

Application of Current Allergy Assessment Guidelines to Next-Generation Biotechnology-Derived Crops

GARY A. BANNON and SUSAN MARTINO-CATT

Monsanto Co., Global Regulatory Sciences, 800 N. Lindbergh Blvd, St. Louis, MO 63167

In any single day, our immune systems are exposed to thousands of different proteins from the environment and the food we eat. In a portion of the human population, some of those proteins will stimulate the immune systems to synthesize immunoglobulin E in an allergenic response. The discrepancy between the vast numbers of proteins we encounter and the limited number of proteins that actually become allergens have led scientists on a quest to discover what unique features exist that make proteins destined to be allergens. The information gained from these studies has led to an allergy assessment strategy that characterizes the potential allergenicity of biotechnology products prior to their commercialization. This testing strategy appears to be effective as shown by the fact that there have been no clinically documented food allergic reactions to any of the biotechnology proteins introduced into food crops, to date. The next generation of biotechnology products will most likely contain more complex traits, including nutritionally enhanced food crops, and the question arises as to whether the current allergy assessment strategy will be sufficient to protect the health of the consuming public. In this paper, we discuss general allergen characteristics in order to better understand how proteins become allergens, summarize the current allergy assessment process, evaluate the different aspects of this process for their adequacy in determining the allergenic potential of engineered functional foods, and, finally, we assess the possibility of new technologies having a positive impact on the allergy assessment of nutritionally enhanced crops.

Genetically modified (GM) crops have been in the world's food supply for around 10 years and are currently planted on 250 million acres in 22 different countries. An additional 29 countries have approved their

import. Of the principal food crops grown worldwide in 2006, biotech (Bt) soybean occupies 58.6 million hectares and maize occupies 25.2 million hectares. Herbicide tolerance has consistently been the dominant trait planted in the field, followed by insect resistance and then products containing both of these traits in a stacked combination. In 2006, herbicide tolerance deployed in soybean, maize, canola, cotton, and alfalfa occupied 69.9 million hectares (68%) of the global biotech 102 million hectares, with 19.0 million hectares (19%) planted to Bt crops and 13.1 million hectares (13%) to the stacked traits of Bt and herbicide tolerance. The accumulated hectareage from 1996 to 2006 exceeded half a billion hectares at 577 million hectares (1.4 billion acres), with an unprecedented 60-fold increase between 1996 and 2006, making biotech crops the fastest-adopted crop technology in recent history (1).

All foods derived through biotechnology must undergo a comprehensive safety evaluation as part of the regulatory approval process prior to entering the market and becoming part of the food supply. The general principles serving as the baseline framework for the safety assessment of biotechnologically derived foods have been developed over time with input and guidance from governmental regulatory agencies; academics; international organizations such as the Organization for Economic Cooperation and Development (2), the International Life Science Institute (ILSI; 3), and the Food and Agriculture Organization/World Health Organization (4, 5); and many companies involved in the production of biotechnologically derived crops. Consequently, an integrated, step-wise approach to safety evaluation has been developed that is utilized in different countries to assess the safety of biotechnologically derived foods (6). As part of this overall strategy, the safety of the protein encoded by the introduced gene is evaluated. This assessment of protein safety includes an evaluation of the history of safe consumption, an estimation of the protein's abundance in the consumed foods, bioinformatic analysis of the amino acid sequence for similarity to known toxins, assessment of the protein's stability to proteolytic digestion, and an evaluation of the protein's potential allergenicity. Results from these assessments are used in a weight-of-evidence approach to determine if a protein is likely to pose a risk to human or animal health.

Guest edited as a special report on "Safety and Adequacy Testing of Foods/Feeds Nutritionally Enhanced Through Biotechnology" by G. Sarwar Gilani and Wayne Ellefson.

Corresponding author's email: gary.a.bannon@monsanto.com

The primary allergy risk to consumers from GM crops may be associated with 3 primary scenarios. The first scenario is the transfer of a known allergen or immunoglobulin E (IgE) crossreacting protein into a food crop. This scenario represents the highest risk to the allergic consumer because it increases the allergenic potential of a food crop to an already sensitive population. The second scenario, which represents a relatively low risk to the allergic consumer, is the potential for an increase in the expression level of allergens that are already present in an allergenic crop like soy (7). The last scenario involves expression of novel proteins that may become new allergens in man. This last scenario represents a relatively low risk to the consumer, because very few proteins of the thousands encountered every day become allergens, although the concept has received much attention of late (8). In order to mitigate the 3 categories of potential allergy risk associated with biotechnology crops, all genes introduced into food crops undergo an extensive series of tests. These tests include determination of the source of the introduced protein; identification of significant similarity between the amino acid sequence of the protein and known allergens; its susceptibility to enzymatic degradation; and, if the protein is similar to known allergens or comes from an allergenic source, serum screens using documented sera from allergic individuals (9). This testing strategy has worked well, as shown by the fact that, to date, there have been no clinically documented food allergic reactions to any of the biotechnology proteins introduced into food crops (10–13).

The first generation of biotechnology crops involved the insertion of a single gene that conferred a trait that the plant did not possess, such as herbicide tolerance or insect resistance. The next generation of biotechnology crops will most likely include engineered functional foods that involve the insertion of genes that have more general effects such as modifying endogenous metabolic pathways (14). For example, recent advances in engineered functional plant foods include genetic modifications to increase a precursor to vitamin A in rice (15, 16); genetic modification of oil crops to produce more heart-healthy oils (17–19); and metabolic engineering of legumes (20) and tomatoes (21) for high content of bioflavonoids, known for their antioxidant, anticancer, and estrogenic properties. In this paper, we will discuss general allergen characteristics that allow a better understanding of how proteins become allergens and summarize the current allergy assessment process. In particular, we will discuss the different aspects of the allergy assessment process for their adequacy in determining the allergenic potential of engineered functional foods. Finally, we will assess the possibility of new technologies having a positive impact on the allergy assessment of nutritionally enhanced crops.

Allergen Characteristics

The vast number of biological and chemical compounds that individuals encounter in a single day is in direct contrast to the relatively small number of compounds that become

allergens. For this reason, it is believed that the small number of compounds that do become allergens share common characteristics. Examining these proteins at the molecular, biochemical, and biophysical levels is at the core of research that looks towards understanding which proteins have potential to sensitize individuals and cause clinical allergy. Most allergens are proteins and are represented by more than 1250 different amino acid sequences (22). After comprehensive bioinformatic analyses to identify common protein structural and functional properties of allergens, a surprisingly small number of allergic protein families emerges. For example, a set of 157 pollen allergens could be classified into only 29 of a possible 2615 protein families found in seed plants (23). Furthermore, a set of 129 food allergen sequences could be classified into only 20 of a possible 3849 protein families found in all known plants (24). In fact, 65% of the clinically relevant food allergens can be grouped into 4 protein families: prolamins, Bet v 1, cupins, and profilins. These results imply that allergens share some level of structural and/or functional similarity that predisposes them to stimulating IgE production or eliciting significant clinical allergic reactions in sensitive individuals. Some common characteristics of allergens include protein structure, protein abundance in the allergenic source, and stability to degradation (25, 26).

Protein Structure

In an effort to identify common structural characteristics of allergens, a variety of algorithms have been developed that are based on the fact that proteins that share a high degree of primary amino acid sequence similarity throughout their entire length are homologous to one another. Homologous proteins not only share a common primary structure but also share significant secondary structure and common 3-dimensional folds (27). There are a variety of algorithms available that compare the primary amino acid sequences of 2 proteins (28). Most of these algorithms search for optimal alignment between 2 sequences by inserting gaps and then reporting the quality of the alignment. The quality of the alignment is summed up in an expectation score (*E* score). The *E* score reflects the potential random occurrence of aligned sequences and can be used to evaluate the significance of an observed alignment. The calculated *E* score depends on the overall length of gapped local sequence alignments, the quality (percent identity and similarity) of the overlap, and the size of the database (29). When comparing sequences, a very small *E* score (1×10^{-5} or smaller) may suggest a structurally relevant similarity, while large *E* scores (1.0 or greater) are typically associated with alignments that do not represent a biologically relevant structural similarity.

In addition to primary amino acid similarity, homologous proteins are also more likely to share allergenic secondary and tertiary structural similarities, such as crossreactive conformational and linear epitopes, than unrelated proteins. Aalberse (30) has noted that proteins sharing <50% primary amino acid identity across the full length of the protein sequence are unlikely to be crossreactive, but immunological

crossreactivity is likely when the proteins share at least 70% identity. Radauer and Breiteneder (31) reviewed sequence identities among allergenic and nonallergenic homologs of pollen allergens, and reported that the prerequisite for allergenic crossreactivity between proteins using this approach was a sequence identity of at least 50%.

Stadler and Stadler (32) proposed comparing a query protein sequence with allergen motifs, arguing that a more precise method for predicting crossreactive IgE-binding proteins was necessary to prevent an inordinate number of false-positive sequences from being selected. The motifs were generated from known allergens by searching for common structural domains of at least 50 amino acids. Of 779 allergen sequences searched in this manner, 644 were matched by 1 or several protein motifs. When tested on random test sequences or known allergens, the authors claimed that the motif-based method had a 95% precision rate. However, a major limitation with this approach is that not all food allergens have been identified, nor have all that have been identified been studied in enough detail to allow their 3-dimensional structure to be determined. It remains to be determined if certain protein motifs can be associated with a protein's allergenic potential.

The algorithms described above help to classify allergens into protein families, and they provide an important framework for structure-function studies that have the potential to improve our capability to predict potential allergens. It will be of particular interest to compare the structure of allergenic and nonallergenic members of well-defined protein families as they become available to determine if there are any unique features of an allergenic protein (33).

Protein Abundance

Protein abundance is another key factor contributing to a food protein's potential for allergenicity (34, 35). The major allergens of most commonly allergenic foods and pollens represent major protein constituents (>1%) of their respective sources (36). Most allergens are present as major protein components in the specific food, representing from 2–3% up to 80% of total protein (35). For example, the major allergen of ragweed, Amb a 1, represents about 6% of the total protein in ragweed pollen (37). The major allergen of shrimp, Pen a 1, comprises 20% of the total shrimp tail protein (38). Also, the major peanut allergens, Ara h 1 and Ara h 2, comprise 2 and 6% of the total protein in peanut, respectively (39).

Protein Stability

One biophysical property shared by many, but not all, food allergens is resistance to degradation (i.e., protein stability) in the gastrointestinal (GI) tract. This biophysical aspect of some food proteins can be used to predict potential allergenicity based on the premise that the longer significant portions of the protein remain intact, the more likely they are to trigger an immune response. There also appears to be a correlation between protein stability and allergenic potential (40), but this correlation is not absolute (41). It should be noted that protein stability is not a predominant characteristic of aeroallergens,

primarily because their route of sensitization is through the respiratory tract, where they would not be expected to encounter the harsh conditions of the GI tract (25).

Investigators tested the correlation between protein stability and allergenicity by disrupting the secondary and tertiary structures of the major allergens from milk and wheat (42, 43). This work demonstrated that the allergens were strikingly sensitive to pepsin digestion, thus losing their ability to elicit allergic reactions. Another food allergen, peanut allergen Ara h 2, is stabilized by disulfide linkages that, when intact, protect a portion of the protein from degradation. Amino acid sequence analysis of the resistant protein fragments indicated they contained most of the immunodominant IgE-binding epitopes. These results provide a link between allergen structure and the most allergenic portions of the protein (44, 45).

Current Allergy Assessment Process

The current allergy assessment process is designed to identify the potential risks associated with the introduced protein as well as the overall allergenic risks associated with a transformed food crop. The current allergy assessment process follows recommendations made by Codex Alimentarius Commission (www.codexalimentarius.net/web/index_en.jsp). Codex is an intergovernmental body representing 168 member states responsible for protecting the health of consumers and facilitating trade by setting international safety standards. The Codex recommendations for allergy assessment include evaluation of the introduced protein with respect to origin (from a known allergenic source or not), sequence homology to known allergens, stability in in vitro digestion assays, and, when appropriate, IgE-binding capacity in in vitro and in vivo clinical tests.

Assessing the Allergenic Potential of the Introduced Protein(s)

The following sections outline several approaches that are currently utilized in the assessment of the introduced proteins themselves.

Source of the introduced gene(s).—The source of the introduced gene is the first variable to consider in the current allergy assessment process. If a gene transferred into a food crop is obtained from a source known to be allergenic, it is assumed to be an allergen until proven otherwise. The 8 most commonly reported food allergic reactions are to peanuts, tree nuts, cow's milk, hen's eggs, fish, Crustacea, wheat, and soybeans (46). Additionally, adverse reactions to plant-derived foods are very common in birch pollen allergic subjects (47). Typical birch pollen-related food allergies include apple, stone fruit (such as peach, apricot, and cherry), hazelnut, carrot, celery, and soybeans (48). It should be noted that allergic reactions to foods are not as prevalent as those to allergic rhinitis (common hay fever). Food allergies only affect up to 4% of adults and 6% of children (49, 50), while allergic rhinitis affects >10% of adults and up to 40% of children. While food allergies are less prevalent, they are more

likely to elicit severe clinical symptoms that may result in life-threatening conditions.

Bioinformatic analyses.—The purpose of bioinformatic analyses is to describe the biological and taxonomical relatedness of a query sequence to other functionally related proteins. In the context of allergy, the goal is to identify the level of amino acid similarity and structural relatedness between a protein of interest and sequences from known allergens in order to determine whether the query protein is similar to known allergens or has the potential to crossreact with IgE directed against known allergens. Because candidate genes for transfer into commodity crops could be from a variety of sources, most allergen databases contain all known allergens including aeroallergens, food allergens and proteins implicated in celiac disease. For example, the Food Allergy Research and Resource Program (FARRP) Allergen Database (22) contains all known allergen, gliadin, and glutenin protein sequences. The protein sequences in the FARRP allergen database were assembled and evaluated for evidence of allergenicity by an international panel of allergy experts, making this one of the more highly curated, publicly available allergen databases. High percentage matches between a query sequence and a sequence in the allergen database suggests that the query sequence could crossreact with IgE directed against that allergen. To distinguish among many matches, criteria can be used to judge the ranked scores produced by programs such as FASTA-All (FASTA). For example, the Codex Alimentarius (9) recommended an identity score of at least 35% matched amino acid residues of at least 80 residues as being the lowest identity criteria for proteins derived from biotechnology that could suggest IgE crossreactivity with a known allergen. The quality of sequence alignments that are detected between a query protein and an allergen can also be evaluated. This aspect of the current allergy assessment process has proven effective in protecting against the transfer of biotechnology proteins that share structural similarities to known allergens and should continue to be used to evaluate introduced proteins for nutritionally enhanced food crops.

An additional bioinformatics approach can be taken by searching for 100% identity matches along short sequences contained in the query sequence as they are compared to sequences in a database. These regions of short amino acid sequence homologies are intended to represent the smallest sequence that could function as an IgE-binding epitope (51). If any exact matches between a known allergen and a transgenic sequence were found using this strategy, it could represent the most conservative approach to predicting potential for a peptide fragment to act as an allergen. Critical to this type of search algorithm is the selection of the overlapping sequence length (i.e., the sliding window). As the length of this window of overlapping amino acids to search with is shortened, the chance for random, false-positive matches becomes higher. Although different window lengths have been recommended, a length of 8 amino acids has been shown to be informative without acquiring a majority of matches based on random chance (52–54).

There exist clear limits to the utility of performing sequence searches based on potential epitopes, with a major limitation being the lack of a comprehensive database of confirmed IgE-binding sequential epitopes for existing allergens. Development of this type of database represents a challenging task due to the fact that many allergens that bind IgE in patient sera and are known to cause clinical allergy symptoms do not have B- and T-cell epitopes described for them in the scientific literature (52). At this time, there is no database of epitope sequences that can fully describe epitopes for all of the known protein allergens. This makes assessment of biotechnology food protein sequences with an epitope database impractical at this time and is not recommended as a safety assessment strategy (54). Thus, further research regarding epitope identity and sequence length is required in order to make short amino acid search strategies informative beyond the theoretical identity-matching strategy currently available (54, 55). Moreover, it must also be noted that many IgE-binding epitopes are conformational in nature (51), not just a string of primary amino acid sequences. The analysis of conformational IgE epitopes is difficult and involves methods such as site-directed mutagenesis of the full-length allergen (56), mimicking conformational IgE-binding sites by short-phage displayed peptides (57), or even structural analysis of allergen immune complexes (58).

Protein Stability

A factor that increases the likelihood of allergic oral sensitization to proteins is the stability of the proteins to GI digestion. Protein allergens tend to be stable to the peptic and acidic conditions of the digestive system, thus allowing them to reach and pass through the intestinal mucosa to elicit an allergenic response (39, 40). Proteins that are rapidly digestible are highly correlated with a significantly decreased likelihood to cause sensitization or allergic reaction when consumed.

Protein stability assessment includes analysis of the digestibility of the protein in a simulated gastric fluid (SGF) assay containing pepsin (59). A relationship between digestibility in SGF and the likelihood of being an allergen has been previously reported for a group of proteins consisting of both allergens and nonallergens (39). Recently, the ILSI standardized the SGF assay protocol based on results obtained from an international, multilaboratory ring study (59). This test showed that the results of in vitro pepsin digestion assays were reproducible when standard protocols were followed.

In addition to SGF, simulated intestinal fluid (SIF) is also used for in vitro studies to assess the digestibility of food components (60, 61). SIF is an in vitro digestion model where proteins undergo digestion at neutral pH by a mixture of enzymes known as pancreatin. However, the relationship between protein allergenicity and protein stability in the in vitro SIF study is limited because the protein has not been first exposed to the acidic, denaturing conditions of the stomach, as would be the case in vivo (62).

Assessing the Overall Allergenic Potential of a Transformed Food Crop

In addition to examining features of the introduced proteins themselves, it is also essential to examine the overall allergenic potential of the transformed food crop. Most allergenic food crops contain multiple allergenic proteins, making it technically impossible to assess the status of each individual protein. To circumvent this difficulty, IgE from allergic patients is used to assess the overall allergenic potential of a food crop. The rationale behind this approach is based on the assumption that allergic patients have IgE to all the major allergens of the food crop and, if the assay is designed correctly, it should be possible to determine whether the overall allergenicity has been modified without knowing the status of specific allergens. Based on the current Codex guidelines, serum IgE testing is necessary when an introduced protein has similarity to other known allergens or to assess the potential for an increase in the endogenous allergenicity of an already allergenic crop. IgE-binding assays such as radioallergosorbent tests (63), enzyme-linked immunosorbent assays (64), or immunoblotting assays are available for this type of testing. Examples utilizing each of these different techniques to determine the IgE-binding capacity of transgenic versus nontransgenic foods and biotechnology proteins have appeared in the literature (65–67).

An important caveat to the serum IgE testing studies is that the usefulness of any data generated is highly dependent on the quality of the IgE used in the study. Therefore, it is critical that serum donors be carefully screened and selected. Selection of serum donors must be limited to those with relevant, clinically proven allergies based on a positive, double-blinded, placebo-controlled food challenge test to a specific food or allergen, or a documented case history of anaphylactic reactions to that food or allergen (68, 69). The IgE in the sera must also be characterized by determining the total amount of IgE present and also the amount of IgE that is specific to a food extract or to a specific allergen. This characterization is usually accomplished using capsulated hydrolytic carrier polymer-fluoroenzyme immunoassay (CAP-FEIA; Phadia, Uppsala, Sweden). CAP-FEIA is a detection method that has been used to assess food-specific IgE concentrations that are indicative of a patient being allergic to an allergenic food (70, 71). This type of information provides important linkages between clinical symptoms, the amount of total IgE, and the amount of specific IgE. Assessment of the overall allergenic potential of a transformed food crop is an important aspect of the current allergy assessment process that will also be useful in assessing the allergenic potential of nutritionally enhanced crops.

New Methods and Technologies for Assessing Allergenic Risk

Although the current methods and protocols for assessing allergenic risks appear to be efficient and robust, there is a constant drive to identify new and improved methods and

technologies. Below is a brief discussion of novel methods and their potential use.

Animal Models of Allergic Disease

The potential for animal models to mimic the human disease process makes them an invaluable tool for potentially predicting allergenicity of nutritionally enhanced crops. Most of the allergy animal models developed to date have been designed to test reagents for immunotherapeutic treatment of allergic disease and predict the potential allergenicity of proteins (72). These 2 disparate goals, identifying effective treatment regimens and predicting potential human allergenicity, require many of the same variables to be considered in the development of an effective animal model. To date, animal models of food allergy developed to test immunotherapeutic reagents have seen some success (73–75). On the other hand, animal models developed to predict allergenicity are not as prevalent (76, 77) and have yet to be widely accepted.

There has been considerable interest in the development of animal models that would permit a more direct evaluation of the sensitizing potential of novel proteins. The development of a predictive animal model could help to address the third category of public health risk posed by introduction of GM proteins into food crops—that of a novel protein becoming an allergen. In this context, attention has focused on the production of IgE in response to the novel protein, and a wide variety of organisms are being developed for this purpose, including rodents (78–80), dogs (81), and swine (82). Many variables are being tested in the development of each model organism, including route of sensitization, dose, use of adjuvant, age of organism, diet, and genetics. Unfortunately, there are currently no validated models available for assessing the allergenic potential of specific proteins in naïve subjects. This is due in part to the extremely complex nature of the immune response to foods and proteins and also in part due to the fact that most animal models of food allergy were originally developed to understand the mechanisms of allergenicity rather than assessing the allergenic potential of novel proteins. The development of an animal model that can accurately predict human allergenicity would be an invaluable tool for assessing the allergenicity of nutritionally enhanced food crops. However, while some progress is being made in select models (83, 84), there remains much work to be done before there is confidence that any one model will provide positive predictive value with regard to protein allergenicity in humans.

Proteomics

Proteomics is a high-throughput technology platform that substantially increases the number of proteins that can be detected simultaneously. This type of approach has the potential to relate complex protein mixtures to complex effects in the form of protein expression profiles.

Generally speaking, proteomics relies on the ability to separate/fractionate complex protein mixtures and then to accurately annotate those proteins based on properties such as mass and amino acid sequence. Separation of complex protein

mixtures is most often done using some variant of the classical 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) method originally described by Laemmli (85). Steady improvements to this methodology have evolved to include more precision in the isoelectric focusing with narrow range pH gradients (86), improved staining and detection protocols (87, 88), and methods to quantitatively analyze proteins within a gel-based format (89). Most of the problems with the 2D-PAGE gels stem from poor reproducibility due to methodological problems (90). While the main strengths of 2D-PAGE are good separation and visualization of complex samples, the resulting image and data analysis tend to introduce additional sources of variation. There have also been good advances in nongel-based methods for protein fractionation, particularly in the area of affinity chromatography applications (91).

Once proteins have been adequately fractionated, identification of the proteins is typically performed using mass spectrometry. There are various types of mass spectrometers that can be used to identify proteins, ranging from the basic matrix-assisted laser desorption/ionization systems that detect peptide masses to complex multistage systems, such as tandem time-of-flight instruments that in addition to mass detection are also able to detect structural features of the peptides [see ref. 92 for detailed comparisons of available mass spectrometers]. Mass spectrometers differ in their mass accuracy, resolving power, sensitivity, and dynamic range, so choice of the instrument is very dependent on the biological question being studied.

Although technology for protein fractionation and identification is continuing to improve, there continue to be challenges that must be resolved before it will become routinely acceptable to apply these technologies to determining the allergenicity of nutritionally enhanced food crops. The main technical challenges include technology variability (differences in platforms, methodology, sensitivity, and reproducibility); data management (storage and visualization of complex gel images and mass spectra); and data analysis (incomplete databases of protein structure information, poor quality deoxyribonucleic acid sequence data, and incomplete sequence annotation).

Another key challenge limiting the routine application of this technology to nutritionally enhanced food crops is the need to define the natural variability of allergen abundance in food crops with different genetic backgrounds and grown in different environments. Since protein abundance is a characteristic of known allergens, before making informed decisions on whether a protein's abundance has significantly increased, we must first know the normal range of protein abundance that is seen in nature. Without this point of reference, it is impossible to interpret the effect of any changes that are detected in protein abundance. Another key point to keep in mind is that having the ability to detect a change, especially given the ongoing improvements in sensitivity of the equipment, does not immediately imply that the change will have any biological effect on allergenicity.

Conclusions

As experimentalists, we reduce complex biological systems to their simplest form in order to study and better understand individual aspects of the organism. By studying in detail the physical, chemical, and biological aspects of known allergens, we have gained some insight into what makes an innocuous environmental or food protein an irritating or even life-threatening allergen. From this work, we can accurately determine if a novel protein is similar to known allergens, and we can predict whether it is likely to crossreact with IgE directed against those allergens. We are less sure of predicting whether novel proteins will become new allergens that have not yet been defined.

The allergy assessment testing strategy, as it is presently formulated, is a hazard identification approach that utilizes currently available scientific data regarding allergens and the allergic response. It is important to emphasize that all aspects of the current allergy assessment testing strategy need to be considered when assessing a novel protein, not just the results from a single arm of this strategy. This strategy takes advantage of past assessments, but by its tiered design attempts to place more importance on the "weight of evidence" from each test rather than relying too heavily on one test to determine whether a protein is likely to have allergenic potential. While a hazard assessment approach has served the public interest well, we should continue to pursue new technologies that will allow us to predict potential allergenicity with increased confidence. We conclude that the protective value of current testing approaches and future approaches that adopt sound, science-based risk assessment principles have provided, and will continue to provide, robust assurances to risk managers and consumers alike when assessing the safety of nutritionally enhanced food crops.

References

- (1) James, C. (2006) *Global Status of Commercialized Biotechnology/GM Crops: 2006*, ISAAA Brief No. 35, ISAAA, Ithaca, NY
- (2) OECD (1993) *Safety Evaluation of Foods Derived by Modern Biotechnology: Concepts and Principles*, Organization for Economic Cooperation and Development, OECD Test Guideline 425, Paris, France
- (3) Jonas, D.A., Antignac, E., Antoine, J.M., Classen, H.G., Huggett, A., Knudsen, I., Mahler, J., Ockhuizen, T., Smith, M., Teuber, M., Walker, R., & De Vogel, P. (1996) *Food Chem. Toxicol.* **34**, 931–940
- (4) FAO/WHO (1996) *Biotechnology and Food Safety*, Food and Agriculture Organization/World Health Organization, Geneva, Switzerland, pp 1–27
- (5) FAO/WHO (2000) *Safety Aspects of Genetically Modified Foods of Plant Origin, Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology*, Geneva, Switzerland, 29 May–2 June 2000, Rome, Italy, Food and Agriculture Organization of the United Nations, <http://www.who.int/foodsafety/biotechnology/consult/en/index.html>

- (6) König, A., Cockburn, A., Crevel, R.W.R., Debruyne, E., Grafstroem, R., Hammerling, U., Kimber, I., Knudsen, I., Kuiper, H.A., Peijnenburg, A.A.C.M., Penninks, A.H., Poulsen, M., Schauzu, M., & Wal, J.M. (2004) *Food Chem. Toxicol.* **42**, 1047–1088
- (7) Goodman, R.E., Hefle, S.L., Taylor, S.L., & van Ree, R. (2005) *Int. Arch. Allergy Immunol.* **137**, 153–166
- (8) Lehrer, S.B., & Bannon, G.A. (2005) *Allergy* **60**, 559–564
- (9) Codex Alimentarius Commission (2003) *Proposed Draft Annex on the Assessment of Possible Allergenicity of the Draft Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant DNA Plants*, Joint FAO/WHO Food Standard Program, Appendix IV, pp 57–60
- (10) Burks, A.W., & Fuchs, R.L. (1995) *J. Allergy Clin. Immunol.* **96**, 1008–1010
- (11) Kim, S.H., Kim, H.M., Ye, Y.M., Kim, S.H., Nahm, D.H., Park, H.S., Ryu, S.R., & Lee, B.O. (2006) *Yonsei Med. J.* **47**, 505–512
- (12) Batista, R., Nunes, B., Carmo, M., Cardoso, C., Jose, H.S., de Almeida, A.B., Manique, A., Bento, L., Ricardo, C.P., & Oliveira, M.M. (2005) *J. Allergy Clin. Immunol.* **116**, 403–410
- (13) Hoff, M., Son, D.-Y., Gubesch, M., Ahn, K., Lee, S.-I., Vieths, S., Goodman, R.E., Ballmer-Weber, B.K., & Bannon, G.A. (2007) *Mol. Nutr. Food Res.* **51** (in press)
- (14) Raskin, I., Ribnicky, D.M., Komarnytsky, S., Ilic, N., Poulev, A., Borisjuk, N., Brinker, A., Moreno, D.A., Ripoll, C., Yakoby, N., O'Neal, J.M., Cornwell, T., Pastor, I., & Fridlender, B. (2002) *Trends Biotechnol.* **20**, 522–531
- (15) Potrykus, I. (2001) *Plant Physiol.* **125**, 1157–1161
- (16) Ye, X. (2000) *Science* **287**, 303–305
- (17) Daniell, H., Streatfield, S.J., & Wycoff, K. (2001) *Trends Plant Sci.* **6**, 219–226
- (18) Walmsley, A.M., & Arntzen, C.J. (2000) *Curr. Opin. Biotechnol.* **11**, 126–129
- (19) Bonetta, L. (2002) *Nat. Med.* **8**, 94–95
- (20) Forkmann, G., & Martens, S. (2001) *Curr. Opin. Biotechnol.* **12**, 155–160
- (21) Muir, S.R., Collins, G.J., Robinson, S., Hughes, S., Bovy, A., Ric De Vos, C.H., van Tunen, A.J., & Verhoeven, M.E. (2001) *Nat. Biotechnol.* **19**, 470–474
- (22) Food Allergy Research and Resource Program Allergen Database (2006) University of Nebraska, Lincoln, NE, <http://www.allergenonline.com>
- (23) Radauer, C., & Breiteneder, H. (2006) *J. Allergy Clin. Immunol.* **117**, 141–147
- (24) Jenkins, J.A., Griffiths-Jones, S., Shewry, P.R., Breiteneder, H., & Mills, E.N. (2005) *J. Allergy Clin. Immunol.* **115**, 163–170
- (25) Bannon, G.A. (2004) *Curr. Allergy Asthma Rep.* **4**, 43–46
- (26) Bredehorst, R., & David, K. (2001) *J. Chromatogr. B* **756**, 33–40
- (27) Pearson, W.R. (1996) *Methods Enzymol.* **266**, 227–258
- (28) Pearson, W.R., & Lipman, D.J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2440–2448
- (29) Baxevanis, A.D., & Ouellette, B.F.F. (1998) *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins*, John Wiley & Sons, Inc., New York, NY
- (30) Aalberse, R.C. (2000) *J. Allergy Clin. Immunol.* **106**, 228–238
- (31) Radauer, C., & Breiteneder, H. (2006) *J. Allergy Clin. Immunol.* **117**, 141–147
- (32) Stadler, M.B., & Stadler, B.M. (2003) *FASEB J.* **17**, 1141–1143
- (33) Breiteneder, H., & Radauer, C. (2004) *J. Allergy Clin. Immunol.* **113**, 821–830
- (34) Taylor, S.L. (1992) *Food Technol.* **46**, 146–152
- (35) Fuchs, R.L., & Astwood, J.D. (1996) *Food Technol.* **50**, 83–88
- (36) Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L., & Fuchs, R.L. (1996) *Crit. Rev. Food Sci. Nutr.* **36**, S165–S186
- (37) Wopfner, N., Gadermaier, G., Egger, M., Asero, R., Ebner, C., Jahn-Schmid, B., & Ferreira, F. (2005) *Int. Arch. Allergy Immunol.* **138**, 337–346
- (38) Daul, C.B., Slattery, M., Reese, G., & Lehrer, S.B. (1994) *Int. Arch. Allergy Immunol.* **105**, 49–55
- (39) Besler, M. (2000) *Internet Symposium on Food Allergens 2*, 87–122
- (40) Astwood, J.D., Leach, J.N., & Fuchs, R.L. (1996) *Nat. Biotechnol.* **14**, 1269–1273
- (41) Fu, T.J., Abbott, U.R., & Hatzos, C. (2002) *J. Agric. Food Chem.* **50**, 7154–7160
- (42) Buchanan, B.B., Adamidi, C., Lozano, R.M., Yee, B.C., Momma, M., Kobrehel, K., Ermel, R., & Frick, O.L. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 5372–5377
- (43) del Val, G., Yee, B.C., Lozano, R.M., Buchanan, B.B., Ermel, R.W., Lee, Y.M., & Frick, O.L. (1999) *J. Allergy Clin. Immunol.* **103**, 690–697
- (44) Sen, M.M., Kopper, R., Pons, L., Abraham, E.C., Burks, A.W., & Bannon, G.A. (2002) *J. Immunol.* **169**, 882–887
- (45) Lehmann, K., Schweimer, K., Reese, G., Randow, S., Suhr, M., Becker, W.M., Vieths, S., & Rosch, P. (2006) *Biochem. J.* **395**, 463–472
- (46) Metcalf, D.D., Astwood, J.D., Townsend, R., Taylor, S.L., & Fuchs, R.L. (1996) *Crit. Rev. Food Sci. Nutr.* **36**, S165–S186
- (47) Vieths, S., Scheurer, S., & Ballmer-Weber, B. (2002) *Ann. New York Acad. Sci.* **964**, 47–68
- (48) Mari, A., Ballmer-Weber, B.K., & Vieths, S. (2005) *Curr. Opin. Allergy Clin. Immunol.* **5**, 267–273
- (49) AAAAI (2000) *Overview of Allergic Disease, The Allergy Report* **1**, 1–3
- (50) Sicherer, S.H., & Sampson, H.A. (2006) *J. Allergy Clin. Immunol.* **117**, S470–S475
- (51) Bannon, G., & Ogawa, T. (2006) *Mol. Nutr. Food Res.* **50**, 638–644
- (52) Hileman, R.E., Silvanovich, A., Goodman, R.E., Rice, E.A., Holleschak, G., Astwood, J.D., & Hefle, S.L. (2002) *Int. Arch. Allergy Immunol.* **128**, 280–291
- (53) Silvanovich, A., Nemeth, M.A., Song, P., Herman, R., Tagliani, L., & Bannon, G.A. (2005) *Toxicol. Sci.* **90**, 252–258
- (54) Thomas, K., Bannon, G., Hefle, S.L., Herouet, C., Holsapple, M., Ladics, G., MacIntosh, S., & Privalle, L. (2005) *Toxicol. Sci.* **88**, 307–310
- (55) Bannon, G.A., Astwood, J., Goodman, R., Hefle, S., & Taylor, S. (2004) *Allergy Assessment for Food Biotechnology, in Agricultural Biotechnology*, Bhalgat, Ridley, Felsot, & Seiber (Eds), pp 151–164

- (56) Neudecker, P., Lehmann, K., Nerkamp, J., Haase, T., Wangorsch, A., Fotisch, K., Rosch, P., Vieths, S., & Scheurer, S. (2003) *J. Biochem.* **376**, 97–107
- (57) Mittag, D., Batori, V., Neudecker, P., Wiche, R., Friis, E.P., Ballmer-Weber, B.K., Vieths, S., & Roggen, E.L. (2006) *Mol. Immunol.* **43**, 268–278
- (58) Mirza, O., Henriksen, A., Ipsen, H., Larsen, J.N., Wissenbach, M., Spangfort, M.D., & Gajhede, M. (2000) *J. Immunol.* **165**, 331–338
- (59) Thomas, K., Aalberse, M., Bannon, G.A., Bartels, M., Dearman, R.J., Esdaile, D.J., Fu, T.J., Glatt, C.M., Hadfield, N., Hatzos, C., Hefle, S.L., Heylings, J.R., Goodman, R.E., Henry, B., Herouet, C., Holsapple, M., Ladics, G.S., Landry, T.D., MacIntosh, S.C., Rice, E.A., Privalle, L.S., Stiner, H.Y., Teshima, R., Van Ree, R., Woolhiser, M., & Zawodny, J. (2004) *Regul. Toxicol. Pharmacol.* **39**, 87–98
- (60) Okunuki, H., Teshima, R., Shigeta, T., Sakushima, J., Akiyama, H., Goda, Y., Toyoda, M., & Sawada, J. (2002) *J. Food Hyg. Soc. Japan* **43**, 68–73
- (61) Yagami, T., Haishima, Y., Nakamura, A., Osuna, H., & Ikezawa, Z. (2000) *J. Allergy Clin. Immunol.* **106**, 752–762
- (62) FAO/WHO (2001) *Evaluation of Allergenicity of Genetically Modified Foods, Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology*, Rome, Italy
- (63) Sampson, H.A., & Albergo, R. (1984) *J. Allergy Clin. Immunol.* **74**, 26–33
- (64) Yunginger, J.W., & Adolphson, C.R. (1992) *Standardization of Allergens*, in *Washington DC: American Society of Microbiology*, pp 678–684
- (65) Burks, A.W., & Fuchs, R.L. (1995) *J. Allergy Clin. Immunol.* **96**, 1008–1010
- (66) Kim, S.H., Kim, H.M., Ye, Y.M., Kim, S.H., Nahm, D.H., Park, H.S., Ryu, S.R., & Lee, B.O. (2006) *Yonsei Med. J.* **47**, 505–512
- (67) Batista, R., Nunes, B., Carmo, M., Cardoso, C., Jose, H.S., de Almeida, A.B., Manique, A., Bento, L., Ricardo, C.P., & Oliveira, M.M. (2005) *J. Allergy Clin. Immunol.* **116**, 403–410
- (68) Burks, A.W., Brooks, J.R., & Sampson, H.A. (1988) *J. Allergy Clin. Immunol.* **81**, 1135–1142
- (69) Bock, S.A., Sampson, H.A., Atkins, F.M., Zeiger, R.S., Lehrer, S., Sachs, M., Bush, R.K., & Metcalfe, D.D. (1988) *J. Allergy Clin. Immunol.* **82**, 986–997
- (70) Burks, A.W. (2000) *Immunology/Allergy, XVI Food Allergies*, ACP Medicine Online, D.C. Dale & D.D. Federman (Eds), WebMD Inc., New York, NY, <http://www.acpmedicine.com/>
- (71) Sampson, H.A. (2001) *J. Allergy Clin. Immunol.* **107**, 891–896
- (72) Knippels, L.M., van Wijk, F., & Penninks, A.H. (2004) *Curr. Opin. Allergy Clin. Immunol.* **4**, 205–209
- (73) Morafo, V., Srivastava, K., Huang, C.K., Kleiner, G., Lee, S.Y., Sampson, H.A., & Li, A.M. (2003) *J. Allergy Clin. Immunol.* **111**, 1122–1128
- (74) Pons, L., Ponnappan, U., Hall, R.A., Simpson, P., Cockrell, G., West, C.M., Sampson, H.A., Helm, R.M., & Burks, A.W. (2004) *J. Allergy Clin. Immunol.* **114**, 915–921
- (75) Srivastava, K.D., Kattan, J.D., Zou, Z.M., Li, J.H., Zhang, L., Wallenstein, S., Goldfarb, J., Sampson, H.A., & Li, X.M. (2005) *J. Allergy Clin. Immunol.* **115**, 171–178
- (76) Buchanan, B.B., Frick, O.L. (2002) *Ann. NY Acad. Sci.* **964**, 173–183
- (77) Dearman, R.J., Stone, S., Caddick, H.T., Basketter, D.A., & Kimber, I. (2003) *Clin. Exp. Allergy* **33**, 1586–1594
- (78) Li, X.M., Schofield, B.H., Huang, C.K., Kleiner, G.I., & Sampson, H.A. (1999) *J. Allergy Clin. Immunol.* **103**, 206–214
- (79) Dearman, R.J., & Kimber, I. (2001) *Toxicol. Lett.* **120**, 181–186
- (80) Akiyama, H., Teshima, R., Sakushima, J.I., Okunuki, H., Goda, Y., Sawada, J.I., & Toyoda, M. (2001) *Immunol. Lett.* **78**, 1–5
- (81) Frick, O.L. (1996) *Adv. Exp. Med. Biol.* **409**, 1–7
- (82) Helm, R.M., Furuta, G.T., Stanley, J.S., Ye, J., Cockrell, G., Connaughton, C., Simpson, P., Bannon, G.A., & Burks, A.W. (2002) *J. Allergy Clin. Immunol.* **109**, 136–142
- (83) Ermel, R.W., Kock, M., Griffey, S.M., Reinhart, G.A., & Frick, O.L. (1997) *Lab. Anim. Sci.* **47**, 40–49
- (84) Li, X.M., Serebrisky, D., Lee, S.Y., Huang, C.K., Bardina, L., Schofield, B.H., Stanley, J.S., Burks, A.W., Bannon, G.A., & Sampson, H.A. (2000) *J. Allergy Clin. Immunol.* **106**, 150–158
- (85) Laemmli, U.K. (1970) *Nature* **227**, 680–685
- (86) Hoving, S., Gerrits, B., Voshol, H., Muller, D., Roberts, R.C., & van Oostrum, J. (2002) *Proteomics* **2**, 127–134
- (87) Unlu, M., Morgan, M.E., & Minden, J.S. (1997) *Electrophoresis* **18**, 2071–2077
- (88) Rabilloud, T. (2002) *Proteomics* **2**, 3–10
- (89) Tonge, R., Shaw, J., Middleton, B., Rowlinson, R., Rayner, S., Young, J., Pognan, F., Hawkins, E., Currie, I., & Davison, M. (2001) *Proteomics* **1**, 377–396
- (90) Voss, T., & Haberl, P. (2000) *Electrophoresis* **21**, 3345–3350
- (91) Lee, W.C., & Lee, K.H. (2004) *Anal. Biochem.* **324**, 1–10
- (92) Domon, B., & Aebersold, R. (2006) *Mol. Cell Proteomics* **5**, 1921–1926