

Allergenicity of Foods Derived from Transgenic Plants

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Introduction

Direct and stable gene transfer into plants was first reported in 1984 [1, 2]. Since then, many economically important crops have been genetically engineered, usually via *Agrobacterium* or particle gun technologies [3]. The variety of traits being introduced into crops is impressive, and includes insect protection, delayed ripening, virus resistance, modified starch, herbicide tolerance, modified oils, disease resistance and male sterility [4]. Most traits introduced into crops result from the expression of one or more new proteins. Typically these proteins are expressed at low levels and represent a minor percentage of the total plant protein. Several transgenic plant products have been commercialized and many others will arrive in the marketplace in the near future (table 1) [5]. Prior to market introduction, each of these products is subjected to extensive food, feed and environmental safety assessments. We present the current approach to assess the allergenic potential of foods derived from transgenic plants.

Plants tissues can express at least 100,000 discrete proteins. Yet, despite this large variety, allergy to food proteins is uncommon – affecting perhaps 5% of infants and up to 2% of adults [6]. Where food allergy is indicated clinically, patients usually are allergic to only one or two taxonomically related foods. Nine foods or food groups (peanuts, soybeans, tree nuts, milk, eggs, fish, crustacea, mollusks and wheat) account for over 90% of food allergies [7]. The majority of allergy sufferers exhibit immunoglobulin E (IgE)-mediated immediate hypersensitivity [8].

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Table 1. Transgenic plants in or nearing the marketplace

Genetic trait	Crop	Primary introduced gene	Company
Delayed ripening fruit	Tomato	Antisense PG	Calgene
	Tomato	ACC deaminase	Monsanto
	Tomato	Antisense PG	Zeneca
	Tomato	Antisense ACC synthase	DNA Plant Tech.
Insect resistance	Potato	<i>B.t.t.</i> [<i>cryIIIA</i>]	Monsanto
	Cotton	<i>B.t.k.</i> [<i>cryIA(c)</i>]	Monsanto
	Corn	<i>B.t.k.</i> [<i>cryIA(b)</i>]	Monsanto
	Corn	<i>B.t.k.</i> [<i>cryIA(b)</i>]	Ciba-Geigy/Mycogen/ Northrup King
Virus resistance	Squash	Viral coat proteins	Asgrow Seed
	Tobacco	Viral coat protein	China
Herbicide tolerance	Cotton	BXN TM nitrilase	Calgene
	Cotton	CP4 EPSPS	Monsanto
	Soybean	CP4 EPSPS	Monsanto
	Flax	Acetolactate synthase	Univ. Saskatchewan
	Corn	Basta tolerance	AgroEvo
	Canola	Basta tolerance	AgroEvo
	Canola	CP4 EPSPS/GOX	Monsanto
Male sterility	Canola	Barnase/Barstar	Plant Genetic Systems
Oil quality modification	Canola	Thioesterase	Calgene

ACC = 1-Aminocyclopropane-1-carboxylic acid; *B.t.t.* = *Bacillus thuringiensis* subsp. *tenbrionis*; *B.t.k.* = *Bacillus thuringiensis* subsp. *kurstaki*; BXN = bromoxonyl; CP4 EPSPS = 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4; PG = polygalacturonase.

Assessment of the allergenic potential of foods derived from new plant varieties developed through genetic engineering should focus on three components: (1) the source from which the gene was obtained; (2) physicochemical and biological comparisons of the introduced protein with commonly allergenic proteins combined with previous consumption information, and (3) potential changes in the endogenous allergens of the host plant (if applicable).

A key physicochemical factor likely to be important to food allergenicity is stability of proteinaceous allergens to digestion by the gastrointestinal tract. We provide evidence that stability of allergens relative to other food proteins in a simulated gastrointestinal digestion model is a valid tool suitable for use in an

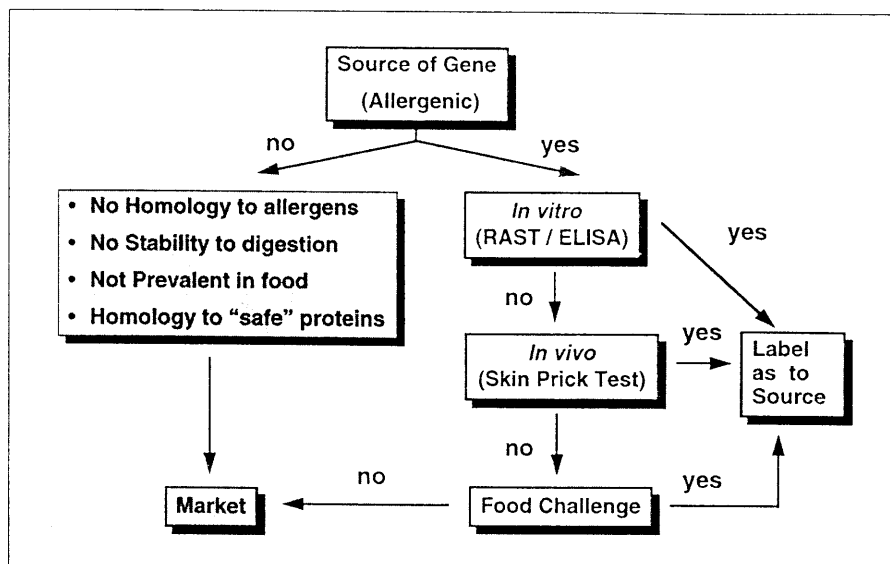


Fig. 1. Allergenicity assessment of foods derived from transgenic plants.

allergenicity assessment strategy. Moreover, we consider this parameter to be a key component in a rational assessment strategy employing careful stepwise decisions. The totality of these assessments provides assurance that foods derived from transgenic plants do not introduce allergenic concerns beyond those present in the current food supply.

Allergenic Sources of Genes

If a gene transferred into a food crop is obtained from a source known to be commonly allergenic, data should be generated to assure that the gene does not encode an allergen. The US Food and Drug Administration (FDA) recognized this need [9]. This part of the assessment (fig. 1, right side) should be a decision tree approach where each subsequent level of evaluation represents a greater degree of certainty that the source protein is *not* an allergen.

The first level in the decision tree employs *in vitro* assays such as radioallergosorbent tests (RASTs) [10], enzyme-linked immunosorbent assays (ELISA) [11] or immunoblotting assays [12] to detect the presence of an allergen(s) in the transgenic plant or as a purified gene product. These assays should use IgE fractions of pooled serum from individuals that are actively sensitive (allergic) to the

food from which the transferred gene was derived. Serum donors should meet clinically relevant criteria which includes a convincing history and/or positive responses in double-blind, placebo-controlled, food challenges (DBPCFC) [10, 13]. Data from one or more of these in vitro assays provides strong evidence of allergenicity of a given gene product. A clear positive result from the in vitro tests would require that any food including the introduced gene be labeled as containing a gene from that source. This is also the recommendation of the FDA [9].

If no differential response is observed during in vitro testing, experiments proceed to in vivo skin prick testing [10]. A positive result from this in vivo test would raise the same concerns as the in vitro tests above.

If the protein does not illicit a positive response in either of the above tests, a final evaluation could involve performing DBPCFCs with sensitive and non-sensitive patients under controlled clinical conditions. Patients who are known to be allergic to proteins from the source organism would be tested directly for hypersensitivity to food containing the protein encoded by the gene from the allergenic source. Food from nontransgenic lines would be used as a negative control. The ethical considerations for this type of assessment would include, but not be limited to, factors such as likelihood of inducing anaphylactic shock in test subjects, availability of appropriate safety precautions, and approval of local institutional review boards. Finally, if there were a reaction by sensitive patients, food derived from crops containing the protein would require labeling. If no adverse reactions were observed in this decision tree testing, it would be concluded that the gene obtained from the allergenic source did not encode one of the endogenous allergens and the product could be marketed without labeling.

An example illustrating the effectiveness of this approach was the demonstration that a gene encoding a Brazil nut 2S albumin storage protein which had been engineered into soybean [14] and canola [15] encodes a major allergen. The Brazil nut 2S albumin storage protein contains 18.8% of the essential amino acid methionine [16] and was perceived to be an ideal candidate gene for enhancement of nutritional seed quality [17]. However, Brazil nut is known to cause anaphylactic reactions [18, 19] and would be described as an allergic source of a gene. The Brazil nut 2S protein expression in soybean represented a significant fraction of total soybean seed protein. Subsequent RAST and immunoblot assays established that sera from 8 of 9 Brazil nut-allergic patients reacted with transgenic soybeans containing the Brazil nut gene [20, 21]. If marketed, this product would have to be labeled as containing a protein from Brazil nut to alert Brazil nut-allergic individuals. This example demonstrates the value and effectiveness of using in vitro assays to identify the accidental transfer of known allergens by genetic engineering.

In cases where suitable immunological reagents are not available for in vitro or in vivo testing, then the introduced protein should be assessed in the same manner as proteins from nonallergenic sources.

Nonallergenic Sources of Genes

There appears to be no single, predictive assay to assess the allergenic potential of proteins from nonallergenic food sources [9, 22]. However, comparing the important physicochemical and biological properties of an introduced protein with known allergenic proteins provides the best scientific basis for an assessment (fig. 1, left side). The physicochemical characteristics of common food allergens have been described [7, 23, 24]. Briefly, food allergens are typically stable to proteolysis, may be glycosylated, tend to be abundant, and tend to be resistant to food processing.

There are two approaches to address the allergenicity of a protein prospectively. First, when a candidate gene is being considered for transfer into a plant, the gene is usually well characterized – the DNA and amino acid sequences are known. As a starting point, a search for amino acid sequence homology of the introduced protein with known allergens can be performed.

Second, an analysis of the physicochemical properties of the introduced protein is informative. A key prerequisite for food protein allergenicity appears to be stability to the proteolytic and acid conditions of the human digestive system. The high concentrations of allergens in foods that cause allergy and the stability of allergens to processing into specific foods products are additional important factors contributing to the allergenicity of a protein. For example, soybean and peanut allergens retain their allergenic potential after the processing steps used in preparation of food products such as peanut butter [7].

Amino Acid Sequence Similarity to Known Allergenic Proteins

The amino acid sequences of many allergens, including food allergens, have been reported [25], and the list is expanding with time. Many important T-cell, B-cell and IgE-binding epitopes of allergens have been mapped [26–37] but the distinction between allergenic and nonallergenic epitopes is obscure [38]. The optimal peptide length for binding is between 8 and 12 amino acids [39]. T-cell epitopes of allergenic proteins and peptide fragments appear to be at least 8 amino acids in length [39]. Exact conservation of epitope sequences is observed in homologous allergens of disparate species [34, 40]. Indeed, conservative substitutions introduced by site-directed mutagenesis can reduce epitope efficacy [41].

Based on this information, an immunologically relevant sequence comparison test for similarity between the amino acid sequence of the introduced protein and known allergens has been defined: a significant sequence similarity requires a match of at least 8 contiguous identical amino acids. In the absence of specific epitope information, this test determines whether there is a theoretical possibility that the introduced protein shares an epitope with a known allergen. Thus, two issues are addressed in a sequence comparison test: (1) whether the introduced gene encodes a known allergen or an evolutionary related (homologous) protein and (2) whether the introduced gene could encode an immunologically relevant epitope. Additional analysis is required if a positive result occurs in either case.

We have searched the amino acid sequences of allergens present in public domain genetic databases (GenBank, EMBL, PIR, and SwissProt) for similarity to sequences of each of the proteins expressed in transgenic plants shown in tables 2 and 3 using the FASTA [42] computer program. With the test criteria above, no homology and no immunologically relevant sequence similarity was observed with allergens. We conclude (1) that the genes introduced into these foods do not encode known allergens or their homologues, and (2) that none of the introduced proteins share linear epitopes with known allergens.

Stability to Digestion

The ability of food allergens to reach and to cross the mucosal membrane of the intestine are likely prerequisites to allergenicity. Clearly, a protein which is stable to the proteolytic and acidic conditions of the digestive tract has an increased probability of reaching the intestinal mucosa. Intact proteins are capable of crossing the mucosal membrane of the gut and of entering the circulatory system [43]. Many allergens exhibit proteolytic stability [7, 23, 24, 44–46], although the majority remain untested directly. Thus, physicochemical properties which favor digestive stability can be used as an important indicator of allergenic potential.

We have used simulated gastric and intestinal models of mammalian digestion [47] to systematically compare the relative digestive stability of a number of common food allergens to proteins engineered into plants. These digestion models have been used to investigate the digestibility of plant proteins [48, 49], animal proteins [50] and food additives [51]. A similar model has also been used to examine the stability of milk allergens [52, 53].

The gastric digestion model consisted of an incubation of a test protein in a solution of pepsin at acid pH, and was referred to as simulated gastric fluid. The model of gastric protein digestion was initially characterized by evaluating the digestibility of two proteins, spinach leaf ribulose bis-phosphate carboxylase/oxygenase (Rubisco) and soybean Kunitz trypsin inhibitor (SKTI) under a set of standard conditions. As assessed by SDS-PAGE and Coomassie Brilliant Blue

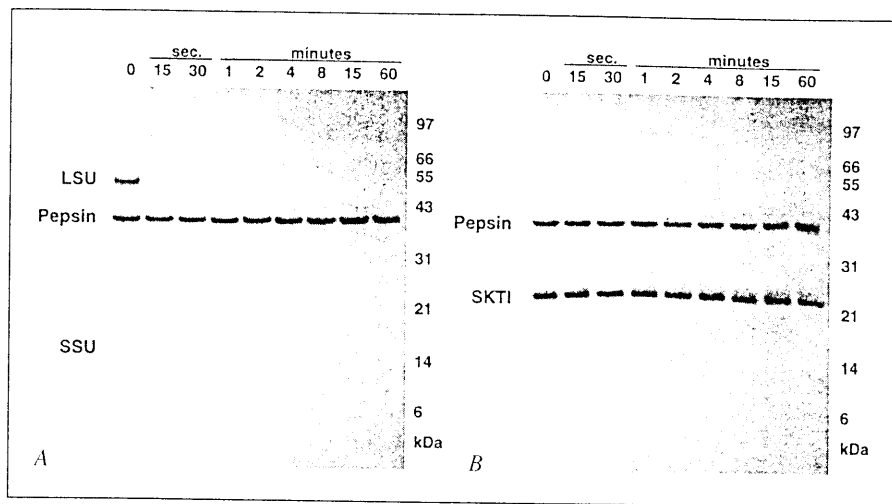


Fig. 2. Characterization of a gastric model for protein digestion. Gastric digests were performed essentially as described previously [57] with the following modifications: 170 ng/ μ l of protein was digested in 200- μ l aliquots of simulated gastric fluid composed of 0.3% (w/v) pepsin, 0.03 M NaCl, pH 1.2. Digests were quenched by neutralization with 75 μ l 0.16 M Na_2CO_3 at the following times: 0, 15 and 30 sec, and 1, 2, 4, 8, 15 and 60 min. Digestion of proteins was evaluated by SDS-PAGE (10–20% acrylamide with tricine buffers [66]) and visualized by Coomassie Brilliant Blue colloidal staining [67]. Incubation times are indicated above, protein labels are on the left and molecular weight markers are on the right. 500 ng of protein was loaded in each lane. SDS PAGE (A) of digestion of Rubisco large subunit (LSU) and small subunit (SSU). SDS-PAGE (B) of digestion of soybean Kunitz trypsin inhibitor (SKTI).

colloidal staining, Rubisco was digested completely within 15 sec whereas SKTI was stable for 60 min (fig. 2). To establish whether pepsin activity was limiting and whether pepsin activity could be saturated by the test protein, pepsin concentration was varied. A wide window of pepsin concentrations yields similar results. The range of test protein concentration was determined to be between 1 and 200 ng/ml of simulated gastric fluid.

To test whether a complex protein mixture effects the digestion of a specific protein, a soybean flour extract was prepared and introduced into the gastric model. Then, the digestion of β -conglycinin, a major protein component of soy flour extract, was monitored by immunoblot and compared to the digestion of purified β -conglycinin alone. The α -subunit of β -conglycinin was stable for 2 min whether present in a soy flour mixture or digested alone (fig. 3). The β -subunit was stable for > 60 min in both cases. This experiment suggests that a com-

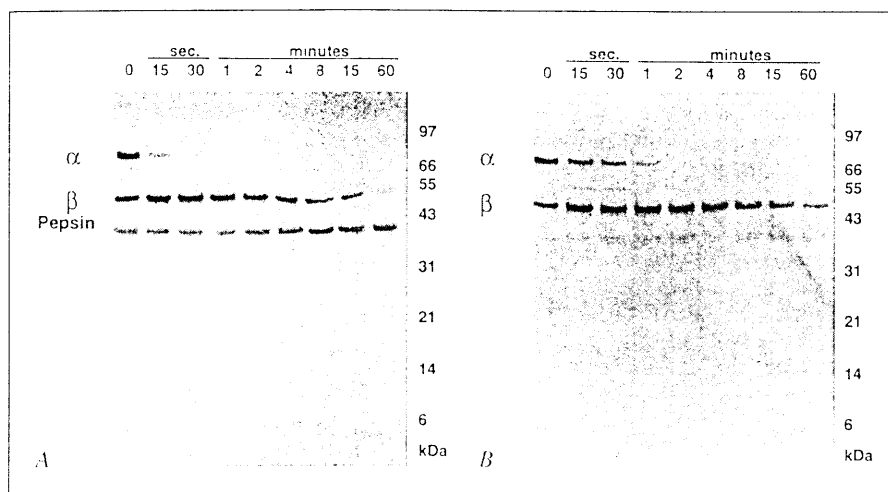


Fig. 3. Effect of complex protein mixtures on digestion of soy β -conglycinin. 170 ng/ μ l of protein was digested in 200- μ l aliquots of simulated gastric fluid composed of 0.3% (w/v) pepsin, 0.03 M NaCl, pH 1.2. Digests were quenched by neutralization with 75 μ l 0.16 M Na_2CO_3 at the following times: 0, 15 and 30 s, and 1, 2, 4, 8, 15 and 60 min. Digestion of β -conglycinin was evaluated by SDS-PAGE or immunoblotted by standard procedures [68], and proteins were detected with rabbit anti- β -conglycinin sera and visualized with alkaline phosphatase-linked goat anti-rabbit second antibody. Incubation times are indicated above, protein labels are on the left and molecular weight markers are on the right. SDS-PAGE and Coomassie staining (A) of purified β -conglycinin subunits (α) and (β). 100 ng of purified β -conglycinin was loaded in each lane. Immunoblot (B) of β -conglycinin subunits (α) and (β) in soy flour digested in a gastric model. 500 ng of total soy flour extract was loaded in each lane.

plex protein mixture like soybean flour extract does not effect the stability of a protein in this gastric model.

We have surveyed 17 common food allergens, including the major egg, milk, peanut, soybean and mustard allergens, for digestibility in simulated gastric fluid. The data presented in table 2 shows that these common allergens are, without exception, stable to digestion in this gastric model. In cases where the food allergen was less stable (i.e. not stable for 60 min), fragments were generated which were stable for a substantial period of time. In contrast, we have also shown that common plant proteins are rapidly digested in the gastric model (table 2).

Proteins that have been expressed in transgenic plants have been tested for stability with this model. In contrast to food allergens, all eight proteins expressed in transgenic plants were rapidly degraded (table 2). The time required

Table 2. Summary of allergen and protein stability in a gastric model

Protein	% total protein ¹	Stability, min ²	
		whole protein	fragments
<i>Egg white allergens</i>			
Ovalbumin (<i>Gal d 2</i>)	54	60	—
Ovomucoid (<i>Gal d 1</i>)	11	8	—
Conalbumin (<i>Gal d 3</i>)	12	0	15
<i>Milk allergens</i>			
β-Lactoglobulin	9	60	—
Casein	80	2	15
BSA	1	0.5	15
α-Lactalbumin	4	0.5	2
<i>Soybean allergens</i>			
β-Conglycinin (β-subunit)	18.5 ³	60	—
Kunitz trypsin inhibitor	2–4	60	—
Soy lectin	1–2	15	—
β-Conglycinin (α-subunit)	18.5 ³	2	60
Glycinin	51	0.5	15
Gly m Bd 30K	2–3	0	8
<i>Peanut allergens</i>			
Ara h II	6	60	—
Peanut lectin	1.3	8	—
<i>Mustard allergens</i>			
Sin a I	20	60	—
Bra j IE	20	60	—
<i>Common plant proteins</i>			
Rubisco LSU (spinach leaf)	25 ³	0 (<15 s)	—
Rubisco SSU (spinach leaf)	25 ³	0 (<15 s)	—
Lipoxygenase (soybean seed)	<1	0 (<15 s)	—
Glycolate reductase (spinach leaf)	<1	0.25	—
PEP carboxylase (corn kernel)	<1	0 (<15 s)	—
Acid phosphatase (potato tuber)	<1	0 (<15 s)	—
Sucrose synthetase (wheat kernel)	<1	0 (<15 s)	—
β-Amylase (barley kernel)	<1	0 (<15 s)	—

to degrade these proteins was between <10 and 30 s in simulated gastric fluid. Allergens or their fragments were stable for at least 2 min (and the major allergens were stable for > 60 min) in simulated gastric fluids, with various stabilities in simulated intestinal fluids (data not shown). Interestingly, those allergens with

Table 2 cont.

Protein	% total protein ¹	Stability, min ²	
		whole protein	fragments
<i>Introduced proteins</i> ⁴			
<i>B.t.t.</i> insecticidal protein	<0.01	0 (<30 s)	—
<i>B.t.k.</i> HD-73 insecticidal protein	<0.01	30 s	—
<i>B.t.k.</i> HD-1 insecticidal protein	<0.01	30 s	—
CP4 EPSP synthase	<0.1	0 (<15 s)	—
Glyphosate oxidoreductase (GOX)	<0.01	0 (<15 s)	—
ACC deaminase (ACCd)	0.4	0 (<15 s)	—
β- <i>D</i> -Glucuronidase (GUS)	0.01	0 (<15 s)	—
Neomycin phosphotransferase II (NPTII)	<0.01	0 (<10 s)	—

¹ Percent protein adapted from references 54–59.

² Stability represents the last time point at which the protein or a proteolytic fragment was observed; — = no fragments.

³ Represents the total proportion of protein for combined subunits.

⁴ Stability data for introduced proteins adapted from references 57, 69.

low stability in simulated gastric fluids tended to have at least some stability in simulated intestinal fluids (data not shown). Many allergens were stable for 24 h in simulated intestinal fluids.

Rapid degradation of the proteins expressed in transgenic plants greatly minimizes the likelihood that these proteins will be absorbed and hence elicit an allergic response. The human digestive system provides a very effective mechanism to remove these proteins before they have the opportunity to reach the intestinal mucosa. The simulated gastric model provides a valuable tool to assess concerns about the allergenic potential of proteins introduced into food crops.

Prevalence in Food and Sequence Similarity to Food Proteins

Many food allergens are present as major protein components in the specific food, typically ranging between 1 and 80% of total protein [54] (table 2). In contrast, the introduced proteins listed in table 2 are expressed in plants at levels ranging from approximately <0.001 to 0.03% of the raw product on a fresh weight bases or <0.01 to 0.4% of the protein content [55–59].

The low expression levels, combined with digestive lability relative to that for known food allergens, suggest a very low probability of any of these proteins

reaching the intestinal mucosa during consumption. These proteins are not likely to sensitize individuals consuming foods containing them.

The final component used in the assessment for the allergenic potential of a newly expressed protein is the amino acid sequence similarity to nonallergenic food proteins. The presence of biologically homologous proteins in food, especially a food with similar levels and types of consumption, decreases concerns of potential allergenicity. Most of the introduced proteins listed in table 2 show biologically relevant amino acid sequence homology to proteins already present in the food supply.

Potential Untoward Effects of Genetic Engineering on the Host Plant

If the host plant being genetically engineered is known to contain specific endogenous allergens and sera are available from allergy patients, the food derived from the new plant variety should be analyzed to assure that the composition and level of endogenous allergens were not altered during the genetic engineering process. Although there is no precedent to suggest that the genetic engineering process itself could change either the composition or level of endogenous proteins, to confirm this expectation a direct assessment is prudent for food products from transgenic plants. Of course, if the plant has no history of causing allergy or a limited history that precludes the availability of sera, this assessment cannot be performed. Immunoblotting and/or ELISA methods could be used for this assessment. Although the ELISA provides quantitative assessment of the total allergenic protein composition, the immunoblotting approach provides more detailed information on the composition of specific endogenous allergens and therefore is the preferred method [60]. Using this immunoblot approach with IgE from soybean-sensitive and normal individuals, Burks and Fuchs [60] showed no qualitative or quantitative differences in the endogenous allergens in transgenic glyphosate-tolerant soybeans compared with the parental soybean variety or with soybeans from commercial varieties. Assessing the endogenous allergens in transgenic glyphosate-tolerant soybeans was the first application of this approach and confirmed that the genetic engineering process caused no changes in the composition or levels of endogenous allergens.

Future Prospects

Genetic engineering provides a unique opportunity to reduce the levels of specific allergens in the food supply. By introducing genes in the antisense orientation (the opposite orientation required to produce a protein), the levels of

Table 3. Characteristics of proteins introduced into transgenic plants and common food allergens

Characteristic	Food allergens	ACCd	B.t.t.	B.t.k. HD-1	B.t.k. HD-73	CP4 EPSPS	GOX	GUS	NPTII
From allergenic source	yes	no	no	no	no	no	no	no	no
Homology to allergens	yes	no	no	no	no	no	no	no	no
Stable to digestion	yes	no	no	no	no	no	no	no	no
Prevalent protein in food	yes	no	no	no	no	no	no	no	no
Homology to 'safe' proteins	n/a	no	yes	yes	yes	yes	no	yes	yes

n/a = Not applicable.

protein produced by the 'sense' orientation can be dramatically reduced. This is the technique used to produce the delayed-softening, Flav-Savr™ tomato. By inhibiting the production of a polygalacturonase enzyme that normally caused softening in tomato, the shelf life of tomato was extended [61]. This same approach has been used to significantly reduce the primary allergen in rice. Matsuda et al. [62] cloned the gene encoding the 16-kD allergenic protein from rice, and introduced the gene encoding this protein in the antisense orientation into rice. The levels of the 16-kD protein were significantly reduced in rice seed from 312 to 60 µg/seed [63]. Further studies are underway to achieve greater reductions in this allergen. The antisense approach could be used in other food crops such as peanuts and soybeans to selectively reduce the levels of specific allergens, although the presence of multiple allergens and multigene families makes this prospect more complex.

Conclusions

The assessment strategy presented above is consistent with guidance from public institutions such as the FDA and the Environmental Protection Administration in the United States [9, 64], the Organization for Economic and Cooperative Development [65] and the World Health Organization [66]. The approach presented provides greater detail and justification for the combination of factors that should be addressed. It is important to remember that no single property or

assay alone can predict allergenicity. However, if a protein lacks all of the important characteristics of an allergenic protein, no further information should be necessary prior to marketing the new plant variety. For proteins that share one or more characteristics of allergenic proteins, a synthesis of all the information discussed above (table 3) should be considered when deciding if further research or characterization is prudent. In the end, this balanced approach will assure the safety of foods derived from transgenic plants.

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