Review

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Assessing Genetically Modified Crops to Minimize the Risk of Increased Food Allergy: A Review

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Abstract
The first genetically modified (GM) crops approved for food use (tomato and soybean) were evaluated for safety by the United States Food and Drug Administration prior to commercial production. Among other factors, those products and all additional GM crops that have been grown commercially have been evaluated for potential increases in allergenic properties using methods that are consistent with the current understanding of food allergens and knowledge regarding the prediction of allergenic activity. Although there have been refinements, the key aspects of the evaluation have not changed. The allergenic properties of the gene donor and the host (recipient) organisms are considered in determining the appropriate testing strategy. The amino acid sequence of the encoded protein is compared to all known allergens to determine whether the protein is a known allergen or is sufficiently similar to any known allergen to indicate an increased probability of allergic cross-reactivity. Stability of the protein in the presence of acid with the stomach protease pepsin is tested as a risk factor for food allergenicity. In vitro or in vivo human IgE binding are tested when appropriate, if the gene donor is an allergen or the sequence of the protein is similar to an allergen. Serum donors and skin test subjects are selected based on their proven allergic responses to the gene donor or to material containing the allergen that was matched in sequence. While some scientists and regulators have suggested using animal models, performing broadly targeted serum IgE testing or extensive pre- or post-market clinical tests, current evidence does not support these tests as being predictive or practical. Based on the evidence to date, the current assessment process has worked well to prevent the unintended introduction of allergens in commercial GM crops.

Introduction

Development of genetically modified crops (GM) began in the 1980s with the development of techniques of biotechnology and improvements in plant cell culture and tissue differentiation [1]. As defined here, GM plants are produced by directly altering the DNA of the genome,

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chloroplasts or mitochondria through the direct introduction, removal or rearrangement of DNA using methods such as electroporation, particle bombardment or infection with recombinant vectors such as *Agrobacterium tumefaciens*. Prior to the development of these techniques, new or improved plant varieties were developed through natural or human induced crossing of sexually compatible plants that had random genetic mutations, followed by natural or cultivated selection [2]. Improvements in crops were limited by the chance occurrence of genetic mutations that expressed an advantageous phenotypic trait. In the past 70 years, radiation and chemical mutagens have been used to increase the speed and diversity of random mutations of undefined characteristics and with potentially many unintended effects [2]. In contrast, biotechnology allows the introduction of complete, well-characterized genes with specific transcriptional regulatory elements that could not be introduced through previous methods.

Biotechnology may be used to improve plant disease resistance, improve yield, and reduced requirements for fertilizers or water. Current commercial GM products include varieties resistant to specific insects and varieties tolerant to specific herbicides, allowing more efficient and environmentally beneficial agricultural practices. Some potential products in development have enhanced nutritional properties, such as the introduction of vitamin A precursors in rice [3]. Plants have been transformed with genes encoding industrial or pharmaceutical proteins and could dramatically reduce production costs compared to microbial fermentation or purification from natural sources [2, 4]. Industrial and pharmaceutical products will require effective controls to ensure isolation from food crop production [5–7]. The allergenicity of a food crop may be reduced by suppressing the synthesis of or modifying the major allergens [4, 8, 9]. While more than 88 species of plants have been transformed for research or for potential commercial production, 10 species of plants have been approved for commercial food production in at least one country. The first GM plants were evaluated by US regulators in 1994–1996 prior to commercial sales and included: delayed-ripening tomato, herbicide-tolerant soybean, insect-resistant and herbicide-tolerant cotton, insect-resistant and herbicide-tolerant maize, insect-resistant potato, virus-resistant papaya and virus-resistant squash [10]. In 2003, approved GM crops (soybean, maize, cotton and canola) were planted on approximately 68 million hectares globally [11].

Regulation of GM crops in the United States is based on a legal notification and a recommended safety assessment process developed by the US Food and Drug Administration (FDA) [12]. Similar regulations (see http://www.agbios.com/main.php) govern growing of GM crops and importation of commodities or foods derived from GM crops in the EU, Japan, Canada and many other countries [13].

While there is no documented case of an adverse health affect due to the consumption of food from any GM crop, there continues to be some public skepticism expressed by some scientists and political activists, even though current commercial GM crop varieties have been far more extensively tested and evaluated than any conventionally bred crop [2].

The focus of this review is to provide information about the development practices of the allergenicity assessment process and their relevance to the current state of knowledge for predicting the allergenicity of conventional foods and proteins. Potential shortcomings of the process will also be discussed.

Rationale for the Allergenicity Assessment Process

The overall goal of the allergenicity assessment is to minimize the possibility that food from the GM crop will be more allergenic than food produced from equivalent non-GM crop varieties. The primary focus is on the safety of any newly expressed protein. The most important steps in protecting those at risk of allergic reactions is to determine if the protein is already known to be an allergen, or is so similar to any allergen that exposure of allergic individuals may cause allergic cross-reactions. The primary risk would be to those who are already sensitized to the allergen, as they could experience serious or life-threatening allergic reactions if they consume such a protein.

The risk of serious and life-threatening allergic reactions associated with the consumption of specific proteins in highly allergenic foods such as peanuts [14], milk [15], eggs [16], wheat [17] and soybeans [18] is well recognized as an important health issue for a small percentage of individuals in the population. While the prevalence of food allergy is not precisely known, estimates from various clinical studies and surveys indicate that 1–3% of adults and 6–8% of children experience some food-allergic reactions with an overall prevalence of up to 4% of the US population having IgE-mediated food allergies [19]. A recent epidemiology study in France indicated approximately 3.5% of their population has food allergy [20].

Eight allergenic foods or groups of foods (peanuts, soybeans, tree nuts, milk, egg, fish, crustaceans, and wheat) are thought to cause nearly 90% of food-allergic reactions in the US [21]. Based on limited data extrapolated to the US population of 280 million, it is estimated that 30,000 individual cases of IgE-mediated food-allergic reactions require emergency room treatment per year in the US, and there may be 150–200 deaths per year due to any food allergy [22]. Most life-threatening reactions are caused by peanuts or tree nuts in the US [23, 24]. The exact incidence and causes of serious and fatal allergic reactions are uncertain because of the diverse causes of food allergy and asthma, the rare occurrence of fatalities, the lack of definitive tests for unlabeled allergenic foods (until recently) and the lack of reporting requirements [25–27]. Data from the US, United Kingdom and Sweden indicate that the allergens most commonly responsible for fatalities are peanuts and tree nuts and less frequently milk, egg, fish and soybeans [27–29]. Results of a recent scientifically conducted telephone survey indicate that 2.3% of the US population may be allergic to fish or shellfish [30], placing seafood allergies ahead of peanut as the most common cause of food allergy. Dietary practices play a major role either in sensitization or elicitation of food allergy, as evidenced by apparent differences in prevalence of specific allergies to foods in different cultures. In Europe, sesame seeds [31], celery root (celeriac) [32] and mustard [33] have now been shown to be common causes of food allergies in addition to the major eight allergens recognized in the US. In Asia, buckwheat is a major food allergen that can cause severe reactions [34].

Differences in responses to various allergens and across individual patients sensitized to the same allergen are poorly understood. Relatively few allergenic proteins had been characterized by the early 1990s. Diagnosis and identification of the specific causative allergenic material can be complex, typically requiring careful clinical histories, temporary elimination diets, skin prick test (SPT), specific in vitro IgE tests and even direct food challenge [18, 19, 35]. Major allergenic proteins have been identified for a number of important allergenic foods. For example, in peanuts the most important appears to be Ara h 2 [36, 37], although both Ara h 1 and Ara h 3 are also potent and abundant allergens [38]. Yet an additional eight peanut allergens have been identified in the literature and are listed in the Allergome database (http://www.Allergome.org/), primarily based on limited in vitro IgE binding studies. Clearly various allergenic proteins differ markedly in potency and in the frequency that they cause allergies.

Based on the scientific and clinical understanding of allergy and allergens, the US FDA (1992) recommended that the allergenicity assessment of GM crops focus on testing to ensure that the allergenicity of the GM variety is not greater than that of the traditionally produced crop [12]. In 1996 those recommendations were elaborated as a detailed assessment strategy with a decision tree by a panel of experts sponsored by the International Life Sciences Institute/International Food Biotechnology Committee (ILSI) [21].

As described in the ILSI approach, the assessment of each new GM crop should evaluate the known allergenicity of the source of the gene [21]. If the source is known to be allergenic, protein-specific in vitro serum IgE tests and clinical tests (SPT and food challenges) would be performed with appropriately allergic subjects to ensure the protein encoded by the transferred gene is not an allergen.

The sequence of any newly transferred protein should be compared to those of known allergens by FASTA or BLAST to identify possible homologues and by searching for matches of eight or more contiguous amino acids identical to a segment of any allergen. If the results of either FASTA or matching of eight amino acid segments indicate a significant match, serum IgE testing and, if needed for clarity, in vivo clinical challenges (SPT and/or food challenges) would be performed using subjects with allergies to the matched allergen to evaluate potential cross-reactivity [21].

The encoded protein should be tested for stability at acidic pH in the presence of pepsin using an in vitro assay as a number of important food allergens have been shown to be stable [39–42]. Finding that a protein is stable would indicate a need for further evaluation including consideration of the quantity of the protein in potential food products [40].

If the results of this assessment indicate the protein is an allergen or likely to be cross-reactive, the protein should not be transferred into a food crop, or foods produced using GM crop material containing this protein would be labelled, so the added allergen is readily identified by consumers.

Other characteristics for consideration that were not well defined and include the 'stability' of the protein to heating or processing, and the abundance of the protein in food material from the GM crop [21]. The rationale follows the empirical observation that a number of the major food allergens remain allergic after boiling or roasting and that many potent allergenic proteins are abundant in the food source [40, 42–44]. Some investigators

have suggested testing the allergenicity of proteins in animal models, although no animal model has been rigorously tested with a variety of allergenic and non-allergenic materials to evaluate the predictive value [21].

If the host crop (gene recipient) is a major allergen (peanut, tree nut, soybean or wheat), in vitro IgE binding, or in vivo challenges should be performed to ensure that the introduction of the gene did not significantly increase the endogenous allergenicity of the crop [21, 45].

Evaluation of the GM Allergenicity Assessment Process

Since 1996 there have been a number of scientific opinions and summaries regarding the allergenicity assessment process [2, 46–48]. Some reports, like that of the expert panel for the Food and Agricultural Organization/World Health Organization (FAO/WHO) of the United Nations in 2001 [49], have suggested significant changes to the original allergenicity assessment. The FAO/WHO report was produced as an advisory to the Codex Alimentarius Commission (Codex), the organization charged with providing food guidelines for the joint FAO/WHO program at the United Nations. In developing the final consensus document [50], the Codex convened a panel of international food safety regulators from member countries to review the FAO/WHO 2001 recommendations and other evidence relevant to the allergenicity assessment. The final Codex guidance calls for a weight of evidence approach rather than a specific decision tree approach as they recognized that there are uncertainties associated with any single test. The approach is based on evaluating the source of the gene, sequence similarities to known allergens, stability of the protein in pepsin at acidic pH and, if appropriate, in vitro IgE binding using sera from specifically allergic individuals. If necessary, in vitro IgE binding tests may be supplemented by SPT or ex vivo cell-based assays [50]. The Codex document recognizes that improvements in scientific methods occur over time, but because there has not been significant progress in the ability to predict allergenicity over what was known in 1996, the allergenicity assessment process in 2004 is similar to the earlier guidance [21]. The European Food Safety Authority has just finalized their guidance for assessing the safety of GM organisms [51] and the recommendations are consistent with the Codex [50].

Allergenic Source

The purpose of considering the allergenicity of the source of the gene that is transferred to a GM crop is to evaluate whether there is a group of individuals who would be at a higher risk from exposure to a given protein from that source due to preexisting allergies. If individuals having allergies (food, contact or inhalation) to the source of the gene (e.g. peanuts, latex or mugwort pollen) can be readily identified, it should be possible to obtain serum from a statistically valid number of consenting individuals in order to test for specific IgE binding to the protein encoded by the transferred gene [50]. While it is hard to estimate the total number of allergenic sources, a few relatively comprehensive lists of allergens can be found on the internet. One already mentioned, http://www.Allergome.org/, currently lists nearly 800 species including a number of allergenic sources for which no individual allergenic protein has been identified. Another, http://AllergenOnline.com/ lists approximately 620 allergenic proteins (1,191 including isoforms) from 210 species where the sequence of at least one allergen is known. Additional electronic database resources (e.g. Entrez PubMed, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi, and Google, http://www.google.com/) should be searched for recent reports of potential allergenicity of the source.

Among the allergic population, some allergens are reported as common causes of allergic reactions in people, e.g. birch pollen (inhalation), peanut (food) or latex (contact). Other allergen sources are known only from one or a few reports, e.g. inhalation allergy to iguana scales [52] or food allergy to lingonberry [53]. The Codex guidelines recognized that it is unlikely that a sufficient number of individuals with specific allergies to uncommon allergens could be identified to provide predictive test results regarding the potential allergenicity of the transferred protein [50]. In such cases, the aggregate risk of allergenicity could be considered low, particularly since the most simple of eukaryotic organisms is estimated to have 400 genes [54], while a complex organism such as rice has >50,000 genes [55] and the mouse has approximately 20,000 genes [56]. In contrast, the number of allergenic proteins identified in even the most commonly allergenic organisms rarely exceeds 10 (http://www.Allergome.org/ and http://www.AllergenOnline.com/). Therefore, by random chance, the probability of selecting a gene encoding an allergen to transfer is small.

Characterization of the GM Protein and Test Materials

The protein as expressed in the GM plant must be well characterized and consistent with the expected protein based on characteristics of the introduced DNA sequence [50, 51]. Additionally, any protein produced as a surrogate test material, e.g. as expressed through any heterologous expression system in bacteria, in cell culture including yeast, or virally produced, must be appropriately characterized to ensure it represents the sequence and complete structure of the plant-produced protein. The full-length amino acid sequence of a protein is rarely obtained by N-terminal sequencing (Edman degradation). However, mass-spectrum data provide additional evidence that the protein matches the expected sequence based on the full-length DNA sequence of the insert. The protein produced in plants should be evaluated for potential asparagine-linked glycan. Any glycan structure on a test protein, as produced in a different plant, bacculovirus or yeast, should match that found in the GM crop [50, 51]. The mass or molecular weight of the heterologous test protein and any biological or biochemical activity (e.g. insect toxicity or enzyme activity) should be equivalent to that of the protein produced in the GM crop [50, 51]. If the test proteins are not fully characterized, the reliability of some or all of the assays in the safety assessment may be questioned as occurred during the US Environmental Protection Agency review of the analyses of Cry9C in StarLink maize [57].

Bioinformatics (Amino Acid Sequence Comparison)

The purpose of comparing the sequence of the introduced protein to that of known allergens is first to determine if the protein is already known to be allergenic, and second to evaluate whether the protein is sufficiently similar to an allergen to suspect an individual sensitized to an allergen might suffer a cross-reaction if exposed to the new protein [21, 49–51]. Two important questions are often raised about the bioinformatics screen. What specific bioinformatics method should be used for the comparison? And what degree of matching is likely to be biologically meaningful?

The list of known allergens has grown since the first GM crop was evaluated. At that time an allergen database containing 219 allergenic and celiac inducing protein sequences was compiled from public protein and amino acid databases into a database for the allergenicity assessment by searching the general sequence databases and public literature databases using key words such as 'allergen' [1]. Compilation of the first internet-based allergen sequence list was described in 1998 [58]. Similar methods were used to compile the AllergenOnline database (http://www.AllergenOnline.com) that currently (2004) lists 1,191 sequences representing 619 allergenic proteins including homologues and isoforms of allergens from more than 200 species of organisms.

The ILSI assessment recommended identification of any exact matches of eight or more contiguous amino acids between the introduced GM protein and any known allergen as the primary criterion for potential cross-reactivity [21]. The stated assumptions were that T-cell epitopes are between 8 and 12 amino acids long and B-cell (IgE) epitopes are generally longer [21]. A FASTA sequence search was also suggested, but without definition of a relevant match. A few IgE binding peptides as short as five amino acids have been identified [59, 60], however high-affinity IgE binding is more likely when the peptides are greater than eight amino acids in length [59, 61]. While allergen-specific T cells are required to help B cells make IgE, other T cells are required for the induction of IgG and IgA antibodies and T-regulatory cells that suppress immune responses. Therefore T-cell receptor binding is not likely to be predictive of allergenicity. While there are few unequivocal studies, it appears that proteins sharing greater than 70% identity over the entire molecule are often cross-reactive, while those of less than 50% identity are rarely cross-reactive [62]. There are no clear reports of two proteins sharing only one short identical region of six to eight amino acids and being cross-reactive. As more information became available about the structure and folding of proteins and allergic cross-reactivity, the emphasis in regulatory submissions for GM crops has shifted toward the use of FASTA or BLAST algorithms to identify potentially cross-reactive proteins based on alignments of segments with 80 or more amino acids that are at least 35% identical [50, 63, 64].

A number of bioinformatic studies have evaluated the predictive value of searching for short segments of exact matches of six or eight amino acids, compared to using the FASTA alignment or other methods [50, 64–67]. Based on published data, 41 out of 50 randomly chosen proteins from maize matched at least one allergen or putative allergen using criteria of an exact match of six contiguous amino acids [64]. Only seven of the 50 matched sequences contained a match of at least eight contiguous

amino acids compared to an allergen. In contrast, nine of the 50 were found to share greater than 35% identity to an allergen over any segment of at least 80 amino acids by FASTA3 comparison using a BLOSUM 50 scoring matrix, a gap penalty of 12 and an extension penalty of two. Only five shared greater than 50% identity with any known allergen, and all of those also matched at least one allergen with a segment consisting of eight contiguous amino acids, although in some cases with different allergens [64]. These data clearly suggest that a six-amino-acid match is unlikely to be predictive for allergenic cross-reactivity [64]. A comparison of the entire set of protein sequences within the Swiss-Prot database indicated that 67% of them (>100,000 proteins) matched an allergen with a six-amino-acid match [67]. Approximately 7% of the proteins in Swiss-Prot matched an allergen if the segment was extended to 12 amino acids, calling into question the predictive value of an eight-amino-acid match [67]. Recent results from studies evaluating cross-reactivity by in vitro inhibition of specific IgE binding support the suggestion by Aalberse [62] that proteins sharing greater than 70% overall identity, as predicted by FASTA or BLAST, often share serological cross-reactivity (shared IgE reactivity) consistent with clinical responses to biological material from taxonomically related species, while those with less than 40–50% identity are unlikely to share significant IgE or allergic cross-reactivity [68–71].

Other approaches have been evaluated to improve the predictive value of sequence comparison. One group used the FAO/WHO [49] criteria for matches of six contiguous amino acids as a preliminary screening tool, followed by a literature survey for IgE-binding epitopes and finally a theoretical evaluation of antigenic sites using the Hopp and Woods algorithm of the allergen-matched specific segment in order to reduce the false-positive rate [66]. However, the predictive value of the combined method has not been evaluated. Antigenicity prediction algorithms have not proven highly predictive for antibody-binding epitopes [72], although ongoing studies on the identification of antigenic epitopes of pathogenic viruses may lead to better predictions [73]. A 'motif-based allergenicity prediction' method has been proposed to compare sequences based on protein structure by classifying 779 known or putative allergenic sequences into 52 distinct motifs [67] based on sequence similarity instead of a short-sequence peptide match. A comparison was made between the two methods using a randomly chosen dataset of protein sequences selected from the Swiss-Prot sequence database. They were screened matching six contiguous amino acids and identified 200 proteins that matched at least one allergen over six contiguous amino acids even though there is no published evidence of allergenicity for 199 out of the 200 proteins [67]. The same dataset of proteins was evaluated using the motif identification method with results showing the motif method falsely identified 9 out of 10 proteins as allergens [67]. While the motif method had nearly a tenfold lower rate of false-positives than the six-amino-acid match, a 90% false-positive rate is still quite high. Another refined sequence and antigenicity motif prediction algorithm has been developed for the prediction of IgE-binding sites based on previously identified sequences and structures [74]. However, neither of these motif prediction algorithms has been compared to FASTA algorithm predictions, but since the algorithms of both [67, 74] are based to a large extent on sequence similarity, it would be surprising if the results from those two methods identified markedly differed from those obtained with a direct FASTA (or BLAST) comparison.

Another group has worked to refine the FASTA3 search comparison by evaluating combining the percent identity matrix value and the overlapping sequence match length as vectors using nearest neighbor analysis in a 'supervised learning system' [75]. Further analysis with this approach tested two scoring matrices, BLOSUM 50 and BLOSUM 80, to statistically evaluate apparent true- and false-positive matches [76]. Further evaluation of this modified method is necessary with data of cross-reactive and non-allergenic protein to understand if it is more predictive than a simple identity score.

It is important to note that at this time a FASTA search of a comprehensive database that includes food allergens, contact sensitizers, airway allergens and celiac associated glutens is quite efficient at identifying proteins that are likely to be cross-reactive, those that share greater than 50% identity over a major portion of their length [62]. The approach is simple and results are easy to interpret. The question remains as to whether the conservative criteria of a 35% identity match over 80 or more amino acids [49] provide too many false-positive or false-negative results. Importantly, no computer search and prediction tool will be 100% accurate at predicting whether a protein will become an allergen or is cross-reactive. The goal of bioinformatics should remain the identification of proteins that may be cross-reactive and would require further evaluation by serum IgE tests.

Serum IgE Binding Tests

Antigen-specific serum IgE tests have been used to confirm the identity of allergenic foods, airway and contact sensitizers as well as a screening tool to identify potential allergenic proteins since the identification of IgE as the reagenic, or allergy-inducing antibody [77, 78]. Reagents and methods for such tests are now common place, although assay validation and interpretation of results are complex.

Allergen-specific allergic donor serum binding studies were used to determine that the gene from Brazil nuts, which was inserted into soybeans as one of the first potential GM products, produced an allergenic protein that is now known as Ber e 1 [79]. Although the protein was not known to be an allergen before this study, Brazil nuts were known to cause allergic reactions in some people. In vitro tests demonstrated serum IgE from Brazil-nut-allergic donors bound to the transferred protein and to proteins in an extract of the GM soybeans, but not to proteins from non-GM soybeans. SPT were used to further demonstrate the allergenic reactivity of the protein. Development of the Ber-e-1-containing soybean product was stopped prior to commercial production [47]. Serum IgE binding tests were also conducted to evaluate the potential increase in endogenous allergens in glyphosate-tolerant soybeans by Monsanto (St. Louis, Mo., USA) compared to non-transgenic soybeans [45]. Recently, IgE binding tests were performed by other investigators using sera from allergic individuals having high apparent soybean-specific IgE, but negative clinical challenges. The results did not show any differences in IgE binding to a number of varieties of the GM and non-transgenic soybeans [80].

Allergen-specific IgE provides the selectivity of the allergic response. Effective binding requires a strong interaction (high affinity or avidity) between the antigen recognition site (paratope) on the antibody and the binding site (epitope) on the antigen. The presence of measurable antigen-specific IgE from a blood or serum sample indicates that the individual has become sensitized to the antigen (or a protein that has a similar epitope). If the individual has IgE to a second epitope on the antigenic protein, the antigenic protein is linked to an identical protein, or another protein with an IgE epitope that is recognized by the individual, exposure and absorption of the protein may lead to cross-linking of high-affinity FcεRI (IgE) receptors on the surface of mast cells or basophils. If a sufficient number of receptors are cross-linked, the mast cell or basophil will be stimulated to release histamine, leukotrienes and other effector molecules, leading to symptomatic responses of an allergic reaction. Most in vitro IgE tests simply measure the level of IgE that is bound to a protein, and do not distinguish between antigens that bind only one IgE molecule and those that bind more than one. Further, direct binding assays do not measure affinity (or avidity) and sometimes weakly cross-reactive IgE antibodies bind to proteins in vitro that do not induce allergic responses. Thus a positive result from an in vitro IgE binding assay suggests that a protein is an allergen, but is not proof that the protein will cause an allergic reaction for the individual, though very high levels of specific IgE correlate well with clinical reactivity [19]. In addition, a negative result may occur for individuals that are allergic to the protein, either because the test material in the assay is not representative of the protein in the allergenic source material, or because the individual has not been exposed to that particular allergenic source for a significant period of time and the level of free IgE in blood samples has fallen below the level of detection of the assay.

Reliable diagnosis of allergen specificity using in vitro IgE binding assays requires the use of test materials that are well characterized. The assays should be validated with sera from multiple individuals with specific clinically defined allergic reactions, as well as non-allergic subjects in order to demonstrate specificity and criteria for positive results. When practical, assay validation should be performed according to internationally recognized guidelines [81, 82], and the assay calibrated to a known amount of international IgE standard or antigen-specific IgE standard [81, 83]. For some commonly allergenic proteins, in particular a number of food allergens, clinical reactions, in vivo IgE challenges (SPT and food challenges) and quantitative antigen-specific in vitro IgE binding data have been evaluated to understand the diagnostic value of the tests and in some cases with the identification of true- and false-positive and -negative values [19, 82, 84, 85]. However, because of the low prevalence of allergy to many specific allergens and the small amount of serum or plasma that can be drawn from allergic subjects, especially children, it may not be possible to perform a full validation for each antigen-specific IgE assay system.

Various direct binding assays, including immunoblotting, ELISA, RAST, and various commercial testing formats, can provide a measurement of the identity (qualitative) or relative abundance (quantitative) results of IgE binding to specific targets. There will always be a degree of uncertainty regarding the predictive value of in vitro

IgE binding tests for predicting allergenicity. Comparisons with clinical effects are needed to demonstrate false-positive and false-negative results as well as true-positive and -negative results [19, 82, 85]. The imperfect predictive values may be due to the inability of the assays to differentiate between low-affinity and high-affinity binding or to discriminate between multivalent and monovalent binding. Inhibition assays can improve diagnostic applications or studies designed to identify new allergens as competitive binding with other relevant and irrelevant proteins will verify specificity. If multiple concentrations of inhibitor are tested, inhibition assays will provide a relative measure of the avidity. The results should be evaluated along with clinical data and historical exposure of the individual subjects. SPT or histamine release assays may be needed to help evaluate the potential biological activity of the allergens [70, 80, 85–89]. For instance, inhibition of bovine-milk-allergic patient IgE binding to bovine and human β-caseins showed roughly 100-fold greater inhibition of binding with the bovine protein, demonstrating greater affinity by the allergenic protein even though the sequences are approximately 60% identical and few individuals are thought to be allergic to human milk [86].

Few studies have attempted to define statistically significant predictive values for allergen-specific in vitro IgE assays or SPT that would circumvent the need for in vivo challenge tests. Four things are evident from such studies. First, large numbers of specifically allergic individual test subjects are needed to establish a significant cutoff value; second, the cutoff is specific for that allergen; third, different studies have reported notably different thresholds for the same allergen, and, finally, values indicating a high probability of specific allergy are markedly above the lower limits of detection, e.g. 0.35 kUA/l in the Pharmacia CAP System™ tests or a 3-mm diameter above negative control in SPT testing [19, 85].

In developing antigen-specific in vitro IgE tests to evaluate the safety of proteins introduced into GM crops, non-specific binding of the IgE to membranes used in Western blots, ELISA plates or the solid-phase target proteins are often overlooked. Additionally, binding of detection reagents (e.g. secondary antibodies or biotin/avidin reagents) may be significant and may appear to be specific [90, 91]. Development of highly specific assays often requires evaluation of blocking reagents and binding conditions with a number of positive and negative control sera as well as the detection reagents. Skin test materials should be tested in non-allergic control subjects to test for the presence of non-specific irritants. In summary, the concept of using IgE binding or SPT to evaluate the allergenicity of a protein is simple, but in practice the performance of valid, meaningful assays is much more complex.

While the FAO/WHO advisory group [49] recommended broadly targeted serum screening of IgE binding to the introduced protein, using sera from 50 individuals who are allergic to species distantly related to the source of the gene (e.g. latex-, birch-pollen- and peach-allergic subjects if the gene was derived from a dicotyledon), no testing has confirmed the predictive value of such a test. Based on the low frequency of allergen specificity to any protein, it is highly unlikely that such a test would rigorously test the potential allergenicity of any protein. Therefore, the Codex did not recommend targeted screening as a routine practice for assessing GM products [50], while the European Food Safety Authority suggested targeted screening as an additional method that may be performed in assessing allergenicity [51].

Protein Stability to Pepsin

Since 1996 a few individuals have questioned the relevance or predictive value of assessing the stability of the introduced protein to pepsin at low pH, or the conditions of the assay [49, 92, 93]. Clearly the pepsin assay is not 100% predictive, but many important food allergens are stable in pepsin at pH 1.2, suggesting the assay may be useful in risk assessment [39, 40]. The physiological basis for the correlation is that the stomach offers a remarkably effective organ at denaturing and breaking proteins into small peptides and amino acids that are available for absorption in the small intestine. Denaturation and partial digestion of susceptible proteins reduces the availability of conformational epitopes more efficiently than linear epitopes. Many important IgE-binding epitopes commonly recognized by those with persistent, systemic allergic reactions to foods such as peanuts and milk have been shown to be linear, based on binding to short-immobilized peptide fragments [94, 95]. In contrast, IgE-binding epitopes for a number of proteins from fruits and vegetables, such as the apple, celery and peach, homologues of the birch pollen allergen, Bet v 1, that cause primarily mild oral itching and oropharyngeal swelling, are conformational and the proteins are not structurally preserved through heating or digestion [44, 96]. Fruit and vegetable allergens that are stable to heat, such as the non-specific lipid transfer proteins, are highly cross-linked by disulfide bonds. These proteins are stable to heat and di-

gestion [39, 70]. It is also important to note that some pepsin-stable proteins such as lectins [92], while not allergenic, are important immunogens or immunomodulators in the intestinal tract and should be considered in the overall safety assessment. Pepsin digestion assay conditions have been refined further in an international collaborative study [97] and it appears the assay has a relatively good predictive value for food allergens [40].

Glycosylation

A number of allergenic proteins are glycosylated at asparagine residues separated by one amino acid from either serine or threonine (unless the intervening amino acid is proline), if the protein also contains an amino-terminal signal peptide sequence for processing in the endoplasmic reticulum and Golgi body. While there are many possible complex carbohydrate structures, those that contain an α(1,3)-fucosyl residue, and/or β(1,2)-xylose may act as a cross-reactive carbohydrate determinant (CCD) that can be bound by IgE from individuals sensitized to unrelated (non-homologous) sequences [98]. For some individuals the CCDs do not seem to elicit allergic responses either because the affinity of binding is low, because there are no other IgE epitopes on the protein or for some other reason [69, 99]. In other cases, it appears that the CCD is responsible for eliciting a mast cell response [100–102]. However, it is clear that the rest of the protein structure must play a role in IgE recognition. Effective cross-linking on the mast cells must require both protein sequences and CCDs in such cases or multiple CCDs because individuals with IgE that recognize CCDs do not respond clinically to all CCD-containing proteins. In addition, the carbohydrate may influence the three-dimensional structure of the protein. If the GM protein is glycosylated in the plant, appropriate safety tests (IgE binding, pepsin digestion or bioactivity) must be performed with a similarly glycosylated protein (e.g. purified from the GM plant), rather than an unglycosylated or differently glycosylated protein [51].

Heat Stability

The allergenic activity of most commonly allergenic foods are not reduced when the material is heated, possibly even to boiling [43]. Some allergens (e.g. peanut) may even be rendered more allergenic by heating, either due to covalent modification of the protein through the Maillard reaction, or enhanced protease activity [103]. While heat stability has been suggested as important in assessing the potential allergenic activity of proteins introduced into GM crops [21, 49], there is no universally applicable method to evaluate stability except to test directly for residual allergenic activity in allergic subjects [44, 104]. For heat-stable allergens the IgE epitope, whether conformational or linear, must retain the native form for the allergenic activity to remain when the protein is heated. In some cases, heat-stable proteins are highly cross-linked by disulfide bonds, in other cases there are relatively thermo-stable hydrophobic interactions that cause retention of structure. However, biochemical evidence that the protein unfolds upon heating at relatively low temperatures (e.g. less than 70°C) or looses enzymatic or biological activity at low temperatures is suggestive that it is less unlikely to be a heat-stable allergen.

Abundance

A number of potent food allergens (e.g. shrimp tropomyosin and peanut allergens Ara h 1 and Ara h 2) are abundant proteins in the allergenic source, often representing more than 1% and sometimes more than 10% of the total protein in the allergenic food [1]. But not all abundant food proteins are significant allergens, e.g. actomyosin. Abundance is likely related both to the probability of sensitization and elicitation. Novel proteins in genetically modified foods that are expressed at very low levels are unlikely to become novel allergens. While the minimal dose of a novel protein necessary to elicit allergic sensitization is unknown (and unlikely to ever be determined due to ethical considerations of such clinical experiments), evidence clearly demonstrates that abundance is important as a risk factor in eliciting an allergic response in sensitized individuals. Therefore, understanding thresholds of elicitation, or minimum provoking doses [105], would be useful to consider whether a quantitative threshold dose could be established below which any protein would be unlikely to induce an allergic reaction even if it was able to sensitize. However, such a threshold should not be used as a criterion to automatically reject products with more abundant proteins, because, as noted, not all abundant proteins are allergenic. Important work is currently in progress for a number of food allergens to determine minimal elicitation doses. Additional data from these well-designed clinical studies may contribute to the risk assessment process for GM crops.

Animal Models

Animal models were recognized as a potentially useful tool for evaluating allergenicity at the time of the initial allergy assessments for GM crops [21]. While various mouse, rat, guinea pig, dog and pig models have been developed and evaluated at some level, they appear most useful at identifying mechanisms of sensitization and elicitation [106–111]. Each model has proven highly reactive for some specific allergens, but they have either not been tested with a variety of allergenic and non-allergenic proteins, or they have been shown to react with non-allergenic proteins. Factors that appear to influence the outcome include: genetic differences across the human population and across animal species, possible prior dietary exposure to test proteins or highly similar proteins, the presence of adjuvants, routes and doses of sensitization, challenge procedures, native state of the test materials and prior or concurrent viral infection. Since none of the models have been widely tested and shown to correlate well with allergic reactions to a wide variety of allergens identified in the human population, it has not been possible to identify a single model or even a practical combination of models for the safety assessment.

Potentially Allergenic GM Plants

While major biotechnology companies are aware of the need for the comprehensive allergenicity assessment described here, and regulatory agencies in at least some countries (e.g. the USA) are commending a partial allergenicity assessment (e.g. bioinformatics) before even small-scale field trials are permitted, a number of academic, governmental and small company scientists may be developing GM crops with increased allergenic risks. Some published examples of transformed GM crops that could provide significant benefits in terms of nutritional improvements or reduced insect predation have not been fully evaluated. In one example, maize was altered by the insertion of a gene of 11S globulin from amaranth *(Amaranthus hypochondriachus)* [112]. The developers performed an allergenicity assessment including a bioinformatics search, but have apparently ignored significantly long matches of greater than 40% identity with allergens in buckwheat, Brazil nuts, peanuts and soybeans (based on our search using the FASTA3 search on http://www.AllergenOnline.com/) because they focused only on short 100% matching sequences (6–8 amino acids). In another case, soybean glycinin, which is an allergen, was inserted into rice [113]. It is not clear if the intent was to provide a model to study the feasibility of modifying the protein content or profile of rice, or if it was (is) intended as a potential product [114]. In case of the latter, the potential impact on soy-allergic individuals should be assessed before commercial development would proceed. A third example is the transfer of an insect-resistant protein, α-amylase inhibitor, from beans into peas [115]. The sequence of the protein is just over 35% identical to a protein in peanut and one in soybeans that are probably minor allergens (identified by us in a FASTA search using http://www.AllergenOnline.com/). Finally, the gene encoding the sunflower 2S albumin, which has been shown to bind IgE from sunflower-seed-allergic subjects [116], has been transferred into both a variety of lupine used for animal feed and into rice [117, 118]. Although sunflower seed is not a common allergen, further testing of the protein expressed in the transgenic plants seems warranted. There are many other genes that have been transformed into food crops from other allergenic sources or from sources of unknown allergenic activity. Many of them will never be developed further because they are unlikely to be economically or agronomically valuable. However, in all cases where the plants represent potential products, the potential allergenicity should be evaluated following the Codex guidance [50].

Conclusions

To a great extent, the ability to predict the potential allergenicity of any protein is still limited to knowledge as to whether and how humans have been exposed to it or to highly similar proteins, with or without the development of allergies. No single characteristic or test has been identified that will predict with great certainty whether a specific protein will sensitize individual consumers. However, application of the current allergenicity assessment strategy for GM crops [50] greatly reduces the risk of introducing a known allergen, or a protein that is likely to be cross-reactive as was demonstrated in the analysis of the potential GM soybean that contained the major Brazil nut protein which later became known as the allergen Ber e 1 [47]. There are still a few shortcomings to the process as evidenced by the inability to successfully guide further assessment of the safety of the pepsin-stable Cry9C protein in StarLink maize. There is uncertainty about how to evaluate protein abundance or

heating and processing 'stability' relative to allergenicity and whether a test could be designed that could add predictive power, further reducing potential risk. There is of course some room for improvement in the assessment. The bioinformatic search algorithms and scoring criteria for potential cross-reactivity should be evaluated more thoroughly to provide additional guidance for identifying likely causes of clinical cross-reactivity that would still need to be tested by serum IgE binding or challenges. Additional guidance for methods to test for IgE binding to specific proteins and for method validation would be useful. While such refinements would improve the allergenicity assessment process, it is important to note that there is no evidence that the proteins transferred into any commercially approved GM crop are allergenic or that the overall allergenicity of the endogenous allergens were significantly increased. As discussed here, more complete application of the current assessment to potential products described in the literature should help reduce the possibility of introducing new allergens to food crops. While some authors continue to call for additional tests to predict potential increases in allergenicity, including broadly targeted allergic serum IgE binding studies, animal sensitization and provocation studies and extensive pre-market clinical evaluations to look for potential minor quantitative differences in endogenous allergens or allergenic activity between what amounts to different varieties of the same crop will not markedly improve food safety. The assessment should remain focused on preventing the introduction of new, significant allergens.

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