

Biosafety of Genetically Modified Plants and Microorganisms: Recent Developments in Approaches to Evaluation of Allergenicity

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Abstract

In recent years, a number of new plants and microorganisms have been developed with recombinant DNA technology. Since the transferred genes code for proteins that ordinarily may not be present in these particular plants, there is concern about the potential allergenicity of these new varieties. The safety evaluation of transgenic foods and microorganisms is relatively straightforward if the allergenicity of the gene source is known. The recombinant product can be assayed using traditional *in vitro* allergen detection methods or IgE antibody binding assays. Examples for this approach are recent studies of transgenic soybeans and recombinant corn proteins. However, it is more difficult to assess the allergenicity of transgenic plants when genes coding for proteins of unknown allergenicity are transferred, since there is no generally accepted or established procedure to define a protein's allergenicity based on its molecular characteristics. Although a comparison of the transferred protein's structure and/or biological activity with that of known allergens and allergen epitopes (particularly amino acid sequences) is one approach, any results must be confirmed by IgE antibody binding studies. In conclusion, although most evidence suggests that the vast majority of transgenic plants and microorganisms will be completely safe for the consumer, it is essential that proper guidelines are established and relevant tests performed to assure their safety.

Introduction

Using recombinant DNA technologies, new plant varieties and microorganisms are being developed to improve the quality and quantity of our food supply (Harlander, 1991; Kessler *et al.*, 1992). Since some of the genes governing new traits encode for proteins found in other plant species or in microorganisms but not ordinarily in the species transformed, there is concern about the potential adverse effects of these proteins on individuals exposed to them (Harlander, 1991; Kessler *et al.*, 1992). One major question regarding these new proteins is their potential as allergens (Kessler *et al.*, 1992; Lehrer *et al.*, 1996).

What is food allergy? There are a number of types of adverse reactions observed following exposure to foods that can be confused with true food allergy because they all

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can have similar symptoms. An adverse reaction to a food is a clinically abnormal response attributed to exposure to a food or food additive and includes both immunologic and non-immunologic reactions (Sampson and Metcalfe, 1991). Food allergy, or food hypersensitivity, is an immunologic reaction resulting from the sensitization to a food or food additive. This reaction occurs only in some individuals, generally after a small amount of substance is ingested, and is unrelated to any physiological affect of the food or food additive. Food allergies are mediated by IgE antibodies and symptoms usually occur within minutes of exposure. Non-immunologic adverse reactions to foods are food intolerance, an abnormal physiological response to an ingested food or food additive; food poisoning, which is basically a toxic reaction; and pharmacologic food reactions, due to chemicals that produce a drug-like effect.

For the induction of allergic hypersensitivity, innocuous antigens called allergens enter the body via mucosal surfaces and are taken up by local antigen-presenting cells which process and present them to Th-2 lymphocytes. Recognition between the Th-2 and B antigen-specific cells results in the release of soluble factors that in turn lead to B cell proliferation and differentiation. These events result in production of allergen-specific IgE by the B cells. IgE binds to specific receptors on mast cells or basophils thus sensitizing them. When the allergen subsequently reaches the sensitized mast cell, it cross-links surface-bound IgE, triggering the release of preformed mediators and newly synthesized mediators. The mediators, in turn, elicit the clinical signs and symptoms of allergic diseases such as asthma, eczema, hay fever and anaphylaxis (Roitt *et al.*, 1996).

Theoretically, all types of foods can cause allergic reactions. However, most allergic reactions are due to a few foods such as eggs, cow milk, nuts, legumes, and seafood (Sampson and Metcalfe, 1991; Taylor and Lehrer, 1996; Bush and Hefle, 1996). Allergic foods of plant origin include grains, such as rice, rye and wheat, legumes such as peanuts and soybeans, seeds and nuts, fruits, vegetables, spices, and miscellaneous substances such as honey, fungi, yeast, and cocoa (Bush and Hefle, in press). Some of the best studied of the major food allergens derived from plants are those from peanut, soybean, and rice.

It is difficult to precisely estimate the prevalence of food allergies. In clinical surveys, 2% to 4% of children younger than 6 years display reproducible allergic reactions to foods. Studies in adults suggest that 1 to 2% of the general adult population are sensitive to foods or food additives (Sampson and Metcalfe, 1991). This contrasts dramatically with the public's perception of the importance of allergic reactions to foods. It has been reported that at least one in four atopic adults believes that it has experienced adverse reactions following the ingestion or handling of foods. Similarly, parents believe that one of four of their children has experienced at least one adverse reaction to a food (Sampson and Metcalfe, 1991). Thus the perception of food allergy is much greater than the prevalence. This has important implications for consumer's acceptance of transgenic foods.

Common properties of food allergens

Generally, most food allergens are naturally occurring proteins found in the food. The most common food allergens tend to be regularly consumed foods that have a relatively high protein content. However, some foods that one would expect to be included as major food allergens, such as beef and pork, are not common causes of food allergy. In general, food proteins that maintain their immunogenicity following processing, cooking and digestion are more likely to elicit an allergic response than those that are not as resistant to such processes (Taylor and Lehrer, 1996).

A prerequisite for any allergen is the ability to stimulate an immune response. Why certain proteins do this better and are more allergenic than others is not yet understood (Lehrer *et al.*, 1996; Scheiner and Kraft, 1995). Allergens triggering allergic reactions must be polyvalent, that is, have several IgE-binding epitopes in order to bridge IgE molecules on the surface of mast cells to initiate degranulation; thus, they are somewhat constrained in their molecular dimensions. Most known food allergens have molecular weights between approximately 5-10 to 70 kD. Although smaller molecules could conceivably act as haptens, a molecular weight of approximately 5-10 kD probably represents the lower limit for an immunogenic response. The upper limit for molecular size is probably the result of restricted mucosal absorption of larger molecules (Lehrer *et al.*, 1996). Generally, most food allergens are glycoproteins with an acid isoelectric point. However, these properties are true for many other antigens and are probably not unique for food allergens (Lehrer *et al.*, 1996). While there may be some exceptions, clearly the most potent food allergens are stable molecules resistant to processing, cooking, and digestive enzymes while they remain soluble and absorbable through the intestinal tract (Lehrer *et al.*, 1996; Taylor and Lehrer, 1996).

Many allergens share epitopes with other food or non-food allergens, such as pollens. The clinical relevance of cross-reactivity is a highly debated issue, but in the context of risk assessment, these cross-reactivities may help to identify potential allergenic molecules if homologies between allergenic epitopes and transgenic proteins are detected (Bush and Hefle 1996).

Effects of biotechnology on our food supply

When evaluating the potential effects that biotechnology can have on newly developed food products, two issues must be considered: first, effects of the transfer of known protein allergens into new foods, and second, effects of transfer of recombinant proteins of unknown allergenic activity into new foods (Kessler *et al.*, 1992; Lehrer *et al.*, 1996).

Almost 80 recombinant protein allergens have been produced to date for possible use in diagnosis and treatment of allergy (Scheiner and Kraft, 1995). These recombinant allergens bind IgE antibodies and thus retain their IgE reactive epitopes, suggest-

ing that recombinant allergens expressed in foods will probably retain their allergic activity and must be considered allergenic unless proven otherwise. So the question is: Can recombinant proteins in newly developed food products be detected and if so, how?

Recombinant allergens in transgenic foods can be identified using traditional *in vitro* assays such as Western blotting (Laemmli, 1970; Demeulemester *et al.*, 1987; Kyhse-Andersen, 1984), RAST inhibition (Lehrer and McCants, 1987) or ELISA assays (Taylor and Nordlee, 1996). Inhibition assays are based on the competition of solid-phase bound allergens and free test sample for the binding of allergen-specific IgE. If the test sample and allergen are cross-reactive, the test sample will inhibit the IgE binding to the solid-phase bound allergen. By testing increasing concentrations of homologous allergen to inhibit the RAST or ELISA reactions, a standard inhibition curve can be developed. Food allergens can also be identified by western blotting (Laemmli, 1970; Demeulemester *et al.*, 1987; Kyhse-Andersen, 1984). Electrophoretically separated proteins transferred to a carrier membrane can be probed with sera from allergic subjects in order to determine if IgE antibodies react with any proteins. Although this method is less quantitative than the inhibition assays, it is very useful since it can identify the allergenic proteins among possible hundreds of non-allergenic proteins.

These methods are all well established, specific, sensitive, and reproducible. The only reagents of limited quantity are patients' sera (Lehrer *et al.*, 1996). However, once sensitized individuals are identified, substantial quantities of sera can be obtained and serum pools produced. At least 1 liter of plasma can be obtained from sensitized individuals by plasmaphoresis. This method can provide sufficient reagents for almost unlimited allergen assays. An alternate possibility would be to use allergen-specific monoclonal antibodies that could be produced in practically unlimited quantities. However, monoclonal antibodies may not necessarily bind to IgE-reactive epitopes and the only definitive way one can identify allergens is through the demonstration of IgE reactive epitopes.

Examples of allergen detection in transgenic foods

During the last year there have been two published studies and one unpublished study from our own laboratory investigating the allergenic potential of recombinant food proteins. These studies are good examples of how transgenic food products are assessed for their potential allergenicity.

The first study investigated transgenic soybeans in which a gene was introduced to confer tolerance to glyphosate, the active ingredient in the herbicide Round-up. Such herbicides have both economical and environmental advantages over those currently used. Wesley Burks and colleagues at the University of Arkansas prepared extracts of different wild type and transgenic varieties of soybeans and demonstrated by western blot allergenic proteins through IgE binding (Burks and Fuchs, 1995). There ap-

peared to be no increased binding activity of IgE antibodies from a serum pool of soy-allergic individuals to the transgenic soybean extract as compared to the wild type (Fig. 1). However, no attempt was made to quantify results by densitometry or RAST inhibition.

The second study, performed in our laboratory, has recently investigated allergenic activity of two unique corn proteins. The 10 kD/delta class of zein proteins in corn is of particular interest to biotechnologists since they are sulfur-rich proteins, an exception to the general rule that cereal seed proteins are low in methionine (Higgins, 1984). Genes of two zein proteins - 10kD (Kirihara *et al.*, 1988) and HSZ (Chui and Falco, 1995) were identified. Since current efforts are directed at increasing the expression of these proteins in corn itself or the seeds of other species using regulatory sequences from genes that are more highly expressed, it is of interest to determine if the 10 kD zein or the HSZ has allergenic activity. Such studies are complicated by the fact that zeins are alcohol-soluble in contrast to the majority of allergens that are water-soluble (Higgins, 1984) and corn allergens have not been well characterized. Therefore, 10 sera from corn-reactive (by skin test, RAST, or clinical history) individuals were tested for IgE antibody reactivity to corn proteins by SDS-PAGE immunoblotting against both aqueous and alcohol extracts (Fig. 2). Substantial IgE antibody reactivity to aqueous and alcohol soluble corn proteins was detected (Lehrer, Reese, Krebbers, in preparation). Both extracts contain IgE-reactive proteins. Their molecular weights range from 10-70 kD. The individual patterns vary; up to 15 IgE-reactive water-soluble proteins were detected whereas up to 7 alcohol-soluble proteins bound IgE. The 10kD and HSZ zein proteins were also tested for IgE antibody reactivity; none of the corn reactive sera bound to those proteins (Fig. 3) demonstrating the absence of allergenic activity (Lehrer, *et al.*, 1997).

The third study was reported by Dr. Steve Taylor's laboratory. Nordlee and co-workers (Nordlee *et al.*, 1996) detected an allergen in a transgenic soybean. This protein, originating from Brazil nuts, was expressed in experimental soybeans to increase their sulfur content. It bound IgE from Brazil nut-sensitive individuals, and was identified as a major Brazil nut allergen (Figs. 4, 5). This example illustrates that testing these new products for allergens is possible when proteins are transferred from sources that contain allergenic material.

Strategies to detect allergenicity of proteins of unknown sources.

The transfer of proteins of undetermined allergenicity into potential foods poses a much more serious problem. Are these proteins expressed in such a fashion that they retain allergenic activity? Do they contain epitopes that cross-react with known allergens? Predicting the potential allergenicity of these proteins is a major challenge for the food industry. There are several approaches that might be useful. One can determine the physico-chemical properties of these proteins such as molecular size, stability, solubility, and isoelectric point and compare them with those of major food allergens (Lehrer *et al.*, 1996; Taylor and Lehrer, 1996). However, a definitive answer can only

be based on epitope (portions of antigens/allergens to which immune cells or antibodies react) similarity and IgE antibody reactivity. This is extremely difficult if not impossible to establish if IgE antibody reactivity is not known.

One potential solution is to compare the amino acid sequence of the recombinant protein with those of known allergen epitopes. More and more allergen epitopes are being identified and their amino acid sequences will be available in computer data banks. However, one must be cautious since amino acid sequence similarity does not necessarily mean similar IgE reactivity (Reese *et al.*, 1996). Thus, amino acid sequence similarities do not prove allergenic reactivity and such findings, of course, must be confirmed by IgE antibody reactivity using *in vitro* tests.

It has been established that TH-2 lymphocytes are an integral part of IgE antibody production, and that allergens stimulate these cells to produce interleukin 4, a cytokine that is important in IgE antibody production (Roitt *et al.*, 1996). Thus, a novel approach to assess allergenicity of a recombinant protein might be to measure its ability to stimulate TH-2 lymphocyte proliferation and production of cytokines including interleukin 4. Certainly, this is an approach that warrants further investigation.

An experimental approach might be to compare the ability of recombinant food proteins to stimulate IgE antibody production in a murine animal model to that of known allergens and hypoallergens. Mice have been well studied for IgE reactivity. This model might provide a very useful preliminary step for assaying allergenic reactivity. However, there is no general agreement on the use of animal models, and certainly, any future animal model requires further study.

A suggested strategy to assess whether or not the recombinant protein may have allergenic activity is summarized in Fig. 6 (Lehrer *et al.*, 1996). If the protein is suspected to be a known allergen or comes from a source that contains known allergens, standard *in vitro* assays using patient's IgE antibodies can be used to determine if the protein expresses allergenic activity. Such assays and reagents required are developed and available.

The main problem is assaying recombinant proteins from sources with undetermined allergenic activity. There is no definitive way to conclusively establish whether or not these proteins have allergenic activity. Several useful approaches include comparison of their properties with those of known food allergens, comparison of their amino acid sequences with those of known allergen epitopes, and measurement of their ability to stimulate IgE antibody responses in animal models. However, any suggested allergenicity must be confirmed by *in vitro* assays testing for IgE antibody reactivity. Such approaches should help avoid potentially serious allergic reactions in consumers to recombinant proteins.

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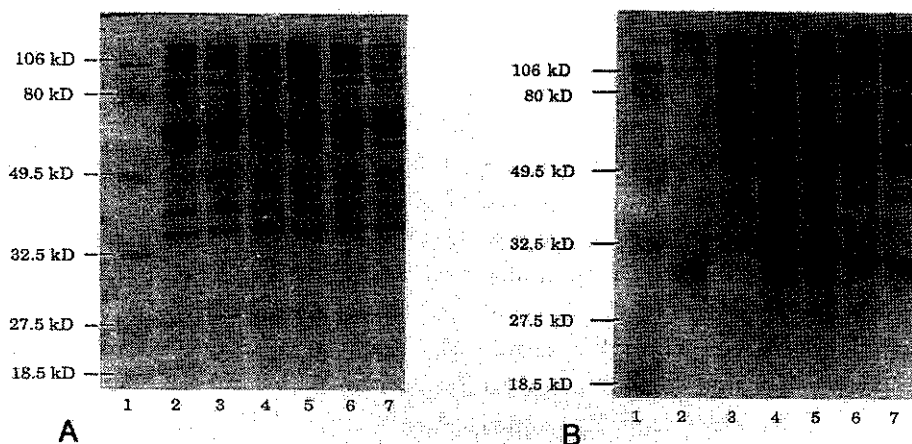


Fig. 1 IgE reactivity of soy-sensitive subjects to transgenic and non-transgenic soybeans

Panel A Coomassie blue-stained SDS-PAGE gel.

Panel B IgE reactivity of soy-sensitive subjects.

Lane 1 - prestained molecular weight markers;

Lanes 2, 4 - extracts from transgenic soybean;

Lane 3 - parental soybean;

Lanes 5,6,7 extracts of other wild type soy lines.

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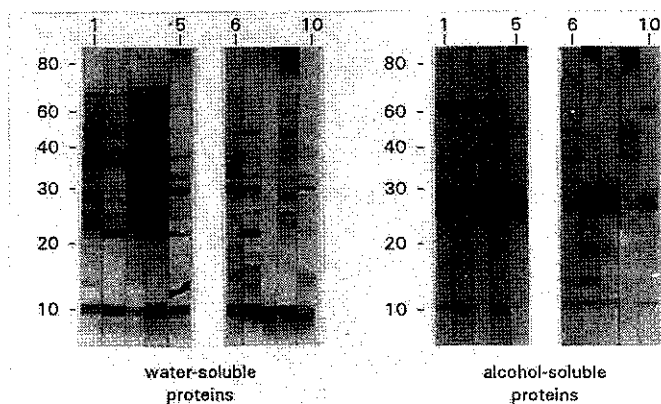


Fig. 2 IgE- reactivity of corn-reactive subjects with water and alcohol-soluble corn proteins

Corn proteins, separated by SDS-PAGE, were blotted onto nitrocellulose membranes and probed with sera from corn-reactive subjects. These sera react to corn proteins in the molecular range of 10 to 70 kD.

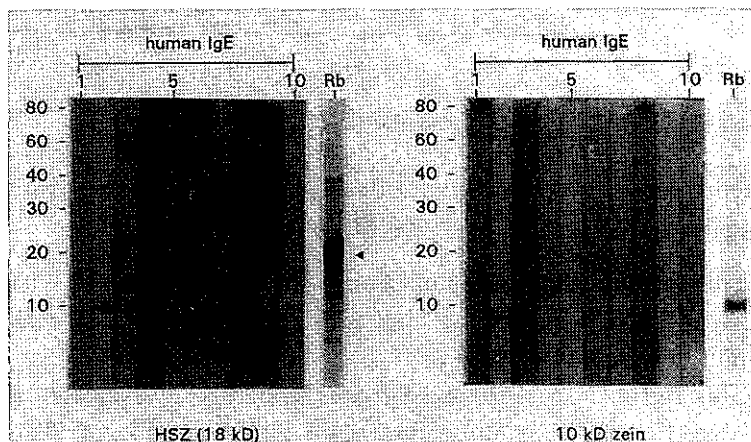


Fig. 3 Lack of IgE-reactivity of corn-reactive sera with the HSZ and 10 kD corn zein proteins

Ten sera, selected on the basis for IgE antibody binding to alcohol-soluble corn proteins, were tested for reactivity to 10 kD and HSZ zein proteins. None of the sera reacted to these proteins. Rabbit anti-sera (Rb) raised against the HSZ or 10 kD proteins were used as positive control.

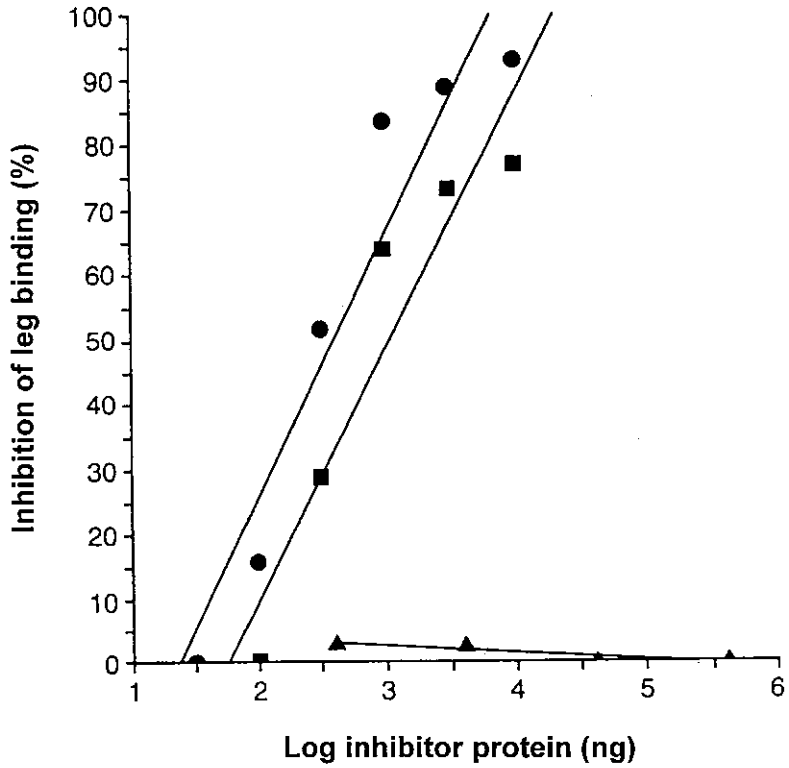


Fig. 4 RAST inhibition of IgE binding to Brazil nut extract by non-transgenic soybeans (▲), transgenic soybean (■), and brazil nut (●)

Substantial inhibition of brazil nut RAST was obtained with transgenic soy or brazil nut extracts. No inhibition was obtained with non-transgenic soybean.

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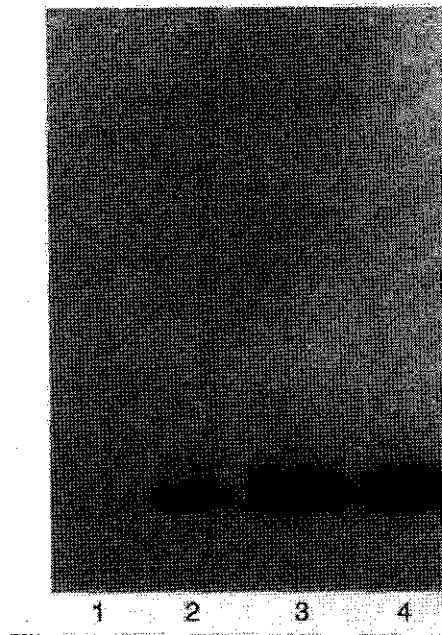


Fig. 5 Reactivity of Brazil nut allergic serum to a 2S albumin Brazil nut allergen

Lane 1 - non-transgenic soybean,

Lane 2 - transgenic soybean,

Lane 3 - Brazil nut extract,

Lane 4 - 9 kD 2S albumin Brazil nut protein allergen.

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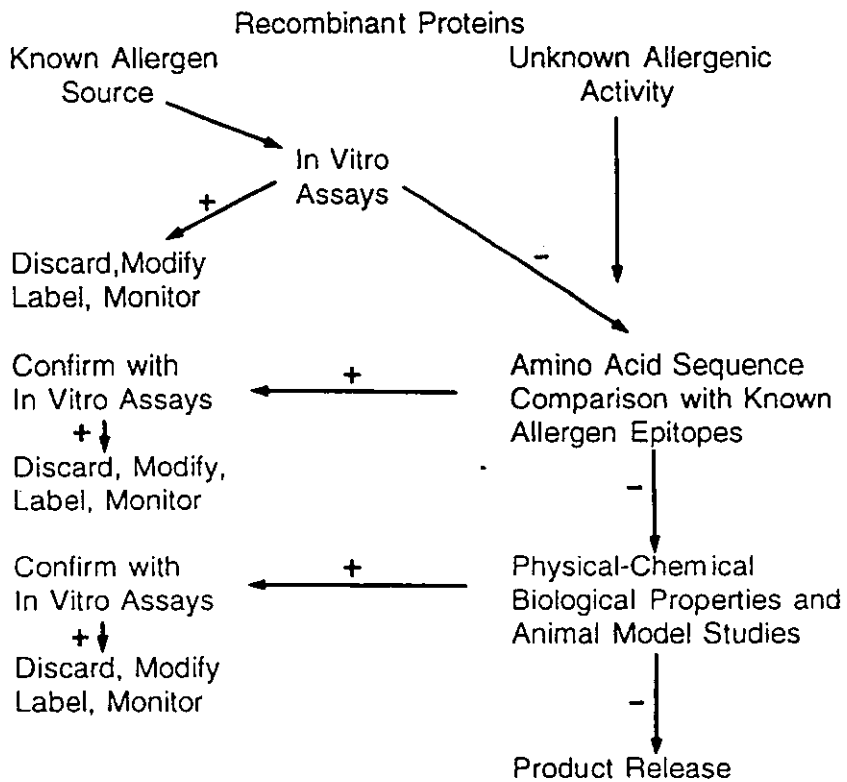


Fig. 6 Decision making tree for assessing the potential allergenic activity of transgenic crops

This is based on a comparison of physical chemical, biochemical, and immunochemical properties of recombinant proteins with allergens. Any similarities must be confirmed by *in vitro* IgE antibody reactive assays. This figure is reproduced from the article by Lehrer *et al.*, Clinical Reviews in Food Science and Nutrition 1996; 33:553-564. Reprinted with the permission of the CRC Press.