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Allergenicity assessment of genetically modified crops—what makes sense?

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Abstract
GM crops have great potential to improve food quality, increase harvest yields and decrease dependency on certain chemical pesticides. Before entering the market their safety needs to be scrutinized. This includes a detailed analysis of allergenic risks, as the safety of allergic consumers has high priority. However, not all tests currently being applied to assessing allergenicity have a sound scientific basis. Recent events with transgenic crops reveal the fallacy of applying such tests to GM crops.

Genetically modified (GM) crops undergo rigorous safety assessment before being allowed to enter the market. One aspect of GM foods that has drawn a lot of public attention is the assessment of their potential allergenicity. Protecting people with food allergies against accidental exposure to allergens has become an important focus for food manufacturers and regulators responsible for all food safety. A significant focus of the food industry is to keep food products that are not intended to contain a major allergen (e.g., peanut, milk, eggs or wheat) from being contaminated with one of the major allergens. Likewise, the primary focus of the safety assessment for GM crops, as defined by the Codex Alimentarius Commission (Box 1)[1], is to prevent the transfer of a gene encoding a major allergenic protein (from any source), into a food crop that did not previously contain that protein.

The producers of GM crops and regulatory authorities focus on preventing avoidable increases in the risk of allergy in producing and accepting new GM crops. It should, however, be recognized that absolute avoidance of all risk is not achievable. Thus the assessment that has been developed focuses on avoiding risks that are predictable and likely to cause common allergic reactions.

Before discussing the details regarding the approaches used for assessing potential allergenicity of GM crops and the drawbacks of some steps, it is important to put the risks associated with food allergy into perspective. The prevalence of food allergy is not well established but is estimated to be around 6% in young children and 3% in adults[2].

Known potent allergenic foods like peanut or shrimp are not banned from the market, even though 1% of the population might develop allergic reactions upon exposure. In addition, market introductions in the recent past of novel foods like kiwi have resulted in the development of new allergies. Yet kiwi has not been removed from the market. Some of the major allergenic foods like fruits, nuts and fish are considered essential components of a healthy diet, and nobody would endeavor to deprive 99% of the population of these foods because 1% is at risk of developing food allergy. Instead, food labeling is used to help the allergic consumer avoid exposure to foods that cause their reactions. Similar arguments could be made for new crops developed either by conventional breeding or by genetic modification to, for example, help combat malnutrition in developing countries.

Furthermore, to date there is no documented proof that any approved, commercially grown GM crop has caused allergic reactions owing to a transgenically introduced allergenic protein, or that generation of a GM crop has caused a biologically significant increase in endogenous allergenicity of a crop[3]. However, the potential for the transfer of an allergen was illustrated in the 1996 case of transgenic soybeans into which the gene for a 2S albumin from the Brazil nut had been transferred to enhance the methionine content of animal feed. Although the protein had not previously been recognized as an allergen, a study sponsored by the developer of the crop, Pioneer Hi-Bred International (Johnston, IA, USA) during product development demonstrated IgE-binding with sera from Brazil nut–allergic subjects and positive skin prick tests to the transferred protein[4]. This protein is now known as the major allergen of the Brazil nut, Ber e 1. Despite being developed for animal feed only, the product was abandoned because of the obvious risk.

That experience provided guidance for development of the premarket allergenicity assessment process and demonstrated that specific, appropriate tests can prevent the transfer of a gene encoding a protein that might pose substantial risk. However, whereas absolute protection against all potential allergic reactions to a newly introduced protein can never be given, the allergenicity assessment of GM crops based on scientifically sound protocols should minimize the risks. It should be noted that some scientists and regulators have called for postmarket monitoring of GM crops to identify the development of new allergies associated with the crop. The full Codex guidelines[1], however, outlines the need for an effective premarket evaluation as the most effective tool to protect the public. There are technical, practical and economic issues that would need to be addressed in designing an effective postmarket monitoring system and are beyond the scope of this paper. Here, we focus on the scientific validity of protocols used in the premarket evaluation of the potential allergenicity of GM crops. In particular, we show how three tests that are commonly called for, and which have not been validated, can block development of potentially useful products.

Evolution of guidelines for allergenicity assessment of GM crops

Guidelines for allergenicity assessment of GM crops were published in three sequential documents that have been broadly recognized. The first comprehensive document was published in 1996 by the International Food Biotechnology Council (IFBC, Washington, DC) in collaboration with the International Life Sciences Institute (ILSI, Washington, DC)[5]. This was followed in 2001 by the UN Food and Agriculture Organization (FAO)/World Health Organization (WHO) consultation recommendations[6] and in 2003 by the Codex Alimentarius Commission guidelines[1]. The revised recommendations (FAO/WHO, 2001; Codex, 2003) were meant to correct shortcomings, although further clarifications are possible as we learn more about allergens and gain experience in test methods[7]. Several elements, however, are well established and have remained consistent throughout the three successive sets of recommendations.

All documents agree that introducing known allergens into a different species needs to be avoided as the primary risk is to those with existing allergies. If the source of the gene is a common allergenic food, or if the protein displays significant sequence identity with known allergens, the candidate protein should be evaluated for IgE binding using a sufficient number (e.g., for >95% confidence) of sera from patients allergic to the source of the allergenic food or to the sequence of the matched allergen. Those tests should reveal whether the gene codes for a yet unidentified allergen from a common allergenic source or whether IgE against known allergens cross-reacts with the homologous new transgenic protein. Another parameter included in all three guidelines is resistance of the candidate protein to digestion by pepsin, the rationale being that pepsin-resistant food proteins are more prone to induce systemic, severe symptoms. Perhaps more importantly, such stable proteins are also thought to be more potent sensitizers than proteins that are readily digested in the gut (that is, they are risk factors for induction of new allergies).

The IFBC-ILSI and FAO/WHO guidelines both used a decision tree to evaluate the risk of allergenicity[5,6], as reviewed previously[7]. The IFBC-ILSI document recommended *in vivo* clinical testing (skin-prick tests (SPT) and double-blind placebo-controlled food challenges (DBPCFC)), even if *in vitro* assays had demonstrated a lack of IgE binding for proteins from an allergenic source, if the protein sequence included as little as a single eight-amino-acid match to a known allergen. Even so, the FAO/WHO recommendations designated *in vivo* clinical testing as impractical and perhaps even unethical under most circumstances as a risk assessment tool, and suggested instead that negative serum testing alone, or in some circumstances SPT testing, but not food challenges, might be necessary to demonstrate a lack of risk. Another change recommended by the FAO/WHO[6] guideline was a six-amino-acid match to indicate a risk of cross-reactivity with allergens, rather than an eight-amino-acid match indicated by IFBC-ILSI[5]. Two additional new elements were added to the FAO/WHO (2001) recommendations: targeted serum screening—in which serum samples of patients allergic (or at least sensitized) to allergen sources broadly related to the source of the gene (sharing similar high taxonomic groups; e.g., monocots, dicots or arthropods) are used to detect or exclude potential cross-reactivity—and animal model testing. Targeted serum screening was recommended even when the transgenic protein did not demonstrate significant sequence identity to a known allergen or when the specific serum screening—using sera from subjects allergic to the source or the sequence-matched allergen—was negative. Animal testing was included despite recognition that validated models predicting risk of sensitization in humans do not (yet) exist.

The Codex Alimentarius Commission guidelines abandoned the risk assessment based on a decision tree and adopted a weight-of-evidence approach[1]. A decision tree was found to be too rigid in a situation where no single criterion is sufficiently predictive and evidence derived from several types of information, based on tests with different levels of validation, needs to be taken into account. Codex clearly emphasized the need to use scientifically validated testing, specifically removing the demand for nonvalidated animal tests and targeted serum screens and calling for validation of short-sequence matching routines. Instead, a 35% identity over an 80-amino-acid window was recommended as a sufficiently conservative prediction for potential cross-reactivity. These recommendations have not been accepted by some regulators. Clearly, the existence of multiple documents with diverging recommendations coming from different organizations has resulted in confusion and sometimes arbitrary inclusion of tests upon request from regulatory authorities. In some cases, regulators continued to base their judgment on nonvalidated (e.g., animal models) or even rejected (short-peptide matches) tests.

Box 1. Risk assessment of genetically modified crops

The Codex Alimentarius Commission, under the FAO and the WHO, adopted guidelines in 2003 to harmonize the premarket risk assessment process for plants derived from biotechnology (GM plants) in the global market[1]. The guidelines were approved by the Codex Commission and are intended to guide countries in adopting consistent rules that provide a strong food safety evaluation process while avoiding trade barriers. Each new GM crop requires a premarket safety assessment to evaluate intended and unintended changes that might have adverse human health consequences caused by the transfer of the DNA (genes). The goal is to identify hazards, and if found, to require risk assessment and where appropriate develop a risk management strategy (e.g., do not approve, approve with labeling and/or monitoring, or approve without restriction).

The process is based on the science and requires the use of methods and criteria that are demonstrated to be predictive. New methods should be validated and demonstrated to enhance the safety assessment.

The framework to guide evaluation of potential safety issues requires detailed characteristics of:

- The GM plant and its use as food
- The source of the gene
- The inserted DNA and flanking DNA at the insertion site
- The expressed substances (e.g., proteins and any new metabolites that result from the new gene product)
- The potential toxicity and antinutritional properties of new proteins or metabolites
- The introduced protein compared with those known to cause celiac disease if the DNA is from wheat, barley, rye, oats or related grains
- The introduced protein for potential allergenicity
- Key endogenous nutrients and antinutrients including toxins and allergens for potential increases for specific host plants (DNA recipients)

Certain steps in the assessment require scientific assessment of existing information; others require experiments, in which case assay validation, sensitivity and auditable documentation are required.

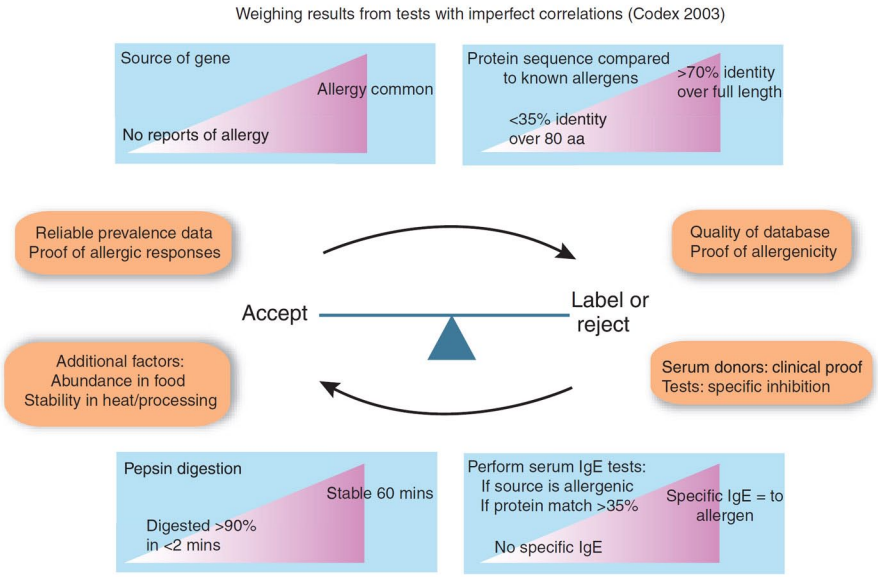

Figure 1. Schematic interpretation of the weight-of-evidence approach described by the Codex Alimentarius Commission Guidelines for Allergenicity Assessment in 2003 (ref. 1). In the figure, the four main areas of evidence are depicted with a graphic representation of the evidence representing maximum risk on the right (high side of the triangles). The weight of the evidence in each of the areas is influenced by the quality of the factors depicted in the yellow boxes. On the basis of the imperfect nature of the test methods available to distinguish between allergenic and nonallergenic proteins, scientific interpretation is necessary to reach a balanced and useful conclusion regarding the potential risks of allergy associated with each new food product.

Assessment protocols

Here, we look at the scientific soundness of the principles and protocols for allergenicity assessment and present some recent case studies to illustrate the inappropriateness of nonvalidated methods for allergenicity assessment, whether part of the FAO/WHO[5] recommendations or the Codex[6] guidelines. Figure 1 outlines the Codex guidance's weight-of-evidence approach to evaluate the potential risk of food allergy.

Gene source. The process begins with an evaluation of the source of the gene. If the source of the gene encoding the new protein is a commonly allergenic food (e.g., peanut, hazelnut, hen's egg or cow's milk), a respiratory allergen (e.g., birch or grass pollen or house dust mite) or a contact allergen (latex), IgE-binding studies using sera from patients allergic to the source are required to ensure that the protein encoded by the gene does not bind IgE from those allergic to the source. For serum selection, demographic factors need to be taken into account. Both age and habitat have been shown to influence the molecular recognition profiles of specific IgE (Box 2). The number of sera needed is dependent on the degree of confidence considered necessary (largely a political and socioeconomic issue) and the prevalence of recognition of the hypothetical allergen. In other words, do we accept a 5% chance of an allergic reaction in 1% of the population allergic to the source or do we want to be more protective and only accept a 1% chance of a reaction in, for example, 0.01% of that population? Choosing to lower the risk requires a higher number of sera.

If the source of the gene rarely causes allergies, it would be difficult or impossible to find enough qualified serum donors to perform statistically valid tests. However, that also means the number of individuals in the population who would be at immediate risk of reactions if the protein were an allergen would be small. In such cases, the number of individuals is not as important as the specificity of the test and evidence of clinical relevance of the allergenic source.

Bioinformatics. The amino acid sequence of all transferred proteins, regardless of the source, are to be compared with known allergens by FASTA or BLAST algorithms to determine if any identity match is sufficiently high to suspect that the protein might cause allergic cross-reactions. This is not meant to be a stand-alone test, but rather to identify proteins that would require serum testing, using donors with specific allergies to the source of the sequence-matched allergen to evaluate potential IgE binding. If the identity match is high (e.g., >70% over most of the length of the protein),

Box 2. Spaniards are different from Dutchmen

Exposure to allergen is an essential prerequisite for sensitization[47]. An exception to this rule is cross-reactivity: for example, exposure to birch pollen can induce allergy to apple, cherry and hazelnut[27, 48, 49]. This is typically seen in those areas of the world where birch pollen exposure is high, such as The Netherlands. In the absence of birch pollen, apple allergy also exists, for example, in Spain. In a recent European multicenter study, almost 400 people allergic to apple from four countries were compared to identify potential cross-reactive causes[48]. As expected Dutch, and others (Austrian and Northern Italian) individuals were allergic to apple because they were allergic to birch pollen. IgE binding the major birch pollen allergen Bet v 1 cross-reacted with the homologous major apple allergen Mal d 1. Symptoms induced by Mal d 1 were almost exclusively mild and restricted to the oral mucosa. Spanish participants had not been exposed to birch pollen and were shown to be sensitized to a non-pollen–related allergen identified as a lipid transfer protein (Mal d 3). Although the majority exclusively have mild symptoms in the oral cavity, it was demonstrated that IgE against lipid transfer protein is a significant risk factor for the development of severe systemic symptoms, as were observed in ~25% of the Spanish individuals[48]. This study clearly illustrates that the outcome of allergenicity assessment of GM crops using serum samples of patients with largely identical clinical symptoms upon consumption of apple is strongly influenced by the geographic origin of the patients. Spanish are simply different from Dutch apple-allergic patients due to differences in exposure or other local environmental factors; variations in genetics of these populations cannot account for the marked differences. In the former case, assessment will focus on non-pollen–related apple allergens; in the latter, on birch pollen–related allergens. Similar patterns have been reported for cherry allergy with Pru av 1, a homolog of Bet v 1, being the dominant allergen, compared with lipid transfer protein in cherry, peach and hazelnuts as the primary allergen in the Mediterranean areas[27, 49]. These studies highlight the need for good patient characterization and selection before the use of their sera in allergenicity assessment protocols, as differences in the prevalence of IgE sensitivity is possible in the same foods, in different populations.

Box 3. Short peptide match: a lot of work for nothing
Pioneer Hi-Bred International and Dow AgroSciences (Indianapolis, IN, USA) developed a GM maize product containing the gene encoding Cry1F, from *Bacillus thuringiensis*. The product was approved for sale in the United States and Canada following full regulatory studies, including assessment of the potential allergenicity of the protein based on Codex guidelines[1]. The protein produced from this gene is toxic to lepidopteran larval pests, such as the European corn borer, but not to mammals[16]. The gene is from an organism not known to cause allergies. The sequence is not significantly identical to any known allergen based on overall FASTA alignment. It is <35% identical to any 80-amino-acid segment of known allergens, which is the primary alignment criterion recommended by Codex[1]. Because of regulatory requests from Taiwan, an additional bioinformatics comparison was performed to identify any six-amino-acid matches with allergens. There was a single six-amino-acid match to the house dust mite allergen Der p 7 (ref. 16). The protein does not have any other alignment similarity to Der p 7, yet regulators from Taiwan required human allergic serum IgE testing to evaluate potential cross-reactivity. The results of the serum IgE test demonstrated a lack of IgE binding to Cry1F using sera from allergic subjects who had clear IgE binding to Der p 7 (ref. 16). The results satisfied the regulators and the product was approved. However, the tests were expensive and there is always a chance of obtaining a weak-positive IgE binding result. Even the slightest amount of binding would likely have led to extensive *in vivo* testing, but would have been unlikely to demonstrate a risk of an allergic response in consumers as at least two IgE binding sites and high affinity are required to effectively cross-link mast cells and trigger an allergic response (as discussed in reference 11).

the potential for cross-reactivity is high and the risk would probably be close to that posed by the matched allergen. Matches sharing between 50% and 70% overall pose a moderate risk of cross-reactivity and should be tested for IgE binding. If the match is <50% identical, the risk of cross-reactivity is expected to be low[8]. Even so, a conservative threshold value of 35% identity over any 80-amino-acid segment of the transferred protein contained in both the FAO/WHO[6] and Codex documents[1] was intended to identify conserved gene segments representing functional motifs, which might retain conformational epitope structure as well. Proteins with higher matching identities (e.g., >35% identity) are recommended for testing of IgE binding.

On the basis of literature searches, only a few examples of endogenous proteins from sources suspected of cross-reactivity demonstrate significant IgE cross-reactivity for proteins sharing between 35% and 50% identity over the entire length of both proteins, and quantitative IgE binding and basophil histamine release (an *ex vivo* test of the circulating effector cells triggered to release histamine by IgE cross-linking) demonstrate only partial reactivity[7]. The lack of known examples of cross-reactivity associated with proteins sharing only 35% identity over 80 amino acids suggests the criterion is too conservative as it would overpredict potential cross-reactivity. One alternative is to focus on overall sequence alignments, as suggested by Ladics *et al.*[9]. Another alternative would be to increase the percent identity for the 80-amino-acid window closer to a level (possibly >50% identity) where there are examples of at least weak *in vitro* cross-reactivity using sera from individuals having allergic symptoms to the sources of both proteins[10].

The bioinformatics step is relatively straightforward and should markedly reduce the risk of transferring even a minimally cross-reactive protein. However, some allergens that may be matched are rarely noted as causing allergies and it would be virtually impossible to identify appropriate serum donors for a well-powered study. In such cases, the risk of potential allergy to the population from that protein is likely to be extremely low and regulators may be willing to waive the requirement for IgE testing. Choosing the appropriate allergen database to search is vital for a reliable sequence comparison[10]. AllergenOnline (http://www.allergenonline.com) is the only database that is currently fully peer-reviewed regarding evaluation of published evidence of allergenicity. Other databases are available and the alternative of searching the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) nonredundant database, with key-word limits can provide more updated sequences, but lacks an accurate screening method for relevance[10].

Although there are not yet any publications reporting validation of the approach using 35% homology over an 80-amino-acid window (or >50% overall homology) to predict likely cross-reactivity, it is clear that it is an improvement over methods using sequence homology over 6 or 8 amino acids[11]. These short peptide matches have not been validated as predictive tools and should be rejected on the basis of extremely high numbers of false-positive hits[11, 12, 13, 14]. The eight-amino-acid match was originally selected without evidence of predictability based on the idea that it would represent both a theoretical B-cell epitope as well as a minimum size for a conserved T-cell epitope[5]. Stadler and Stadler[13] reported that a 6-mer match resulted in more than two-thirds of all proteins in Swiss-Prot being predicted to be allergens, and >40% of the human genome being predicted as such. Obviously, the use of short amino matching searches (6–8 mer) is not a useful approach for allergenicity assessment, but it has never been truly renounced. Consequently, a few regulatory authorities sometimes still require bioinformatics analyses based on 6-mer matches (Box 3).

Serum IgE binding. Serum IgE testing to evaluate proteins from an allergenic source, or proteins with sequence identity (e.g., >35% over an 80-amino-acid widow or >50% overall) to a known allergen works very well if performed properly[15]. Appropriate positive and negative control proteins or extracts of the allergenic source material are required to demonstrate assay validity. The positive test sera must be from clearly diagnosed allergic subjects who react to the gene source or sequence-matched allergen and its source. Negative control sera would typically include donors with allergies to other unrelated proteins as well as nonallergic subjects. A few relatively well-controlled studies have been used to evaluate GM crop safety[4, 15, 16], although the relevance of donor selection has not always been clear[15].

The design and interpretation of assays for specific IgE can be complex. Potential confounding factors include the molecular appearance of the protein (e.g., monomeric versus multimeric, proper folding or misfolding, presence or absence of disulfide bonds, presence or absence of N-linked glycans) and abundance of the protein in the source material (that is, sensitivity). The test material must be representative of the form available in the GM food source. The tests should be capable of detecting IgE binding to linear and conformational epitopes, sometimes requiring two separate assays (e.g., under reducing and native conditions). Demonstration of specificity of binding requires replicate samples with sera exposed to appropriate inhibitors.

Moreover, it must be recognized that there are no absolute thresholds of serum IgE binding that provide absolute measurement of safety or risk. Positive IgE tests without clinical relevance

are common in clinical practice (e.g., due to the presence of cross-reactive IgE to plant N-glycans). To avoid potentially confounding test results, developers may want to remove glycosylation sites before introducing the new gene unless the glycan is needed for functionality. Serum from individuals with strong carbohydrate-specific IgE antibodies should be avoided for GM assessment to ensure selection of appropriate donors who should have IgE directed against peptide epitopes rather than carbohydrate. Otherwise, carbohydrate-binding sera would lead to designating most glycoproteins as an allergenic risk, although it is widely accepted that the glycans are unlikely to cause clinical food allergy[17,18]. In the event the transgenic protein is glycosylated, alternative testing may be required to evaluate glycan structure or if IgE binding is demonstrated, the relevance should be tested by basophil histamine release or *in vivo* allergen testing. Diagnosing allergic disease requires a holistic evaluation of diet, symptoms, SPT and/or specific IgE and elimination diet or challenge test[19]. Likewise, interpretation of IgE binding to GM proteins requires judgment. Strong, specific binding to the protein using appropriate donors should be taken as evidence of risk. However, low levels of binding that are not clearly specific and close in affinity to the suspected cross-reactive allergen may not indicate significant risk. If results are equivocal, SPT or challenges might be necessary to demonstrate the relevance of low levels of apparent specific IgE binding.

Stability in pepsin and abundance. The ability of the new protein to withstand digestion by pepsin is evaluated as a potential risk factor of allergenicity[20,21]. Several potent food allergens are known to be very stable in an *in vitro* pepsin digestion assay, whereas it is thought that most dietary proteins are readily digestible[22]. However, some proteins not known to cause significant food allergies are also stable[23]. And some proteins known to cause food allergy, especially those inducing only oral allergy syndrome—mild tingling or itch in the mouth, without substantial edema—are relatively labile[24]. Thus far, food allergens from this last category are mainly found among cross-reactive allergens, where primary sensitization occurs by inhalation (e.g., pollen or latex). These are therefore usually not designated to be 'true' food allergens[25]. Such proteins are likely to pose little risk to consumers if expressed at low abundance in crops.

Some very stable proteins such as thaumatin-like proteins from apple and grape rarely cause allergy or possibly only mild reactions[26], whereas others, like the lipid transfer proteins from a variety of sources, are very stable and may frequently cause severe reactions[26,27]. Some of these stable proteins are inducible pathogenesis-related proteins and expression is variable in foods, which may complicate their recognition as allergens[28]. There is also evidence that some important pepsin-labile allergens become more stable with minor shifts in pH (e.g., from pH 2.5 to 2.75 for codfish parvalbumin)[29]. Although the increased stability at moderate stomach pH values may help explain the allergenicity of some of these proteins, the use of standard pepsin stability testing at pH 1.2 or 2.0 still has a good demonstrated predictive value[30].

An additional risk factor for food allergy is the abundance of the protein in food, as many major food allergens account for >1% of the protein in high-protein allergenic foods[20]. Others, such as lipid transfer proteins and parvalbumins are less abundant. Abundant, pepsin-stable proteins are more likely to survive digestion in sufficient quantities to facilitate sensitization and become significant food allergens. The typical quantity consumed of specific foods would be expected to have an impact as well, so nonabundant, stable proteins may be potent allergens if a large amount of food is consumed. However, additional scientific data would be required to establish completely objective criteria for acceptance or concern based on stability and abundance. Currently the results are judged relative to common, potent food allergens.

Box 4. Mission impossible: evaluation of changes in endogenous 'hypo-allergenicity'

A transgenic herbicide-tolerant rice, Liberty Link-rice (LLRICE62), was produced by Aventis CropScience (now Bayer CropScience, LP, Research Triangle Park, NC, USA), by inserting the gene for phosphinothricin-*N*-acetyltransferase (PAT) from a bacteria that has not been reported to be allergenic, nor does it share significant sequence identity with any known allergens. The nonglycosylated PAT protein is rapidly digested by pepsin under standard conditions[50]. On the basis of these characteristics, there is no need to test IgE binding to evaluate the potential allergenicity of the PAT protein. US regulators approved the product in 1999 (http://www.agbios.com/dbase.php). However, because rice has been reported (rarely) to cause allergic reactions in humans, the developer performed an *in vitro* IgE binding study of LLRICE62 to compare endogenous allergenicity to a nontransgenic cultivar after their interpretation of the IFBC-ILSI recommendations and based on historical questions from regulatory agencies (pre-1999). Because true (challenge-positive) rice-allergic individuals cannot easily be found, sera of food-allergic subjects with rice-specific serum IgE or skin test–positive reactions to rice extract, or individuals with clinical histories suggestive of rice allergy were used. However, rice-food allergy was not confirmed by food challenge. These individuals were probably sensitized to grass pollen or inhaled rice flour and may be unaffected when ingesting rice based on a paucity of published cases of proven rice allergy and our experiences[51,52].

The unpublished study (personal communication, Donna Mitten, Bayer CropScience, data reviewed by R.E.G.) revealed no significant differences in IgE binding and allergen content between the GM and a genetically similar traditional rice variety. The value of a study based on sera of patients with unconfirmed rice allergy is questionable. Regardless, Canadian authorities approved LLRICE62 in 2006 having been satisfied with the assessment of potential allergenicity that included an evaluation consistent with current guidelines in addition to the results of the serum study (http://www.hc-sc.gc.ca/fn-an/gmf-agm/appro/nf-an90decdoc_e.html). It can be argued that it is not justified to evaluate GM crops for potential changes in endogenous allergenicity for a food with extremely low allergenicity because results will generally be meaningless.

However, the story does not end here. Recently, a small amount of seed from a similar GM event (LLRICE601) was discovered in a commercial 'nontransgenic' rice variety. In order to quickly respond to regulatory requests for information about studies of LLRICE601 safety, Bayer CropScience considered the earlier Canadian request for LLRICE62 and decided to perform a similar study if feasible. However, the original sera used for the previous study were no longer available (personal communication, Donna Mitten). Communications with clinical allergists (including M.E., D.H., H.A.S.) in Australia, Japan, Korea, Taiwan and the United States (by R.E.G) indicated the extreme difficulty in obtaining serum donors with clinically defined allergy to rice (as food), and the study was not performed. But, because people who are allergic to rice should avoid eating it, and so few are allergic, it is not clear that there would be any value in performing such a study.

GM crop allergenicity assessment—what is not (yet) useful?

As the assessment of the allergenicity of GM crops has evolved, scientific evaluation of some tests and criteria included in earlier guidance has demonstrated that some specific approaches are not (yet) particularly useful (e.g., six- to eight-amino-acid matches, targeted serum tests). Additionally, some new approaches have been espoused that are not sufficiently validated in terms of predicting allergenicity for use in regulatory decision making, although in some cases continued research may be warranted.

Endogenous allergenicity. If a transgene is transferred to a commonly allergenic food, it appears logical to monitor the influence on endogenous allergenicity, which was recommended by the various guidelines on GM crop allergenicity assessment[1,5,6]. These documents, however, have not addressed the level of change that would be (un)acceptable. Several studies have been carried out comparing endogenous allergenicity of nontransgenic and GM varieties. Monsanto (St. Louis, MO, USA) performed an evaluation of herbicide-tolerant soybeans using sera from soy-allergic subjects[31], and also of a potential herbicide-tolerant GM wheat product using sera from ten subjects allergic to wheat. In a comparison of IgE binding, neither study demonstrated any significant differences between the GM crop and non-GM controls (R.E.G., poster presentation, World Allergy Organization meeting, Vancouver, BC, 2003). A study by Lehrer and Reese[32], commissioned by Pioneer Hi-bred International, compared conventional and GM high–oleic acid soybeans using sera from five individuals selected for high IgE binding to soybean extract. A radioallergosorbent-inhibition (RAST-inhibition) assay demonstrated similar IgE binding results between the GM and non-GM varieties. However, what is the risk and what should be done if statistically significant differences are detected?

Serum IgE binding and histamine release were tested in a comparison of ten varieties of Roundup Ready soybean (GM) developed by Monsanto and eight cultivars of non-GM soybean[15]. IgE-inhibition tests demonstrated up to fourfold differences in IgE-binding potencies across both the GM and non-GM varieties, but overall the GM and non-GM varieties were not significantly different. That study illustrated that a head-to-head comparison of a pair of randomly selected soybean varieties may lead to statistically significant differences, even though the apparent allergenicity of the individual varieties falls within the range of responses to several commercially available non-GM soybeans. Apart from the fact that serum samples used in this study originated from subjects that were negative to soy by food challenge (or were not challenged), the variable IgE binding results clearly highlight an aspect that should be taken into account when evaluating effects on endogenous allergenicity: natural variation of allergenicity of available food crops due to differences in the genetics of commercial varieties, and interactions with the environment (e.g., nutrient availability, differences in moisture, temperature, plant pathogens). It is unreasonable to be more stringent toward GM crops with respect to changes in endogenous allergenicity than can already be accounted for by natural variability. Basing judgment on statistical significance alone has no clinical meaning if natural variability is larger. Importantly, the whole discussion about endogenous allergenicity has limited relevance because patients allergic to the food will (should) avoid eating it anyway, GM or not, to avoid allergic reactions.

The soy study results[15] suggest that there is wide variation in IgE binding to different varieties of the same species of non-GM crops, but few studies have been performed to study the question in a systematic way. Various groups have addressed differences in allergenicity between non-GM apple cultivars, focusing on two major apple allergens, the birch pollen–related allergen Mal d 1 and a lipid transfer protein, Mal d 3. Differences in allergenicity have been found by IgE-binding and IgE-inhibition studies, immunoassays for quantifying allergens, *in vitro* basophil histamine release and genomic sequence variability, but also by SPT and DBPCFC as illustrated below.

Sequence variability, possibly translating into differences in allergenicity, has been recently reported for both Mal d 1 and Mal d 3 in different apple cultivars[33]. Most studies focusing on differences in allergenicity of apple cultivars have used IgE-based binding (*in vitro* and *in vivo*) as an endpoint. A recent study has evaluated IgE binding and SPT reactivity as well as measuring Mal d 3 content, comparing ten cultivars of apples[34]. The Mal d 3 content varied more than sixfold on a dry material basis across cultivars. The mean wheal area resulting from SPT of the highest Mal d 3 con-

Box 5. A controversial nonvalidated animal model

A gene encoding an *α*-amylase inhibitor 1 (*α*AI) was transferred from kidney bean to field peas to make peas resistant to a bruchid storage beetle[53]. Because of the recommendation for animal model tests by the FAO/WHO[6], the developer tested the product in a mouse model using repetitive intragastric sensitization followed by intratracheal challenge[54]. This model had not previously been used to predict allergenicity of food proteins and we are aware of no other studies that have used an airway challenge or measure of pulmonary cellular infiltration to evaluate food allergenicity. The test results demonstrated stronger eosinophil accumulation in the lungs in mice sensitized and challenged with the GM pea (or *α*AI from the pea), compared with the kidney bean[54]. This supports increased Th2 inflammation, but not necessarily IgE-mediated allergy. The report described structural differences of the *N*-linked glycan on *α*AI expressed in peas compared to kidney bean. There was also evidence of different proteolytic processing of the C terminus of the protein. The authors concluded that differences in post-translational proteolytic processing were responsible for the apparent enhanced immunogenicity of the GM product[53].

The mechanism leading to the altered response in mice is not clear, but more importantly, the model has not been widely tested with allergenic and nonallergenic proteins as would seem necessary based on Codex guidelines[1]. In the case of the GM ∝AI pea, the differences found in glycan structure and protein processing would have been more appropriately investigated by human serum testing to evaluate IgE binding using serum donors with allergies to legumes if regulators wished to have testing beyond the bioinformatics, pepsin digestion and characterization of the protein.

Despite the fact that no scientific evidence was provided for an increased risk of IgE-mediated food allergy in humans, the study aroused a storm of negative publicity for GM crops, being an allergy risk[55]. Although the developer did not report results of a bioinformatics evaluation of the protein, in our hands a FASTA search of AllergonOnline (http://www.AallergenoOnline.com/), version 7.0, revealed one match of 41% identity over an 80-amino-acid segment to peanut agglutinin precursor, a putative allergen. The overall identity was 34.5%. Although this low level match is not likely to indicate cross-reactivity, it is above the Codex criterion. The data suggesting that peanut agglutinin is an allergen should be evaluated in making a final decision on whether to perform human serum-IgE testing, before any regulatory decision to approve the GM crop. In any event, data from a mouse model should not be relied upon to predict allergenicity.

Box 6. Box 6 Balb/c mice no substitute for human IgE recognition evaluation

A gene encoding amarantin was transferred from *Amaranthus hypochondriacus* into maize[56]. Although the protein was digested in the pepsin assay, comparing the sequence to known allergens identified a number of 6-, 7- and 8-amino-acid matches to known allergens[56]. Although noting overall homology to some allergenic proteins, the developer decided to use animal models to evaluate the allergenicity of the GM maize[56]. Comparing the amarantin sequence by FASTA demonstrated up to 70% identity over an 80-amino-acid segment to known allergens and >40% identity for overall alignments to a number of important 11S globulin allergens[7]. Clearly, this should have set off an alarm calling for serum IgE testing, if not immediately convincing the developer that the protein was too risky to transfer. Instead, the immunogenicity of the product was tested in BALB/c mice, with results demonstrating no significant response and the authors suggested there was no significant risk of allergy[56]. Although it is not clear if this potential product has been submitted for regulatory review anywhere, the Codex guidelines (2003) indicate that the amarantin-containing maize would require serum IgE testing with sera from at least a number of buckwheat- and/or Brazil nut–allergic subjects and possibly others.

tent apple variety (~55 mg/g) was significantly higher (~threefold) than the mean wheal area for the two varieties with lower concentration (~10 mg/g) of Mal d 3. One may infer from the SPT results and Mal d 3 quantities that the cultivar with the highest levels of the allergen (Starking) is probably three times more allergenic on a gram basis than those with lower levels (e.g., Golden Delicious). Similar studies[35] were carried out with 88 apple cultivars focusing on both Mal d 1 and Mal d 3, although not all the results are published (R.v.R., unpublished data). In both cases, differences in allergen content differed up to 100-fold between the extremes, both in allergen quantification and IgE-inhibition assays. Some of these differences had been observed in SPT and DBPCFC testing, with about tenfold differences between individual cultivars. These detailed studies demonstrate the wide range of natural variability of allergenicity in a common non-GM food.

Similar tests of soybean varieties by *in vivo* skin reactivity and *in vitro* IgE binding of ten soy cultivars found up to sixfold differences in IgE-binding potencies[36]. Apart from differences between cultivars, natural variability in allergenicity can also occur due to harvest timing and storage conditions[37, 38]. Even between individual apples from a single cultivar and harvest, up to tenfold differences in allergenicity have been reported[39]. Yet clinicians and food safety experts do not recommend avoiding certain apple or soybean varieties, nor is there evidence of significant differences in clinical reactivity for the allergic consumer.

Overall, these studies demonstrate the need to establish natural variability of allergenicity of non-GM crops before demanding evaluation of changes in endogenous allergenicity of GM crops. Nevertheless, some regulatory authorities have interpreted the guidelines so broadly that they demand evaluation of changes in endogenous allergenicity of foods for which it is virtually impossible to find sufficient truly allergic patients for a well-powered study (Box 4).

Of course, in cases where there are specific reasons to suspect a major impact on expression levels of endogenous allergens, special attention has to be given to evaluating allergenicity. This can, for example, be the case when a transcriptional activator is inserted or the transgene is inserted in the coding region for an allergen. Such events should however, not go unnoticed by the developer of a GM crop as detailed molecular characterization of the insert and the protein as well as protein function are required by Codex[1] (Box 1).

Targeted serum screens. The FAO/WHO[6] recommendation for broadly targeted serum screens specifically stated that if the source of the transferred gene was a monocotyledonous plant (class Liliopsida), serum should be taken from 50 individuals with allergies to diverse monocot sources (e.g., some allergic to grass pollen, maize, rice or dates) to identify potentially cross-reactive allergens. However, in the Codex guidelines, this was recognized as unlikely to be predictive[1]. There are four or five structural protein families (prolamins, Bet v 1-relatives, cupins and profilins) with representative clinically cross-reactive allergens from taxonomically diverse sources[40]. Although a few individuals react to material from sources as diverse as representatives of an order (e.g., Fabales) or even higher group, most clinically important cross-reactions are elicited by material from within the taxonomic family (e.g., Fabaceae), tribe (e.g., Phaseoleae) or, more commonly, genus (e.g., *Phaseolus*)[41]. Differentiating between clinical cross-reactivity, cosensitization and irrelevant IgE binding (low affinity or binding to cross-reactive carbohydrate determinants) is often complicated as clinical reactivity is rarely measured, rather some level of skin prick sensitivity or direct *in vitro* IgE binding is used to define cross-reactivity and this is likely to overestimate clinical reactivity[10, 42, 43]. Although validated specific serum tests with samples from clinically well-characterized subjects allergic to the source of the gene—or allergic to a sequence-matched allergen—should be useful when the need is indicated, targeted testing is unlikely to provide reliable data for the assessment.

Animal models. The FAO/WHO[6] recommendations called for evaluating each new GM crop with studies in two separate species of animals and/or using two routes of sensitization in one species, even though the panel recognized that no current animal model is predictive of allergenicity in humans. There are still no validated animal models for predicting allergenicity to food proteins, even though many models have been successfully applied to dissect mechanisms of allergic responses and potential changes due to modification of the allergenic proteins[44, 45]. Even though many authors recognize that different animal models respond to specific proteins differently[46], they still suggest using animal models in the safety evaluation process for GM crops. On the basis of the paucity of correlative data between any one animal model and human food allergenicity, and the complex genetic diversity that predisposes subjects to allergy, it is not clear that any animal model could be useful in predicting the potential allergenicity in humans of a novel protein or GM crop. It is also not clear how one might combine results from two animal model tests to produce a predictive result. An unpublished study coordinated by the ILSI-Health and Environmental Sciences Institute (Washington, DC) reported results from a multi-laboratory test of the most commonly used mouse strains (BALB/c, C3H/HeJ, A/J and BDF-1) using commonly recommended protocols to evaluate IgE and allergic responses to identical samples of common potent allergens of peanut (Ara h 1 and Ara h 2) and milk (beta-lactoglobulin) compared with relatively nonallergenic proteins of spinach (RUBISCO) and soybean (lipoxygenase). The responses to the potent allergens were equivalent or weaker than responses to the weakly or nonallergenic proteins (Thomas, K. *et al.*, 2005 annual meeting poster, American Academy of Allergy Asthma and Clinical Immunology).

On the basis of current knowledge, therefore, we recommend continuing research to evaluate potentially predictive animal models but caution against testing potential products at this time as there is no scientific validation demonstrating predictive values that are acceptable for risk evaluation. This opinion is also reflected in the recommendations of the Codex Alimentarius Commission[1]. Producers as well as regulators are sometimes confused about which recommendations to follow. This is illustrated by two cases in which developers of GM crops used animal models to evaluate potential allergenicity. In one, case results were interpreted as demonstrating likely allergenicity of the GM crop (Box 5) and in another case to suggest absence of allergenicity, even though there is a strong potential for cross-reactivity based on bioinformatics (Box 6). In either case, there is no scientific justification for these conclusions.

Conclusions

The current safety assessment outlined in the Codex guidelines (2003)[1] is based on the current state of knowledge regarding food allergens and risk, and is therefore well-suited to evaluate the potential for increased risk in allergenicity of GM crops compared with the risk of allergy from the conventionally bred crop varieties. The weight-of-evidence approach was adopted in part as it was recognized that there are exceptions to each component in the process. Thus, each product must be reviewed on a case-by-case basis and experienced scientists must be able to interpret results in aggregate. Key elements of this weight-of-evidence assessment are illustrated in Figure 1:

- Source of the gene: common allergen or not?
- Bioinformatics: sequence searches for matches of >35% identity over 80 amino acids (or of >50% overall identity for more realistic risks).
- IgE-testing: does the introduced protein bind IgE-antibodies?
- Stability testing: is the expressed protein highly resistant to digestion by pepsin?
- Abundance: is the protein abundant in the food (and stable)?

The premarket assessment recommended by Codex provides a mechanism to intercept GM crops that are likely to increase the risk of food allergy, as demonstrated by the identification of the Brazil nut 2S albumin transferred to maize, and the amarantin transferred to maize (Box 6) as proteins that would likely present significant health risks for specific populations of allergic consumers. The premarket screening process helps to avoid possible severe reactions in unsuspecting allergic consumers and also prevents subsequent costly food and seed recalls that would be needed to prevent additional reactions.

There is no scientific justification for inclusion of the following tests in allergenicity assessment because their predictive values have not been validated:

- Bioinformatics: short-peptide matches resulting in random false-positive hits.
- Animal models: useful for mechanistic studies but not applicable for prediction of human sensitization to food.
- Endogenous allergenicity: natural variability needs to be taken into account first.
- Targeted serum screens: potentially high rate of false-positive and low probability of true-positive results.

Demanding inclusion of such nonvalidated tests can lead to the rejection of safe and beneficial products, excessive costs and, potentially, disruption of trade without any further reduction of risk. Importantly, the use of inappropriate tests such as unvalidated animal models in place of more appropriate tests could lead to the introduction of a product that does pose substantial risk for a group of allergic consumers.

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