Allergenicity Assessment of Foods Derived from Genetically Modified Plants

Numerous crop plants developed by plant biotechnology are being introduced into the marketplace. Assessment of the allergenic potential of the foods derived from these crops is a critical component of the overall food safety assessment of these products

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MORE THAN 60 DIFFERENT PLANT species, including most economically important crops, have been successfully genetically engineered since 1983, and the list is growing (Fiske and Dandekar, 1993). The variety of traits being introduced into plants is impressive, and includes insect protection, delayed ripening, virus resistance, modified starch, herbicide tolerance, modified oils, disease resistance, male sterility, and many others (WHO, 1993). More than 30 different genetically engineered plant products are predicted to be in the marketplace within the next four or five years (WHO, 1993).

Prior to market introduction, each of these products is subjected to extensive food, feed, and environmental safety assessments. In this article, we present the current approach to assess the allergenic potential of foods derived from genetically modified plants.

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. In contrast, plants contain an enormous number of proteins; some 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 (Sampson, 1992). Where food allergy is indicated clinically, patients usually are allergic to only one or two taxonomically related foods. Nine foods (peanuts, soybeans, tree nuts, milk, eggs, fish, crustaceans, mollusks, and wheat) account for more than 90% of food allergies (Taylor, 1992). The majority of allergy sufferers exhibit immunoglobulin E (IgE)-mediated immediate hypersensitivity (Metcalfe, 1992).

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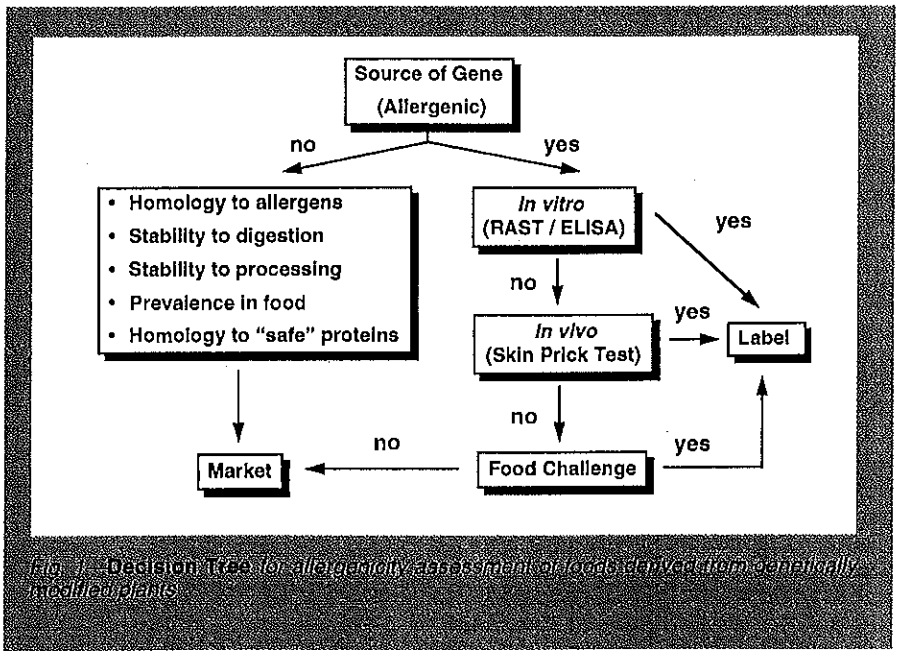

Fig. 1—Decision Tree for allergenicity assessment of foods derived from genetically modified plants

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 present).

A rational assessment of allergenic potential should be conducted in a careful stepwise process, using a decision tree strategy (Fig. 1) based on these factors. The totality of these assessments provides assurance that foods derived from new plant varieties do not introduce allergenic concerns beyond those present in the current food supply.

Allergenic Source of Introduced Protein

If a gene is obtained from a food source known to be commonly aller-

genic, data should be generated to assure that the gene does not encode an allergen. The Food and Drug Administration, in its policy on foods derived from new plant varieties (FDA, 1992), recognized this need. This part of the assessment (Fig. 1, right side) should be a tiered system where each subsequent level of study represents a greater degree of certainty that the source protein is not an allergen.

The first tier employs in-vitro assays such as radioallergosorbent tests (RASTS; Sampson and Albergo, 1984), enzyme-linked immunosorbent assays (ELISAs; Burks et al., 1986), and immunoblotting assays (Burks et al., 1988). These assays should use IgE fractions of pooled serum from individuals who are actively sensitive (allergic) to the food from which the gene was derived. Serum donors should meet clinical criteria which include positive responses in double-blind, placebo-controlled food challenges (DBPCFCs; Sampson and Albergo, 1984). Data from one or more of these in-vitro assays provides strong evidence of whether the transferred gene encodes an allergen. A positive result from the in-vitro tests would require that any food including the introduced gene be labeled as containing a gene from the specific source organism, per FDA guidance (FDA, 1992). Additional analysis could be performed to ensure that processing would eliminate the allergenic response to the source protein.

If no differential response is observed during in-vitro testing, a second tier using the in-vivo skin-prick test (Sampson and Albergo, 1984) could be performed. A positive result from this in-vivo test would raise the same concerns as the in-vitro tests above.

If the protein passes both tier 1 and tier 2, a final test could involve performing DBPCFCs with sensitive and nonsensitive patients under controlled clinical conditions. 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 and availability of appropriate clinical safety. Finally, if there were a reaction by sensitive patients, food derived from crops containing the protein would require labeling as under tier 1 and tier 2. If no adverse reactions were observed in the tier 1, 2, and 3 testing, it would be concluded that the gene obtained from this source did not encode one of the endogenous allergens and the product should be marketed without labeling.

An example which illustrates the effectiveness of this approach is the Brazil nut 2S storage protein which was engineered into soybean to increase levels of sulfur-rich amino acids (Nordlee et al., 1995). Brazil nut is known to contain an allergen that can cause adverse reactions (Gillespie et al., 1976; Arshad et al., 1991). The Brazil nut 2S protein expression in soybean represented a significant fraction of total soybean seed protein (Nordlee et al., 1995). RAST and immunoblot assays established that soybeans containing the 2S gene were as allergenic as Brazil nuts and that the gene obtained from Brazil nut probably encoded the major Brazil nut allergen (Nordlee et al., 1994, 1995). Either this product will not be commercialized or all soybean products derived from this variety will be labeled as containing a protein from Brazil nut. This example demonstrates the value and effectiveness of using in-vitro assays to identify the 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.

Table 1—**Summary of Proteins Introduced Into Crops** *by genetic engineering*

Introduced protein[a]	Crop products[b]
ACC deaminase (ACCd)	Delayed-ripening tomato
B.t.t. insecticidal protein (*B.t.t.*)	Insect-protected potato
B.t.k. HD-1 insecticidal protein (*B.t.k.* HD-1)	Insect-protected corn and tomato
B.t.k. HD-73 insecticidal protein (*B.t.k.* HD-73)	Insect-protected cotton
CP4 EPSP synthase (CP4 EPSPS)	Herbicide-tolerant canola, cotton, corn, soybean, and sugarbeet
Glyphosate oxidoreductase (GOX)	Herbicide-tolerant canola and corn
ß-D-glucuronidase (GUS)	Herbicide-tolerant soybean
Neomycin phosphotransferase II (NPTII)	Delayed-ripening tomato, insect-protected cotton and potato, FlavrSavr™ tomato
Phosphinothricin acetyltransferase (PAT)	Insect-protected corn

[a]Further explanation of abbreviations:
ACC: 1-amino-1-cyclopropane-carboxylic acid
B.t.t.: *Bacillus thuringiensis* subsp. *tenebrionis*
B.t.k.: *Bacillus thuringiensis* subsp. *kurstaki* proteins from strains HD-73 and HD-1, corresponding to the ((Cryla(c)) and (CryIA(b)) proteins according to the nomenclature of Höfte and Whiteley (1989)
CP4 EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4
[b]From Fuchs et al. (1995), Ciba-Geigy (1994), and Noteborn and Kuiper (1995)

Nonallergenic Source of Introduced Protein

According to FDA policy (FDA, 1992) and the scientific community in general (Anonymous, 1994), there appears to be no single, predictive assay to assess the allergenic potential of proteins from nonallergenic food sources. 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 (Taylor, 1992; Taylor et al., 1987, 1992; Metcalfe, 1985) and supported by reports from workshops sponsored by the World Health Organization (WHO, 1993) and the Organization for Economic Cooperation and Development (OECD, 1995).

Amino-acid-sequence homology of an introduced protein with known allergens represents a useful first approximation of allergenic potential. Immunologically relevant sequence similarity, especially similarity to IgE binding epitopes in known allergens, would implicate the introduced protein as a potential allergen.

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

food products are additional important factors contributing to the allergenicity of a protein. For example, soybean and peanut allergens retain their allergenic potential after all processing steps used in preparation of food products such as peanut butter (Taylor et al., 1992).

Allergenic proteins are also typically 10–70 kDa in molecular mass and often glycosylated. However, these two properties are shared by many proteins that are not allergenic and hence are not useful indicators of allergenicity.

The relevance of each of these characteristics will be discussed below. Proteins introduced into the seven different genetically engineered plant products listed in Table 1 will be used to illustrate that the majority of proteins being expressed in these products do not share the properties of known allergenic proteins.

• **Amino Acid Sequence Similarity to Known Allergens.** A comparison of the amino acid sequence of an introduced protein with the amino acid sequences of known allergens is a useful first approximation of allergenic potential. The amino acid sequences of many allergens, including food allergens, have been reported (King et al., 1994), and the list is likely to expand with time.

The important IgE binding epitopes of many allergen proteins have been mapped (Elsayed and Apold, 1983; Elsayed et al., 1991; Zhang et al., 1992). The optimal peptide length for binding is between 8 and 12 amino acids (Rothbard and Gefter, 1991). T-cell epitopes of allergenic proteins and peptide fragments appear to be least 8 amino acids in length (O'Hehir et al., 1991). Exact conservation of epitope sequences is observed in homologous allergens of disparate species (Astwood et al., 1995). Indeed, conservative substitutions introduced by site-directed mutagenesis reduce epitope efficacy (Smith and Chapman, 1995).

Based on this information, an immunologically relevant sequence comparison test for similarity between the amino acid sequence of the introduced protein and known allergens can be defined as follows: An immunologically significant sequence similarity requires a match of at least eight contiguous identical amino acids.

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 introduced proteins shown in Table 1 using the FASTA computer program (Pearson and Lipman, 1988). With the test criteria above, no significant sequence similarity was observed with allergens. We conclude (1) that the genes introduced into these foods do not encode known allergens, and (2) that none of the introduced proteins share immunologically significant sequences with known allergens.

• **Stability to Digestion.** The abilities 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. Many allergens exhibit proteolytic stability (King et al., 1967; Kortekangas-Savolainen et al., 1993; Onaderra et al., 1994; Taylor, 1986, 1992; Taylor et al., 1987; Metcalfe, 1985), although the majority remain untested directly. Intact proteins are capable of crossing the mucosal membrane of the gut and of entering the circulatory system (Gardner, 1988). 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 described in the *United States Pharmacopeia* (Anonymous, 1990) to systematically compare the relative stability of a number of common food allergens to that of proteins engineered into plants. These digestion models have been used to investigate the digestibility of plant proteins (Nielson, 1988; Marquez and Lajolo, 1981), animal proteins (Zikakis et al., 1977), and food additives (Tilch and Elias, 1984). A similar model has also been used to examine the stability of milk allergens (Asselin et al., 1988, 1989).

We have generated data to substantiate that common allergens are, without exception, stable to digestion in the gastrointestinal digestive models. These data establish a correlation between the digestive stability of egg, milk, and soybean allergens and the incidence of individuals with IgE to the specific allergen (referred to as percent allergenicity).

Nine different proteins that have been expressed in genetically modified plants have been tested for stability in the digestion model. In contrast to food allergens, all nine proteins were rapidly degraded (Table 2). The time required to degrade these proteins was 30 sec or less in simulated gastric fluids. Allergens were stable for at least 2 min (the major allergens for >1 hr) in simulated gastric fluids, with various stabilities in simulated intestinal fluids (not shown). Interestingly, those allergens with low stability in simulated gastric fluids tended to have at least some stability in simulated intestinal fluids (Astwood, 1995). Indeed, many allergens were stable for 24 hr in simulated intestinal fluids.

Rapid degradation of the proteins expressed in genetically engineered 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.

• **Stability to Processing.** The stability of a protein to various food processing activities is also important in assessing the allergenic potential of a protein. For example, if a protein was engineered into fresh-market products such as tomatoes, squash, or lettuce, then processing stability is irrelevant, since the product will be consumed fresh. If, on the other hand, a protein was engineered into soybeans, wheat, or rice and each of these products is processed in one or more ways prior to consumption, the stability of the protein to processing conditions should be taken into account. Furthermore, if the only product used for human consumption is free of protein (e.g., oils or carbohydrates), there would be no significant human consumption and the allergenic potential of the expressed protein would be greatly minimized or eliminated.

Studies using direct food challenges with oil derived from several different crops, including soybean, peanut, and sunflower, showed no allergic reaction in patients allergic to these foods (Bush et al., 1985; Halsey et al., 1986; Taylor et al., 1981). This is not surprising, since there is an extremely low or negligible level of protein in crop-derived oils (Tattrie and Yaguchi,1973).

The majority of the biological activity and immunoreactivity of each protein expressed in genetically engineered plants listed in Table 1 is lost during processing typically used for that crop, with the exception of the proteins introduced into tomatoes that are consumed fresh. The majority of the enzymatic and immunoreactivity of both the neomycin phosphotransferase II (NPTII) and 1-amino-1-cyclopropane-carboxylic acid deaminase (ACCd) proteins introduced into tomatoes is lost during the processing used

to produce tomato products such as juice, sauce, paste, and ketchup. Loss of immunoreactivity by processing, as detected using polyclonal antibodies, decreases the likelihood that epitopes on the protein, including those which are required for eliciting allergenic reactions, are maintained. This is especially relevant for conformational epitopes that require tertiary structures.

• **Prevalence in Food.** Many food allergens are present as major protein components in the specific food, typically ranging between 1 and 80% of total protein. Examples of highly abundant allergens include those in milk (Taylor et al., 1987; Baldo, 1984; Lebenthal, 1975; Taylor, 1986), soybean (Shibasaki et al., 1980; Pederson and Djurtoft, 1989), and peanuts (Barnett et al., 1983; Sachs et al., 1981; Barnett and Howden, 1986; Kemp, 1985). In contrast, the nine proteins expressed in plants range from approximately <0.001 to 0.03% of the raw product on a fresh-weight basis or <0.01 to 0.4% of the protein content (Padgette et al., 1996; Fuchs et al., 1993; Perlak et al., 1991, 1993; Reed et al., 1995).

The low levels of these proteins, combined with their processing and 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.

• **Amino Acid Sequence Similarity to Food Proteins.** The final component used in the assessment of the allergenic potential of a newly expressed protein is the similarity of the amino acid sequence to that of 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. Each of the proteins listed in Table 3 show biologically relevant amino acid sequence homology to proteins already present, or approved for use, in the food supply.

Table 2—**Relative Protein and Allergen Stability in a Gastric Model**

Protein	Total protein (%)	Allergenicity[a] (%)	Stability[b]
Egg allergen[c]			
Ovalbumin	54	100	60 min
Milk allergens[d]			
β-Lactoglobulin	9	72	60 min
Casein	80	56	15 min
BSA	1	45	15 min
α-Lactalbumin	4	14	2 min
Soybean Allergens[e]			
β-Conglycinin (β subunit)	18.5	75	60 min
Kunitz trypsin inhibitor	2–4	25	60 min
β-Conglycinin (α subunit)	18.5	20	60 min
Soy lectin	1–2	10	15 min
Glycinin	51	5	15 min
Gly m Bd 30K	2–3	65	8 min
Introduced proteins			
B.t.t.	<0.01	0	0
B.t.k. HD-73	<0.01	0	30 sec
B.t.k. HD-1	<0.01	0	30 sec
CP4 EPSPS	<0.1	0	0
GOX	<0.01	0	0
ACCd	0.4	0	0
GUS	0.01	0	0
NPTII	<0.01	0	0
PAT	nd[f]	0	0

[a]Percent allergic individuals with IgE specific for that protein
[b]Last time point that the intact protein or protein fragments are observed
[c]From Langeland (1982)
[d]From Savilahti and Kuitunen (1992)
[e]From Shibasaki et al. (1980), Ogawa et al. (1991), and Burks et al. (1994)
[f]Not detectable

The Host

If the host being genetically engineered is known to contain specific endogenous allergenic proteins 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 engineering process. Although there is no precedent to suggest that the genetic engineering process itself could affect either the composition or level of endogenous proteins, to confirm this expectation a direct assessment is prudent for the first genetically engineered food products.

Of course, if the plant has either 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 implemented 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 (Burks and Fuchs, 1995).

Using this immunoblot approach with IgE from soybean-sensitive and normal individuals, Burks and Fuchs (1995) showed no qnalitative or quantitative differences in the endogenous allergens in genetically engineered, glyphosate-tolerant soybeans (Padgette et al., 1996) compared to the parental soybean variety or to soybeans from commercial varieties. This was the first application of this approach and confirmed that the genetic engineering process per se caused no changes in the composition or levels of endogenous allergens.

National and International Consensus

Several national and international efforts have stimulated discussion and agreement that the type of information presented in this article is appropriate to assess the potential allergenicity of foods derived from genetically engineered plants. FDA's (1992) policy provides guidance for allergenicity assessment. The Environmental Protection Agency also provided some guidance in its draft guidelines for pesticidal plants (EPA, 1994). FDA, EPA, and the U.S. Dept. of Agriculture cosponsored a conference in March 1994 focused specifically on assessing the allergenic potential of foods derived from genetically engineered plants (Anonymous, 1994).

Recent OECD (1995) and WHO (1995) workshops also provided guidance on allergenicity assessment.

The approach presented in this article is consistent with the guidance from these public institutions and provides greater detail and justification for the combination of components 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 considered necessary prior to introduction of new plant varieties. For proteins that share one or more characteristics of allergenic proteins, a combination of all the information discussed above should be considered when deciding if further research or characterization is prudent.

In the end, a balanced judgment of all the available data generated during allergenicity assessment (Table 3) will assure the safety of foods derived from genetically engineered crops. Using these approaches, the new plant varieties generated by genetic engineering should be introduced into the marketplace with the confidence that new plant varieties developed by traditional breeding have been introduced for centuries.

Reducing Unwanted Proteins

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 protein produced by the "sense" orientation can be dramatically reduced. This is the technique used to produce the delayed-softening, FlavrSavr™ tomato. By inhibiting the production of a polygalacturonase enzyme that normally caused softening in tomato, the shelf life of tomato was extended (Sheehy et al., 1988).

This same approach has been used to significantly reduce the primary allergen in rice. Matsuda et al. (1993) cloned the gene encoding the 16-kDa allergen from rice and introduced the gene encoding this protein in the antisense orientation into rice. The levels of the 16-kDa allergen were significantly reduced in rice seed in a number of the progeny. However, this allergen was not completely eliminated in these modified plants. 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. Some of the methods described above enable the assessment of the effectiveness of this and other approaches to reduce or eliminate individual allergens from crop plants. Thus, the opportunity now exists to significantly reduce or eliminate, through genetic engineering, noxious proteins, such as food allergens, which are currently present in the food supply.

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Table 3—**Characteristics of Presently Introduced Proteins** *compared to common food allergens*

Characteristic[a]	Food allergens	ACCD	*B.t.t.*	*B.t.k.* HD-1	*B.t.k.* HD-73	CP4 EPSPS	GOX	GUS	NPTII	PAT
From allergenic source	yes	no	no	no	no	no	no	no	no	no
Homology to allergens	yes	no	no	no	no	no	no	no	no	no
Stable to digestion	yes	no	no	no	no	no	no	no	no	no
Stable to processing	yes	no	no	no	no	no	no	no	no	no
Prevalent protein in food	yes	no	no	no	no	no	no	no	no	no
Homology to "safe" proteins	na[b]	no	yes[c]	yes[c]	yes[c]	yes	no	yes	yes[c]	yes(?)

[a] Characteristics of food allergens as described in Taylor (1992), Taylor et al. (1987, 1992), and Metcalfe (1985)

[b] Not applicable

[c] Already present in the food supply with no reported allergy

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