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Review

Review of the development of methodology for evaluating the human allergenic potential of novel proteins

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Safety assessment of novel proteins in genetic-engineered foods is a key component of the overall safety evaluation for these products. Since allergens are typically proteins, assessment of the potential allergenicity of the novel proteins in genetically engineered foods is critical. This article reviews methods available to assess the potential allergenicity of novel proteins, as well as problems and deficiencies in the existing methods. The role of bioinformatics and knowledge of allergenic epitopes in developing new approaches to this problem is discussed.

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1 Introduction

Agricultural biotechnology has already had considerable impact on the production of certain agricultural crops. Thus far, the number of crops that have been commercially developed through agricultural biotechnology is relatively small, including corn, potatoes, canola, soybeans, cotton, squash, sugar beets, and papaya [1]. Agricultural biotechnology has had its greatest impact on two staple crops, soybeans and corn. Commercialization of genetically engineered canola and cotton has also been quite successful. While genetically engineered squash and potatoes have been developed and commercialized, the impact remains rather minimal. In some cases, introduction of genetically engineered crops onto comparatively small acreage can have considerable positive impact. For example, the Hawaiian papaya industry that was virtually destroyed by a viral infection has been revitalized through the development of a virus-resistant variety through agricultural biotechnology. Because of the inherent production advantages of crops produced through agricultural biotechnology, these crops have been introduced in an ever increasing number of countries.

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Abbreviations: FAO, Food and Agriculture Organization; **WHO,** World Health Organization

Only a limited number of beneficial traits have been introduced into crops commercially developed through agricultural biotechnology [2]. The majority have either improved insect-resistance or enhanced herbicide-tolerance or both, although the beneficial trait introduced into squash and papaya is virus-resistance. These traits provide primarily agronomic benefits, where the benefits accrue primarily to farmers. The commercial success of crops having enhanced agronomic traits will likely lead to the development and introduction of additional transgenic crops with these and similar benefits. Additional crops that protect themselves from diseases and pests and that prosper under adverse climatic conditions such as heat, cold, and drought are likely in the future.

Currently, consumers likely perceive few benefits from genetically engineered crops. Thus far, consumers have mostly not encountered genetically modified foods having direct consumer benefits. Tomatoes with improved ripening characteristics that provided enhanced flavor attributes were developed [3], but these tomatoes, while introduced into the marketplace, are no longer commercially available. Golden rice with enhanced levels of β-carotene has been developed [4] but is not yet commercially available. Golden rice holds some promise to help eradicate widespread vitamin A deficiency and night blindness in certain Asian populations [5]. Agricultural biotechnology offers the potential for development of many more traits that provide direct benefits to consumers. The consumer benefits that could be developed through agricultural biotechnology include enhanced nutritional and nutraceutical composi-

tion, prolonged shelf-life, resistance to spoilage, improved flavor and appearance, and the elimination of naturally occurring toxicants including allergens.

Obviously, for the benefit of consumers and farmers alike, the safety evaluation of foods produced through agricultural biotechnology is critically important. Since the development of genetically engineered foods often involves the introduction of novel proteins, the safety assessment of the novel proteins is a key component of the overall safety evaluation process. Since allergens are typically proteins, the potential allergenicity of the novel proteins introduced into foods derived through agricultural biotechnology has become one of the pivotal elements of protein safety assessment. Although beyond the scope of this particular review, similar approaches can be used in the safety evaluation of all novel foods – those created by agricultural biotechnology and other novel foods. Novel foods regulations exist in certain regulatory jurisdictions, such as the EU and Canada, and many of these techniques are applied in the safety assessment of all novel foods.

2 Comparative risks from foods containing proteins introduced through agricultural biotechnology

Agricultural biotechnology provides a rather precise method for enhancing the beneficial traits of foods produced from plants, animals, and microorganisms. With these recombinant DNA methodologies, genes can be selected from virtually any biological source and be moved into the genome of some other species [2]. Genes could be selected to provide beneficial traits ranging from improved agronomic characteristics to improved nutritional profiles. Often, only a small number of genes are inserted into the genome of the recipient species to provide the selected beneficial trait. Fewer genes may need to be inserted for certain agronomic traits such as insect-resistance or herbicide-tolerance compared to improved nutritional profiles such as golden rice [6]. Thus, crops produced through agricultural biotechnology will contain one to perhaps four novel proteins.

In contrast, conventional breeding involves the transfer of hundreds of genes from one biological source into another. The identity of all of these genes is often unknown. In the process of selecting for the beneficial trait, other unwanted genes may also appear in the new variety. Also in contrast to agricultural biotechnology, conventional breeding only allows the introduction of genes from closely related biological sources. However, conventional breeding could conceivably lead to the introduction of dozens or perhaps hundreds of novel proteins into the new variety. Historically, only a few episodes have occurred with conventional breeding where the new variety was found to have potentially hazardous components [7, 8]. However, a thorough safety assessment is not typically performed with new varieties produced by conventional breeding and the novel proteins that they contain.

3 Allergenicity assessment of genetically modified foods

Because genetically modified foods contain inserted, novel genes that express novel proteins, the assessment of the potential allergenicity of those novel proteins has become a key component of the overall safety assessment. Food allergies affect an estimated 3 – 4% of the population [9]. The prevalence of food allergies is higher among infants and young children than it is among adults [10], but food allergies afflict individuals of all ages. In general, eight foods or food groups are responsible for more than 90% of all food allergies on a worldwide basis – milk, eggs, fish, crustacean shellfish, peanuts, tree nuts (almond, walnuts *etc.*), soybeans, and wheat [11, 12]. However, some specific food allergies can occur at a higher frequency in some geographic areas probably owing to the presence of crossreacting pollen allergies and the cultural dietary practices in those regions. Examples would include peach and celery allergy in Europe, buckwheat allergies in southeast Asian countries, and sesame seed allergy in several countries with large Middle Eastern populations [13 – 16]. Certainly, some individuals are more susceptible to the development of food allergies than others. Children born to parents with food allergies are at higher risk of the development of food allergies (and other allergies) than children who have parents with no allergies. While genetic differences between individuals are very important, no evidence exists to suggest racial or ethnic differences in the prevalence of food allergies beyond the cultural dietary habits noted above.

The safety, including the potential allergenicity of the novel proteins introduced into foods produced through agricultural biotechnology, is assessed as part of the product development process [17]. Virtually all allergens are proteins but fortunately, only a few of the many thousands of naturally occurring proteins found in foods are allergenic under typical circumstances of exposure [16]. Unfortunately, no single test exists that is fully predictive of the potential allergenicity of any specific novel protein [16]. Thus, an array of tests has been developed for this purpose.

The genetically modified crops thus far introduced onto the market have involved the insertion of a few novel genes that express novel proteins at very low levels. Certainly, the potential hazards including potential allergenicity are going to be lessened when the novel protein is present at a low concentration in the genetically modified food. While the

level of expression of the novel proteins is probably another factor to consider in the safety assessment of genetically modified foods including the allergenicity assessment, this attribute is not directly considered as part of most of the proposed strategies for safety assessment. Metcalfe *et al.* (1996) [18] suggested that the level of expression was probably an important factor to consider in assessing the allergenicity of foods produced through agricultural biotechnology. Future genetically modified crops with enhanced nutritional attributes could likely contain much higher levels of the novel proteins. Emerging evidence suggests that threshold doses exist below which allergic individuals will not react adversely to offending foods. Experience with double-blind, placebo-controlled trials suggests that the threshold dose is individually variable but in the range of 1 mg of the allergenic food for the most sensitive individuals [19]. Apparently, if an allergenic protein were expressed in a food produced through agricultural biotechnology at levels well below 1 mg *per* serving, the hazard for allergic consumers would be minimal [16]. The genes used in agricultural biotechnology are often obtained from sources with no history of allergenicity. Consequently, the threshold dose for sensitization to a novel protein is also an important consideration. Unfortunately, very little information exists on threshold doses for sensitization. Despite this situation, foods produced through agricultural biotechnology are less likely to become allergenic if the novel proteins are expressed at low levels in the edible portion of the modified plant.

4 Historical development of the allergenicity assessment of genetically engineered foods

The critical need to assess the potential allergenicity of the novel proteins in genetically engineered foods may not have been appreciated in the early days of agricultural biotechnology [20]. However, the US Food and Drug Administration and other regulatory agencies recognized this potential risk in the early 1990s [21]. The need for such evaluations was more widely recognized in the early 1990s with the development of a soybean variety with improved nutritional qualities for animal feeding on the basis of enhanced methionine content. To achieve this desired characteristic, a methionine-rich protein from Brazil nuts was cloned into soybeans. At the time, Brazil nuts were a known allergenic food [22] but the identification of the major allergenic protein in Brazil nuts remained unknown. However, the product developers (Pioneer Hi-Bred International) recognized that they must determine whether the methionine-rich protein was an allergenic protein. Using sera from individuals with Brazil nut allergy, Nordlee *et al.* (1996) [23] documented that this high-methionine 2S albumin from Brazil nuts is likely the major allergen of Brazil nuts, *Ber e 1*. As a result, Pioneer Hi-Bred International decided not to commercialize this variety. This remains the only example of the development of a genetically engineered crop with significant allergenic potential. Of course, it is also an example that illustrates that an assessment strategy can identify and eliminate such potential risks.

Subsequently, serious attempts were begun to establish effective approaches for allergenicity assessment of genetically engineered crops. The first of these was the decision tree approach advocated by the International Life Sciences Institute and the International Food Biotechnology Council (ILSI/IFBC) in 1996 [18]. This decision tree approach employed several different tests under the assumption that the overall predictability of the allergenicity of a novel protein would be improved by a combination of tests. The ILSI/IFBC approach focused on evaluating the source of the gene, the sequence homology of the novel protein to known allergens, specific serum testing with sera from humans with known allergies to the source of the transferred gene, and various physicochemical properties of the novel protein such as heat stability and digestive stability [18]. While this approach provided reasonable assurance that the novel protein was unlikely to be or to become an allergen, the ILSI/IFBC approach was subjected to some criticism [24, 25].

As a result of the criticism, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) assembled an expert panel to consider an alternate approach in 2001 [26]. The 2001 FAO/WHO decision tree approach relied upon many of the same elements as the ILSI/IFBC decision tree including the source of the gene, its sequence homology to known allergens, specific serum screening, and comparative resistance to pepsin. Additional approaches were also considered, including target serum screening (the immunoreactivity of the novel protein with serum IgE from individuals with known allergies to species that are broadly related to the source of the transferred DNA) and the use of animal models. However, the Codex Ad Hoc Intergovernmental Task Force on Foods Derived from Biotechnology ultimately concluded that targeted serum screening and animal models were not well-developed approaches that could be used with confidence by governmental regulatory agencies [27]. Therefore, these two approaches will not be discussed further.

As a result, the allergenicity assessment of foods derived from agricultural biotechnology continues to rely on evaluation of the source of the gene, sequence homology to known allergens, specific serum screening, and comparative pepsin resistance. While several different tests continue to be used, a weight-of-the-evidence approach is now favored over a strict application of decision tree strategies.

Additionally, the various tests have been improved over the years leading to greater confidence in their reliability.

5 Source of the novel gene

If the source of the gene introduced into the genetically engineered species is known to be allergenic, the novel protein produced from that gene must be assumed to be the allergen until data are generated to disprove that assumption. Environmental allergens, *e.g.*, pollens, as well as for food sources of allergens are considered because some known food allergens are crossreactive with pollen allergens [28]. With allergenic gene sources, the potential allergenicity of the novel gene product can be determined to a reasonable degree of certainty using specific serum screening, discussed further below, employing blood serum from humans known to be allergic to the source of the gene. Greatest concerns are raised when the gene is obtained from a commonly allergenic source including peanuts, soybeans, tree nuts, and wheat from the plant kingdom or milk, eggs, fish, and crustacea from the animal kingdom [11].

In many cases in agricultural biotechnology, the gene is obtained from a source with no history of allergenicity.

6 Sequence homology to known allergens

The amino acid sequences of many food and environmental allergens are known (www.allergenonline.com and other similar websites). Comparing the amino acid sequence homology of the novel protein to the sequences of known allergens is useful in the determination of allergenic potential regardless of the source of the gene [18, 26]. If sufficient homology exists, then suspicions would be raised regarding the possibility that the novel protein might cross-react with the known allergen and provoke symptoms when ingested by individuals with that particular allergy. The criteria used to determine significant sequence similarity have been a subject of some debate. The ILSI/IFBC approach advocated the use of matches of at least eight contiguous identical amino acids [18]. The strategy proposed by FAO/WHO in 2001 was a match of at least six contiguous, identical amino acids or sequence identity in excess of 35% over sliding 80-amino acid windows [26]. The use of 6-mer matches has been compared to 8-mer matches using corn proteins to document that 6-mer matches would yield numerous false-positive matches [29]. Therefore, guidelines of the Codex Ad Hoc Intergovernmental Task Force on Foods Derived from Biotechnology suggest the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids and that a search for exact matches of short segments of six or eight amino acids may be of value but must be scientifically validated [27].

The criterion for 35% structural identity over 80-amino acid windows is intended to identify proteins that share similar structure [16]. Many common plant allergens fall within a few functional categories including several different types of pathogenesis-related proteins and the most structurally similar are frequently crossreactive [30, 31]. If the novel proteins introduced into foods developed through agricultural biotechnology have greater than 35% structural identity with these known allergens, great caution must be exercised to assure that these proteins are not crossreactive with the structurally similar known allergens.

If the sequence homology tests are positive, either the commercial development of crops containing that novel protein could be halted due to possible allergenicity concerns or the potential crossreactivity with known allergens could be more thoroughly evaluated with serum screening. While bioinformatics is the primary focus of this conference, bioinformatics is best positioned as a key part of a broader allergenicity assessment strategy.

Several limitations exist to the use of bioinformatics in the assessment of the potential allergenicity of novel proteins in genetically engineered foods. Certainly, this approach is limited by existing knowledge of the identity and sequences of food and environmental allergens. However, such basic knowledge is increasing rapidly and the identities and sequences of more than 1100 allergens are now known with varying degrees of certainty. Another limitation is that a high degree of sequence homology can occur without clinically relevant crossreactivity. A good example would be the tropomyosins where chicken and shrimp share about 60% sequence homology but there is no IgE crossreactivity between these two foods [32]. Certainly, sequence homology comparisons cannot be solely relied upon in the assessment of potential allergenicity. Serum screening and other approaches are often needed to confirm IgE reactivity and clinical significance.

7 Specific serum screening

Serum screening is useful when either (1) the gene is derived from a known allergenic source or (2) the search for sequence homology identifies a possible match with a known allergenic source. Serum screening involves evaluation of the immunoreactivity of the novel protein with IgE antibodies from the sera of individuals allergic to the source material [18, 26]. Since the structures of all of the allergens from all allergenic sources are not yet known, specific serum screening is desirable in every case where the gene is derived from a known allergenic source [26]. A specific protocol does not exist for serum screening. Typically, the novel protein would be bound to a solid phase and incubated with blood serum from individuals known to have allergic

reactivity to the relevant allergenic source to determine if the allergen-specific IgE in the serum reacts with epitopes on the novel protein. The availability of sera from well-characterized patients is an important and sometimes challenging issue. Depending upon the allergenic source, well-characterized human blood serum may be difficult to obtain. Sera would be well characterized if the patient had a positive and convincing history of allergy to the gene source, had a positive skin prick test or radioallergosorbent test to an extract of the gene source, and ideally had a positive clinical challenge trial with the source material [16].

False-positive results are a concern with specific serum screening. For example, many plant proteins contain carbohydrate moieties. The existence of IgE binding to carbohydrates is a well-known phenomenon [33]. The clinical significance of these crossreactive carbohydrate determinants is questionable [33]. Thus, the possibility of IgE binding to carbohydrate determinants must be excluded during specific serum screening [26].

Metcalfe *et al.* (1996) [18] advocated the confirmation of positive serum screening tests with additional clinical studies including skin prick testing and double-blind, placebo-controlled oral challenge trials. However, ethics boards may not approve such human trials in some countries. Obviously, these tests could be valuable in areas where they are allowed.

8 Resistance to pepsin

Allergenic proteins must reach the intestinal tract in a form that is sufficiently intact to provoke the immune system. Proteins that are rapidly hydrolyzed by digestive proteases would seem to be less likely to be or become allergens. In general, known food allergens exhibited greater proteolytic stability than known nonallergenic food proteins in simulated gastric and intestinal digestive models [34]. Many of the novel proteins introduced into foods produced through agricultural biotechnology were also rapidly digested in these same model systems [34]. Thus, Metcalfe *et al.* (1996) [18] advocated digestive stability as a criterion for the allergenicity assessment of novel proteins introduced through agricultural biotechnology. However, it is widely recognized that humans vary widely in their digestive capacity and existing *in vitro* tests do not mimic the wide range of human digestive capacity. Thus, FAO/WHO (2001) [26] wisely altered this criterion to comparative pepsin resistance.

The comparative resistance to pepsin proteolysis is likely a reasonable comparative measure in the allergenicity assessment. Novel proteins resistant to pepsin are more likely to become allergenic than proteins that are rapidly hydrolyzed by pepsin. However, the conditions of the pepsin resistance assay are critical in this evaluation. By varying the conditions, some have questioned the value of pepsin resistance [35, 36]. Clearly, a standardized protocol would be advantageous and ILSI-HESI has now established one [24].

Pepsin resistance will never be a perfect indicator of allergenic potential. Some allergens in fresh fruits and vegetables are known to be sensitive to proteolysis [37]. These particular allergens tend to be ones that are crossreactive with known pollen allergens [37] and would thus likely be discovered in the sequence homology testing [16]. Cross-reactive allergenic proteins that are pepsin- and heat-labile are typically quite similar in sequence and structure, and should therefore be identified by bioinformatic analysis.

9 Conclusion

Several tests exist that are quite helpful in the assessment of the potential allergenicity of novel proteins in genetically engineered foods. The application of these tests in a weight-of-the-evidence approach provides reasonable assurance regarding whether the modified crop would elicit a reaction in an allergic individual or induce allergic sensitization in a susceptible individual. Continuing improvements in the various tests provide increasing confidence in their reliability. Several improvements are needed with respect to existing methods including the validation of criteria and the harmonization of methodological approaches. For example, the pepsin resistance method has been improved by the establishment of a standardized protocol [24]. However, standardization remains needed for serum-screening methods. The criteria used for sequence homology comparisons have been improved with validation efforts as noted earlier but further such efforts would be beneficial. The development of additional tests would also be helpful. If a validated animal model could be developed, it would offer tremendous value. And, bioinformatics approaches would benefit from increased knowledge of epitopes.

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