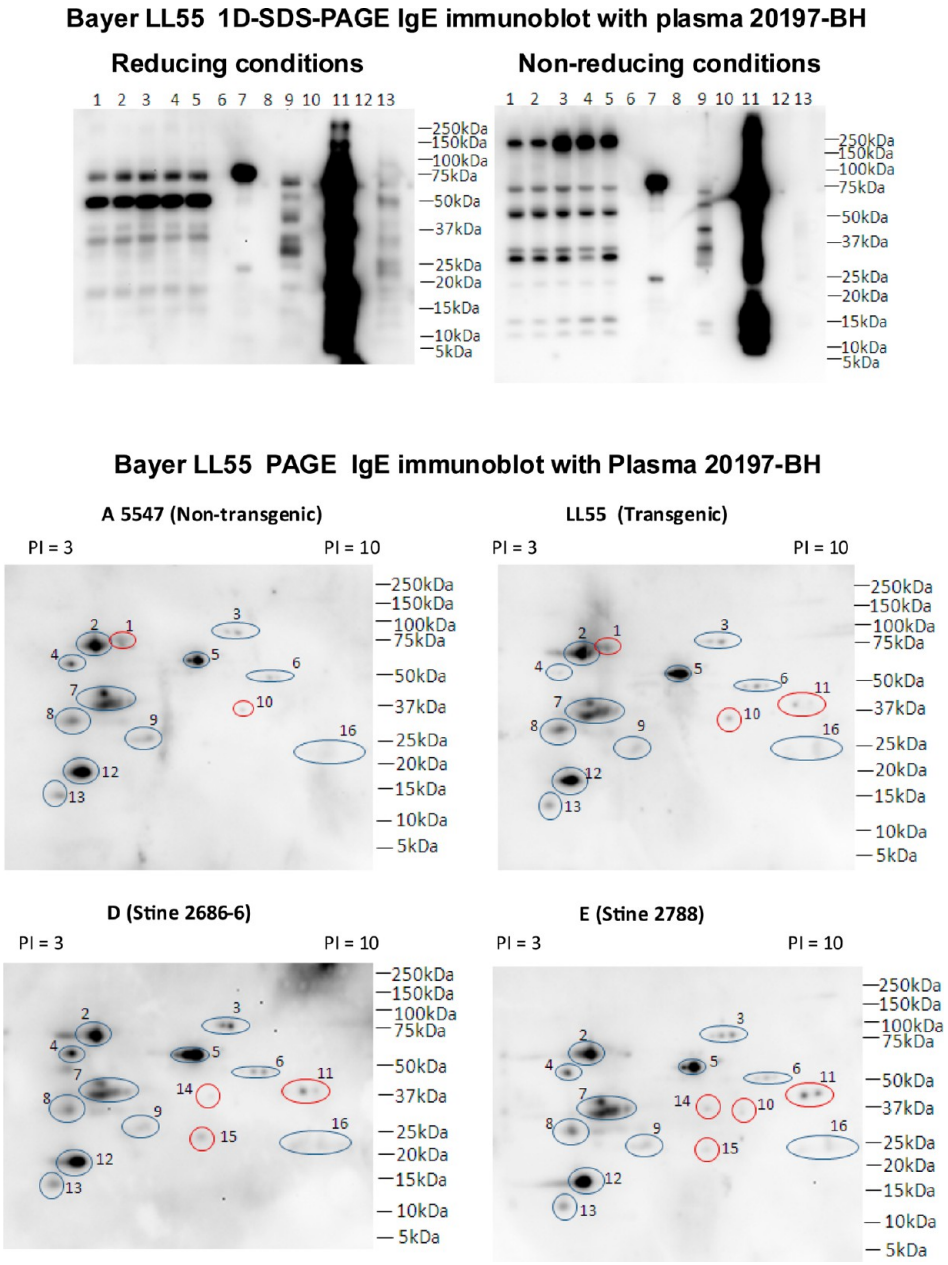

Figure 4. Serum IgE immunoblots to Bayer LL55 soybeans with soybean allergic plasma 20197-BH using 1D SDS-PAGE samples under reducing and nonreducing conditions and 2D PAGE. Sample lanes (1D blots): (1) A5547 (near-isogenic); (2) LL55 (transgenic); (3) Stine 2686-6; (4) Stine 2788; (5) Stine 3000-0; (6) empty; (7) MW marker; (8) empty; (9) navy bean; (10) empty; (11) peanut; (12) empty; (13) maize (corn). No obvious difference in binding between soybean samples was visible from 1D blots beyond the more intense band at 250 kDa in the three commercial lines (lanes 3−5). A few low-intensity spots differed in 2D PAGE blots. Two spots that were visible in LL55 transgenic in circle 11 were not visible in the A5547 nontransgenic line but were also visible in both D and E samples. In addition, the faint spots in circles 14 and 15 were visible in D and E, but not the LL55 transgenic or nontransgenic A5547.

were minor differences of all three of those relative to commercial lines MON8001 (2415-M) and CD217 (3416-C) as described by Panda et al.[14] However, differences were not observed using the three human samples shown in Figure 1A. We previously observed that some anti-IgE secondary detection antibodies can bind to IgG in immunoassays[25] and that many sera contain both IgG and IgE specific to dietary proteins (data not shown). Therefore, the specificity of the SouthernBiotech anti-IgE used in these studies was tested with reciprocal binding to diluted IgE and IgG and demonstrated specificity of >10000 to 1 for IgE (Figure 1B).

Two-dimensional immunoblots of proteins separated first by charge (p*I* value) and second by apparent mass (SDS-PAGE) provide a much more detailed and complicated picture than is obvious from 1D gels. Individual proteins and clusters of proteins are visible in Figure 1C using serum RGLEG103, compared to Figure 1A. Clearly the single visible bands in Figure 1A (reducing) separate into clusters of proteins with multiple p*I* values (e.g., 22, 37, and 47 kDa) that can show minor variations, primarily in intensity, between soybean lines (Figure 1C). However, new spots (circled as 18 and 19 in 2D gel blots Figure 1C) of CD217 (3416-C) were not visible in lane 4 under either reducing or nonreducing conditions from

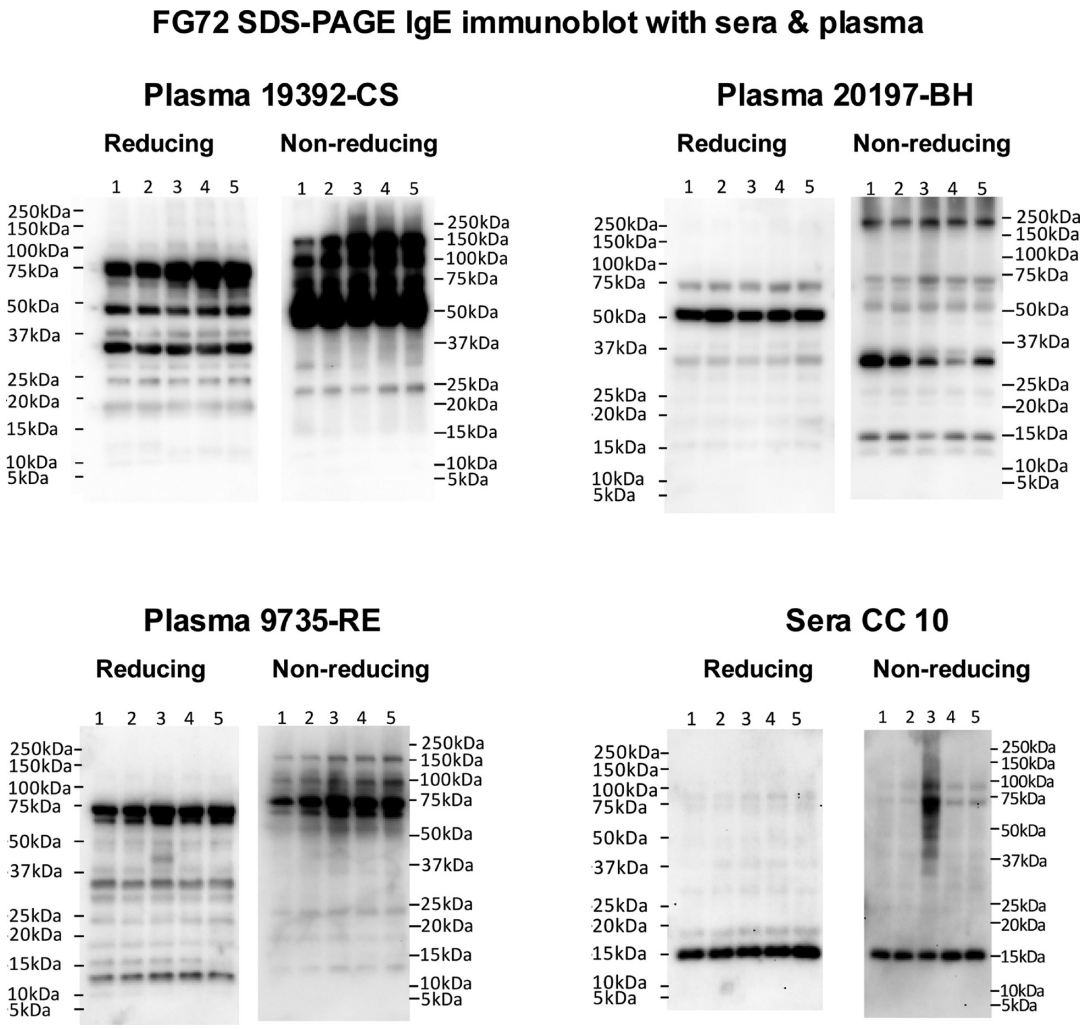

Figure 5. SDS-PAGE IgE Immunoblots from FG72 study using reducing and nonreducing gels. Minor differences in band intensity are obvious between some samples. Soybean sample loading: (1) FG72; (2) Jack (parental); (3) Stine 2686-6; (4) Stine 2788; (5) Stine 3000-0. Control lanes (navy bean, peanut, and maize) are not presented to allow presentation of blots using four soybean allergic human samples (plasma samples 19392-CS, 20197-BH, and 9735-RE; as serum CC10). A minor additional band was present at about 20 kDa in lane 3 (~45 kDa) of reducing sample with plasma 9735-RE. A major higher MW smear and visible band at 75 kDa in lane 3 of nonreducing sample with serum CC 10 is likely due to the presence of a conformational epitope on a 75 kDa protein that is abolished by reduction with 2-mercaptoethanol. That observation was also made with a number of other serum samples (not shown). Other differences seem to represent minor concentration differences of specific protein bands, although in some cases differences may be artifacts of protein transfer. These human samples were used in the serum pool for ELISA inhibition tests.

1D immunoblots (Figure 1A). This commercial line is not known to be more allergenic than the other lines. Additional minor differences in the numbers of spots visible from an apparent series of likely post-translationally modified similar proteins (e.g., circle 4 in Figure 1C) are unlikely to represent any significant difference in overall IgE binding or allergy between soybean lines. Interpretation of these blots is complex. Although other investigators have shown that larger gel formats (13 cm IEF strips compared to our 7 cm strips) increase discrimination of spots, we find it is impossible to obtain a sufficient volume of serum from individual donors to be able to perform multiple immunoblots with the larger format. However, results showing minimal differences in 1D (reducing and nonreducing conditions) and 2D immunoblots as well as ELISA inhibition of the BASF soybean lines led us to conclude it is highly unlikely there are biologically relevant differences in the allergen content of the GM, near-isogenic, parental, or other commercial soybean lines.[14]

Evaluation of the allergen content of Bayer's LL55 was performed by comparison of IgE from soybean-allergic subjects to bind to proteins in the transgenic, parental lines and three other nontransgenic commercial soybean lines by immunoblot comparisons of 1D SDS-PAGE with proteins in extracts separated under reducing and nonreducing conditions. Immunoblots of 2D gel separated proteins of transgenic, parental line and two (of the three) other commercial soybean lines were also tested. Protein separation patterns are demonstrated by Coomassie blue staining in gels shown in Figure 2. The same extracts were separated in identical gels and the proteins transferred to PVDF membranes prior to blocking and incubation with samples from allergic subjects or controls. Bound IgE was detected as described for the BASF study. Representative results are shown in Figures 3 and 4. Plasma 18534-LN revealed minor differences in the intensity of binding to some proteins in the nontransgenic control A5547 and transgenic LL55 in 1D immunoblots, but the most notable difference was the intense binding to a band at approximately

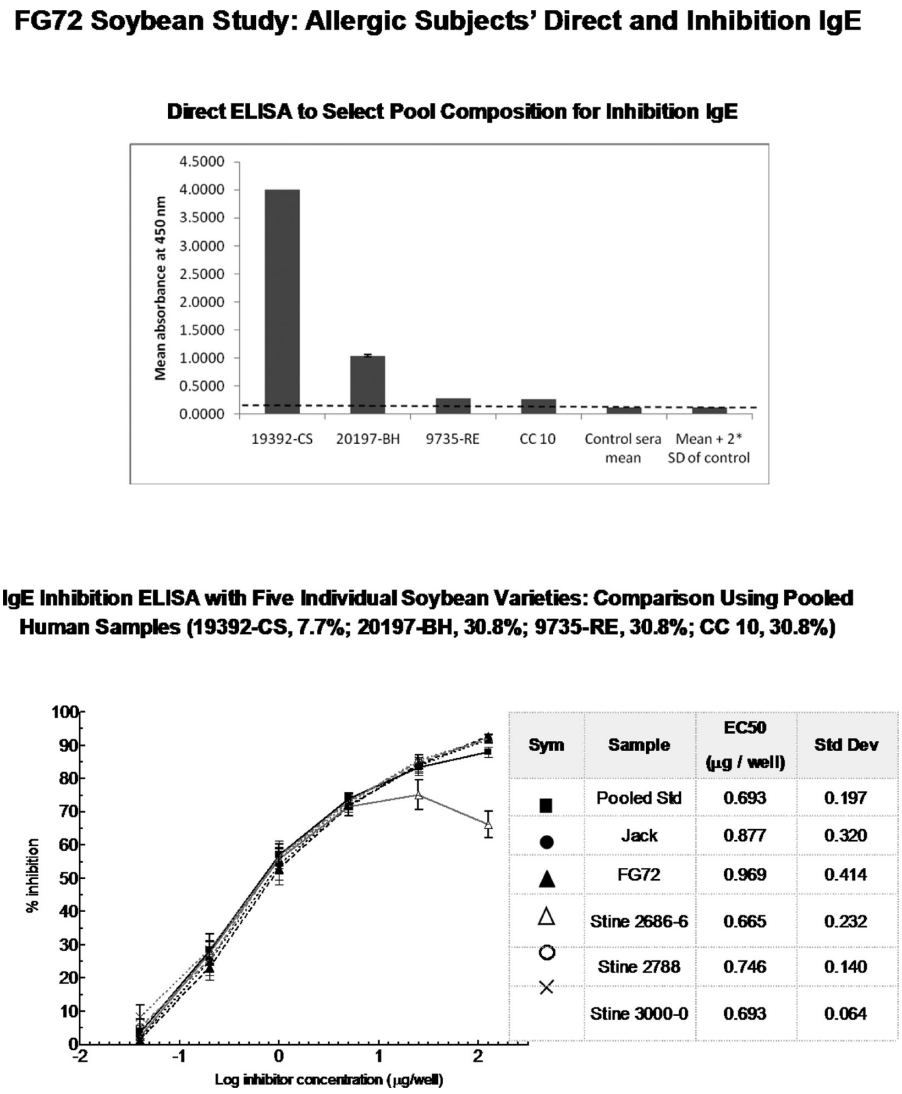

Figure 6. FG72 soybean study: allergic subjects' direct and inhibition IgE ELISA. The four highest IgE binding sera (of 10) by immunoblotting and preliminary ELISA were retested to guide dilution of individual samples for the inhibition pool. Raw optical density (OD) values are reported for individual human samples with direct binding indicated; 19392-CS was 4 times as high in soybean-specific IgE as the second highest binding sample, and immunoblot results demonstrated at least three major bands (Figure 5), whereas the lower binding samples (20197-BH, 9735-RE, and CC10) primarily bound to different single proteins. Inhibition ELISA assays were performed in triplicate using a pool of four subjects (0.25 volume of 19392-CS to 1 volume of each of the other three human samples). ELISA plates were coated with an equal protein pool of the five soybeans (parental, Jack; FG72, Stine 2686-6, Stine 2788, and Stine 3000-0) for both direct binding and inhibition. For inhibition the pooled serum/plasma was preincubated (1 h) with diluted samples of the standard pool or individual soybean extracts prior to incubation with the coated sample wells, then washed and incubated with anti-IgE, washed again, and finally substrate. The amount of protein of each extract required to achieve 50% inhibition of maximum IgE binding is shown in the table and is the reciprocal of "relative potency". Thus, FG72 is the weakest at inhibiting IgE binding, although there were no significant differences between extracts.

45 kDa in reducing and intense multiple bands in nontransgenic sample in lane 3, sample D (Stine 2686-6) of both reducing and nonreducing samples, which was not present in any of the other four soybean lines. The intense binding to a band of approximately 34 kDa in the navy bean sample (lane 9 of both 1D reducing immunoblots) corresponds to phytohemaglutinin (PHA) and likely represents IgE binding to complex carbohydrate determinants on PHA (unpublished inhibition data). By 2D immunoblot, the fairly weak IgE binding spot marked 7 on the A5547 immunoblot was not present in the transgenic or the other two commercial soybean lines. We did not have a sufficient volume of serum to attempt inhibition of binding to the soybean sample Stine 2686−-6 and kidney bean by preincubating the serum with kidney bean extract, which would confirm that CCD was the likely source of binding. Plasma sample 20197-BH (Figure 4) showed slightly weaker binding to the very high MW band at 220−240 kDa in both nontransgenic lane 1 and transgenic lane 2 compared to more intense binding to the same MW band in the three nontransgenic commercial lines (lanes 3−5). Because the reduced samples (left blot) do not show the high MW band (220 kDa) and rather show intense bands at ∼50 and 75 kDa, the high MW band in the nonreduced blot probably corresponds to the trimeric form of β-conglycinin, which is expected to be reduced to two subunits of about 70 kDa and one of about 50 kDa, corresponding to α-, α'-, and β-subunits of β-conglycinin (not yet confirmed by protein sequencing). In the 2D immunoblot of LL55, two moderately faint spots are

visible in circle 11 that are not visible in the nontransgenic A5547 sample. However, the same spots are visible in the two nontransgenic Stine lines, suggesting this is not unique to transgenic LL55. In addition, both Stine soybean lines showed two additional faint spots marked 14 and 15 that are not visible in either nontransgenic A5547 or transgenic LL55. The other eight sera and plasma samples showed similar patterns of IgE binding between the transgenic LL55 and nontransgenic A5547 lines. We did not perform a quantitative analysis (ELISA inhibition) in the study of A5547, but the qualitative results of 1D immunoblots under reducing and nonreducing conditions and 2D immunoblots indicated that differences in IgE binding to LL55 are remarkably similar to binding to nontransgenic comparator line and other commercial soybean lines. It is therefore highly unlikely that there is any significant difference in the allergen content of the transgenic and nontransgenic soybeans for LL-55.

The final study described here evaluated IgE binding to FG72 transgenic soybean line compared to nontransgenic parental Jack and three other nontransgenic commercial lines (Stine 2686-6, Stine 2788, and Stine 3000-0) using 1D immunoblotting and ELISA inhibition. Representative 1D immunoblots (Figure 5) are shown using the four soybean-allergic subjects' serum or plasma samples that were used as a pool in ELISA inhibition. The banding patterns demonstrated very different IgE protein binding patterns between human donors, but qualitatively similar results across soybean lines. Interestingly, IgE from donor 19392-CS bound to a protein of approximately 50 kDa under reducing conditions as did IgE from donor 20197-BH. However, under nonreducing conditions donor 20197-BH IgE bound to lower molecular weight proteins at 32 and 15 kDa, which does not correlate with results from donor 19392-DS. The other prominent bands bound by donor 19392 under reducing conditions were not prominent bands in blots of 20197-BH. Under nonreducing conditions the donor 19392-CS also showed considerable binding to bands at 150, 100, and 50 kDa that were not recognized as prominent binding bands for donor 20197-BH. The rather faint band at 230 kDa in the nonreducing blot of donor 20197-BH may represent the complexed trimeric β-conglycinin under nonreducing conditions, and the 50 kDa band under reducing conditions may represent IgE binding to β-conglycinin. The prominent IgE binding bands from donor 9735-RD at approximately 75 kDa in both reducing and nonreducing blots likely represents a unique, nonseed storage protein that does not include any disulfide bonds. However, the lower MW and modest binding to a 70 kDa protein under nonreducing conditions likely represent binding to a monomeric subunit of glycinin that is reduced to acidic and basic subunits under reduction (35 and 13 kDa). With these four soybean-allergic subjects the differences in IgE binding by immunoblotting were minor across soybean lines for all four donors, with the exception of a 75 kDa band using serum CC-10 (lane 3, nontransgenic control). This band is visible under nonreducing conditions and is not replicated in other soybean lines. It was also not visible under reducing conditions. It is therefore likely to represent a conformational epitope that is dependent on a disulfide bond.

The total IgE binding to native soybean proteins for each clinical subject used to create a pool of serum for quantitation of IgE binding in the FG72 study was measured by direct ELISA (Figure 6, top panel). Clearly, donor 19392-CS had the highest total IgE against soybean proteins with an absorbance value nearly 4 times that of 20197-BH and >10 times higher than those of 9735-RE and CC10. Because of these differences, the volume of plasma 19392-CS was reduced to one-fourth of the volume used from the other three soybean-allergic subjects as the pool for the inhibition tests to reduce bias in the ELISA inhibition assay. These four subjects each presented a different IgE binding pattern in 1D immunoblots, demonstrating different protein recognition with the adjusted volume ratios provided a balanced pool for relative potency comparison in the inhibition assay. Inhibition ELISA tests were performed in triplicate, and results are compiled in the bottom panel of Figure 6 for each soybean sample. Six concentrations of inhibitor were tested for each extract of soybean lines, and the amount of protein from each extract needed to achieve 50% inhibition (EC50) was calculated for each sample as listed in the inset (Figure 6). There were no statistically significant differences in EC-50 values. However, of interest is the tailing off of inhibition using extract Stine 2686-6 at the higher concentrations of extract. This suggests a low-affinity interaction of IgE from the pool for some protein(s) in that extract. This result correlates with the extract (Stine 2686-6) that was lost in reduced samples. This finding of IgE binding to only nonreduced samples by 1D immunoblotting was also observed for most of the other serum samples from the EU (data not shown).

Focusing on the question of comparing IgE binding between the transgenic and nearest genetic soybean line, there were no marked differences in IgE binding to transgenic and nontransgenic soybean lines in the three studies reported here. By including other commercial non-GM soybean lines, we can conclude that the variation was well within the range of binding differences likely to be found in commercial lines of soybeans that are used in foods today. Our conclusion is that there were no data to suggest these three GM soybean lines differ in endogenous allergen content from their nearest non-GM counterparts or other tested commercial lines.

It is important to recognize the complexity of performing these tests. It is very difficult to identify appropriate serum IgE donors that have clearly recognized soybean food allergy as well as significant levels of soybean protein-specific IgE binding. Furthermore, the tests including 1D immunoblots, 2D immunoblots, and ELISA inhibition provide somewhat different answers in binding to proteins. The differences likely depend on the structural epitopes that are bound, whether conformational, sequential, or binding of IgE to CCD epitopes.

Due to the difficulty in obtaining relevant allergic sera and the complexity of interpreting results when using subjects with varied IgE sensitization patterns, some scientists have suggested using non-antibody-dependent proteomic approaches to evaluate potential changes in protein expression in a GM crop relative to near-isogenic or commercial varieties as described by Rouquie et al.[30] A quantitative proteomics approach using labeled peptide controls in a non-gel-based evaluation method demonstrated high accuracy in measuring concentrations of eight allergenic soybean proteins.[31,32] However, the methods are technically challenging and measure only specific peptide fragments from identified allergens, not IgE binding or allergenicity. Measurement of allergens across four genetically different soybean lines and in another study across growing conditions demonstrated that some allergenic proteins varied little, whereas one of the proteins varied up to 10-fold across environments.[31] To be useful in the context of endogenous allergen evaluation, one should know all of the

allergenic proteins in a complex commodity and preferably the relative allergenic potency of the individual components. The methods should be capable of measuring all variables in protein structure that might be relevant to IgE binding and elicitation of allergy. Even more importantly, these tests demonstrated that there is wide variation in allergen expression in non-GM soybean varieties and lots used in commerce today. Regulatory tests for allergen levels are not required for non-GM soybean varieties.

In light of the complexity of performing tests to evaluate endogenous allergens and the reality of commodity crop food production, trade, and food processing, we do not believe the types of studies we have described here add substantial information to the safety assessment of GM commodity crops except in cases when there are reasons to suspect differences. If the transgene inserted into a GM crop was a transcription factor or the transgenic event was intended to reduce the allergenicity of the commodity crop, then tests evaluating endogenous allergen content should be required.

At this time there is not good agreement about the exact methods that should be used to perform tests to compare endogenous allergen content. There has also not been discussion about how much difference is too much or about developing data to set boundaries of acceptability on potential changes in allergen content of commodity crops. At the current time, those involved in testing and those involved in regulation of GM crops have rather quietly been looking for "statistically significant differences" between the quantitative IgE binding to proteins in the GM and near-isogenic line, as well as an absence of qualitative differences (no new bands or spots), to conclude that there are no concerns that a GM event is acceptable regarding allergy. The expectation stems from the principles of substantial equivalence that were intended to ensure a new GM crop would be suitable in nutritional and antinutritional properties to the crop varieties in current use as discussed by Codex (2003).[1] However, we do not have data to demonstrate heightened risk of food allergies based on differences of allergen expression between crops. One could argue that the same criteria used to evaluate standardized allergenic extracts (pharmaceutical products) might be suitable to use as a very conservative guide for food safety. Regulators of standardized allergen extracts used for diagnostic testing or immunotherapy in the United States and EU have established limits of acceptance of approximately 2-fold for claims by manufacturers of those products that they are equivalent for diagnosis or immunotherapy.[27,33] Those criteria were established for allergenic extracts intended for diagnosis or treatment (immunotherapy) of allergic disease. However, even a 2-fold difference is arguably too small for defining food safety if the variation in allergen content is at least 15-fold for LTP in commercial corn and uncertain for most allergenic crops. An additional consideration is that most of the common and potent food allergens (proteins) are major components of the food source. Thus, there are limits to the amount of increased accumulation the major peanut allergens (Ara h1, Ara h2, Ara h3, Ara h6) or soybean allergens (glycinins and β-conglycinins) that can occur in a given food grade material. Many of these proteins represent 2−20% of the protein in the food. Clearly, expression of any major component cannot increase more than a few-fold without influencing the expression of other proteins or the characteristics of the food material.

Perspective could also be gained from procedures used by recognized clinical food allergists and scientists involved in double blind, placebo-controlled tests attempting to define threshold doses for food allergy for individuals as presented by Crevel et al.[34] The common guideline is to start food challenges of individuals suspected of having severe allergies at a very low dose of total allergenic food (e.g., peanut), such as 10 μg. Dose, and observe for 20 min, then increase the dose by from 2- to 5-fold at each succeeding step of active (allergen) dose. Controlled clinical studies following this protocol have been deemed safe by ethical boards and clinicians and rarely lead to systemic anaphylaxis if stopped at the dose that caused objective allergic symptoms in the individual.[34] This information suggests that a 2−5-fold increase in total allergen dose in a given commodity crop is highly unlikely to significantly increase the risk of food allergy for those with severe allergic disease.

The process of clinical risk management for food-allergic subjects over the past 20 years has been to identify the specific food causing allergy for the individual and have them avoid consuming foods that contain any of the allergenic food, whether it is peanut, soybean, milk, eggs, wheat, or corn. Food companies are required to label ingredients of packaged foods with special attention given to any ingredient that might contain proteins from the most common allergic foods. The allergic consumers are informed about foods to avoid and how to interpret food labels. Nonallergic consumers can consume large quantities of the same food without adverse effects. There is risk of severe food allergy, but that is from unlabeled or inaccurately labeled foods, not from minor differences in the expression of allergens within the allergenic commodity crop.

It seems quite logical, then, to use the collective information in deciding on the appropriate need to test for changes in endogenous allergen expression in GM crops relative to the non-GM counterpart. It should rarely be necessary, based on public health risks, as food sources should be labeled and allergic consumers should avoid consuming foods containing their allergen. In cases when regulators deem such tests are necessary, limits should be established that reflect the natural variation of allergens in commonly used varieties of the same food crop. A doubling of allergen content would be acceptable for a pharmaceutical product intended to be used to diagnose or treat those with allergies. An additional factor that should be recognized is the most likely and deleterious risk of food allergy that might occur from creation of a new GM crop would be introducing an existing allergen or highly cross-reactive protein from a different source.[12,35] The evaluation process and testing to prevent the risk of transferring an allergen is well established and markedly limits the likelihood of increased food allergy from GM crops.

■ AUTHOR INFORMATION

Corresponding Author

*(R.E.G.) E-mail: rgoodman2@unl.edu.

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Notes

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■ ABBREVIATIONS USED

CCD, cross-reactive carbohydrate determinants; ECL, electrochemiluminescence; GE, genetically engineered; GM, genetically modified; HRP, horseradish peroxidase; IEF, isoelectric focusing; mLTP, maize lipid transfer protein; NFDM, nonfat dry milk; OD, optical density; PBS, phosphate-buffered saline; PBST, PBS with 0.2% Tween 20; 1D, one-dimensional; 2D, two-dimensional

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