SAP Report No. 2000-03B
September 28, 2000

REPORT

FIFRA Scientific Advisory Panel Meeting, June 6-7, 2000, held at the Sheraton Crystal City Hotel, Arlington, Virginia

Sets of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

Session II - Mammalian Toxicity Assessment Guidelines for Protein Plant Pesticides

Note: The report for Session I - Consultation: National Drinking Water Survey Design for Assessing Chronic Exposure at the June 6-7, 2000 FIFRA SAP meeting was previously released.

NOTICE

This report has been written as part of the activities of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP). This report has not been reviewed for approval by the United States Environmental Protection Agency (Agency) and, hence, the contents of this report do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the Federal government, nor does mention of trade names or commercial products constitute a recommendation for use.

The FIFRA SAP was established under the provisions of FIFRA, as amended by the Food Quality Protection Act (FQPA) of 1996, to provide advice, information, and recommendations to the Agency Administrator on pesticides and pesticide-related issues regarding the impact of regulatory actions on health and the environment. The Panel serves as the primary scientific peer review mechanism of the EPA, Office of Pesticide Programs (OPP) and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. Food Quality Protection Act Science Review Board members serve the FIFRA SAP on an ad-hoc basis to assist in reviews conducted by the FIFRA SAP. Further information about FIFRA SAP reports and activities can be obtained from its website at http://www.epa.gov/scipoly/sap/ or the OPP Docket at (703) 305-5805. Interested persons are invited to contact Larry Dorsey, SAP Executive Secretary, via e-mail at dorsey.larry@.epa.gov.

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Mammalian Toxicity Assessment Guidelines for Protein Plant Pesticides

Mr. Paul Lewis
Designated Federal Official
FIFRA Scientific Advisory Panel
Date:_____

Mary Anna Thrall, D.V.M.
FIFRA SAP Session Chair
FIFRA Scientific Advisory Panel
Date:_____

Federal Insecticide, Fungicide, and Rodenticide Act
Scientific Advisory Panel Meeting
June 7, 2000

SESSION II - Mammalian Toxicity Assessment Guidelines for Protein Plant Pesticides

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Oral statements were made by:
Bruce Hammond, Ph.D. on behalf of Monsanto Company
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Written statements were received from:
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INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP) has completed its review of the set of scientific issues being considered by the Agency regarding mammalian toxicity assessment guidelines for protein plant pesticides. Advance notice of the meeting was published in the *Federal Register* on May 17, 2000. The review was conducted in an open Panel meeting held in Arlington, Virginia, on June 7, 2000. The meeting was chaired by Mary Anna Thrall, Ph.D. Mr. Paul Lewis served as the Designated Federal Official.

The Agency solicited guidance from the Panel on the assessment of the potential mammalian toxicity of proteins expressing plant-pesticides. John Kough, Ph.D. (EPA, Office of Pesticide Programs) and Michael Watson, Ph.D. (EPA, Office of Pesticide Programs) discussed protein biochemistry characterization, amino acid homology comparison, and animal models of toxicity to assess mammalian toxicity of protein plant pesticides.

CHARGE

1. Does the Panel agree that the maximum hazard dose approach is generally adequate to address protein toxicity? If not, what additional and/or other approaches would be appropriate? As an alternative for proteins with low expression in plant tissue, would it be possible to demonstrate an acceptable level of mammalian safety by testing the purified protein at, for example, 10,000 times the expression level in the plant? For proteins that show no toxicity in a maximum hazard dose toxicity study, do the negative results of the maximum hazard dose also address the issue of the potential for toxicity following multiple and/or long-term exposures to these proteins?

2. The Agency believes that longer-term testing is not applicable for digestible protein plant-pesticides that display no toxicity in the acute oral toxicity tests. Under what circumstances, if any, should the Agency require repeat dose (30 day) feeding studies for protein plant-pesticides? For repeated dose studies, what animal model test system and anticipated effects or toxic endpoints would the Panel suggest be considered?

3. Assume that repeated dose (30 days) testing is indicated and that the test animal diet is appropriately adjusted to provide a healthy diet. Does the Panel agree that whole-food testing with plant-pesticide containing food products does not provide a means to apply an appropriate margin of safety in these studies? Would the plant-pesticide expressing food crop in question amended with pure protein plant-pesticide to yield a higher dose be an appropriate test substance? Or would the purified protein as test substance alone be more appropriate?

4. What is an accepted method of amino acid homology/similarity analysis that can be used to screen for a protein function like mammalian toxicity ? Are there any analyses that examine higher levels of protein organization (i.e., secondary, tertiary and quantriary) that could also be incorporated in these structural comparisons?

5. Are peptide fragments that result from the breakdown of proteins more toxic than the intact proteins from which they originated? Are there examples of post-translationally modified proteins which have different toxicity compared to the non-post-translationally modified proteins?

6. How does the breakdown of proteins differ in infants and individuals with digestive disorders compared to those with "normally" functioning digestive systems? Would risk from break-down products of an otherwise digestible plant-pesticide protein differ for these digestion impaired individuals compared to that posed by any other digestible proteins in their diet?

7. Other than the predominant oral route of exposure, are there any additional routes of exposure of concern for the toxicity of plant pesticides? Are there any combinations of different routes of exposure that may result in an enhancement of potential adverse effects? If so, what tests should be considered to evaluate this combined effect?

8. Does the Panel believe there is any other area of toxicity that should be routinely examined for the safety of protein plant-pesticides? What new areas of enquiry should be considered for research into the safety of protein plant-pesticides?

DETAILED RESPONSE TO THE CHARGE

The specific issues to be addressed by the Panel are keyed to the Agency's background document "Mammalian Toxicity Assessment Guidance for Protein Plant-Pesticides", dated May 15, 2000, and are presented as follows:

1. Does the Panel agree that the maximum hazard dose approach is generally adequate to address protein toxicity? If not, what additional and/or other approaches would be appropriate? As an alternative for proteins with low expression in plant tissue, would it be

possible to demonstrate an acceptable level of mammalian safety by testing the purified protein at, for example, 10,000 times the expression level in the plant? For proteins that show no toxicity in a maximum hazard dose toxicity study, do the negative results of the maximum hazard dose also address the issue of the potential for toxicity following multiple and/or long-term exposures to these proteins?

The Panel concurred that the maximum hazard dose approach is generally adequate to address protein toxicity. It acknowledged that, given the low levels of pesticidal proteins produced in transgenic crops, it generally will not be possible to feed test animals the large amounts of the plant pesticide required to ensure a margin of safety in humans. Therefore, tests must be designed to evaluate toxicity by either concentrating plant proteins in the pesticidal plant, for example by lyophilization, or by testing the concentrated candidate protein produced in an alternative host, such as a bacterial expression system.

In addition, as an alternative approach, the Panel agreed that it would be possible to assess mammalian toxicity with the purified protein at exponential multiplicities of the concentrations produced in the plant, provided the designated test mammal (e. g., rat) is capable of ingesting such an amount of the candidate pesticidal protein in a balanced diet, and that the candidate protein has met the criteria established for substantial equivalence, as stated in question 3. For many of the plant pesticidal proteins evaluated to date, the candidate proteins produced in bacteria have been tested at levels in the range of 1,000 to 10,000 times the level produced in plants. For example, in feeding tests against rats, preparations of *B. thuringiensis* subsp. *kurstaki* that contained Cry1Aa, Cry1Ab, Cry1Ac and Cry2A at a combined level of approximately 2 gm of endotoxin protein kg/day, no toxicity or infectivity was observed after two years, although there was weight loss in the treated group. In some cases, higher levels have been tested with no indication of toxicity.

Multiple exposure and long-term studies may be needed to consider whether negative results at the maximum hazard dose are also applicable to address toxicity following multiple and/or long term exposures. Data currently available indicate that most plant pesticidal proteins are rapidly degraded (within 2-30 minutes) to smaller non-toxic peptides and amino acids in mammalian gastric juices. These cleavage products in essence become nutrients, and there is no evidence that they bind to or accumulate in mammalian tissues. There is no reason to think that under the circumstances of multiple or repeated long-term exposures that the basic physiological processes of the mammalian digestive system will change, and thereby make these proteins toxic.

One Panel member also commented on general issues of mammalian toxicity to plant pesticidal proteins. Risk may only occur at or above a certain threshold, and in that situation, depends on the level of exposure to the hazard (toxic pesticidal protein). The existence of the hazard does not *per se* imply an appreciable risk. The risk may be threshold and in that situation, a safety evaluation involves deriving a level of exposure on a body weight basis, which if continued daily for a lifetime, would not result in appreciable risk. Conversely, for non-toxic

pesticidal proteins, a threshold of toxicity cannot be demonstrated or assumed and it may be necessary to estimate the risk quantitatively associated with given dietary levels of the proteinaceous plant pesticidal compound (for example Cry1Ab5).

2. The Agency believes that longer-term testing is not applicable for digestible protein plant-pesticides that display no toxicity in the acute oral toxicity tests. Under what circumstances, if any, should the Agency require repeat dose (30 day) feeding studies for protein plant-pesticides? For repeated dose studies, what animal model test system and anticipated effects or toxic endpoints would the Panel suggest be considered?

While the consensus of the Panel generally concluded that studies beyond the 30-day acute oral toxicity testing are not necessary for digestible protein-plant pesticides that display no toxicity, there are some exceptions. There is a tendency to consider the pesticidal proteins as being biochemically highly similar, although clearly there are differences among them. For example, the DNA sequence similarity among insecticidal proteins can be rather low (as low as 25% among Bt Cry proteins, Feitelson et al, 1992; Crickmore et al., 1998).

Therefore, the toxicological requirements for proteinaceous plant pesticides need to be considered on a case-by-case basis. Demonstration of the lack of amino acid sequence homology to known protein toxins/allergens and their rapid proteolytic degradation under simulated mammalian digestion conditions is generally appropriate to confirm the safety of these proteins (Joint FAO/WHO Consultation, Rome, 1996).

In consideration of their structural composition, pesticidal proteins should not be evaluated in the same manner as additives or chemical xenobiotics. Dietary proteins have not been associated with teratogenic, mutagenic or carcinogenic effects in rodent models. In addition such effects would not be expected to be based on their structure/function. Thus, genotoxicity studies (*in vitro and* short-term *in vivo* studies for mutagenicity/clastogenicity), chronic toxicity (usually in diet or drinking water), carcinogenicity (usually 2 years in rodents); reproductive toxicity (single/multiple dose studies during pregnancy), and multigeneration studies (dietary administration prior to and during mating, gestation and suckling) should not be required.

However, the testing program described by the Agency may not be sufficient for all pesticidal proteins. Some proteins are toxic such as bacterial toxins (i.e. cause acute toxic effects ranging from GI-tract discomfort to life threatening dehydration, paralysis and death), protease inhibitors and lectins/hemagglutinins. Thus, the circumstances that require a repeat dose (30 day) feeding study for the protein under consideration are: 1) the specificity and biological function/mode of action of the newly introduced protein is only partly known or unknown; 2) the protein is implicated in mammalian toxicity (e.g. PHA-E agglutinates erythrocytes), 3) human exposure to the protein is not documented, or 4) novel proteins are created via modification of the primary structure of the naturally occurring protein pesticide.

The safety of a novel pesticidal protein could be demonstrated on a case-by-case basis using a tiered approach by combining biochemical and animal models which could include the following studies (Noteborn et al. 1995, 1998; Kuiper and Noteborn, 1996):

(1) comparison of the plant protein with the bacterial protein by considering items such as molecular weight, full length amino acid sequence, amino acid composition/sequence, post-translational modification and functional characteristics;

(2) homology to known toxic proteins and by comparison of the sequence of the pesticidal protein to known protein toxins and allergens using data bases, predicted 3D-structure, and amino acids sequence in regions of the protein that are critical to allergenic or toxicological properties;

(3) investigation of enzymatic/functional activity looking at pH, substrate, and temperature dependency;

(4) digestibility and stability of the protein in simulated gastric and intestinal fluids and ex vivo gastric fluid (e.g., pig, cattle, dog) and in vivo models to reflect the physiological situation in the digestive tract, with these tests being validated for predictive value;

(5) acute oral toxicity study limited testing in a rodent species using the protein at one high dose level (i.e., OECD guideline 401 (three-dose method) or via OECD guideline 420 (fixed dose-method)) is not essential but it will allow to study the nature of the pathogenicity of the plant pesticidal protein based on mechanism of action in mammals (e.g., receptor binding and acute target effects) and required ADME parameters. In addition, the perfusion of ligated intestinal segments and other in vivo models using animal species such as rabbits, rats and suckling mice should be used for enteric toxic assessment [Sears and Kaper, 1996];

(6) sub-acute repeated dose feeding study (30 day) in rodents including a tier I immunotoxicity screen (modified OECD guideline 407) to establish dose-response characteristics (i.e., LOAEL and/or NOAEL), as follows [OECD Guideline 407: Acute Oral Toxicity, OECD Paris, 1987; Modification of OECD Guideline 407: Repeated Dose Oral Toxicity - Rodent: 28-day study (OECD Paris, 1987) by van Loveren and Vos: RIVM Report no. 158801001, Bilthoven, 1992; Dutch Health Council: Committee of Immunotoxicology: Immunotoxicity of Compounds, The Hague, 1991].

Modification of the OECD guideline 407 would include an extended testing in the rodent model with focus on a Tier I screening (i.e., differential leukocyte counts, histopathology of spleen, total IgM/IgG/IgA and IgE in serum, weight and histology of thymus/spleen/mesenteric lymph nodes, histology of GALT, bone marrow cellularity, cytofluorometry of lymphocyte subpopulations in the spleen, natural killer activity). It is the intention of this modified OECD protocol to identify any potential immunotoxicity and provide an indication for a Tier II screen to

predict whether the pesticidal protein will reduce the ability of the immune system to defend the host to infections etc.

Therefore, all the investigations recommended in OECD guideline 407 should be performed, including additional endpoints linked to the mode of action and specificity of the pesticidal protein under consideration. The triggers for these additional tests/observations should be assessed in the repeated dose study (e.g., gut toxicity, receptor binding etc.). In addition, immunotoxicity Tier I assessment and Ad hoc studies on special end-points where indicated (i.e., developmental toxicity, immunotoxicity (tier II screening); neuro-behavioural toxicity etc.) should be considered.

(7) immunotoxicity studies using animals models <u>if indicated</u> via the IFBC/ILSI decision-tree.

Despite the complete immunological cross reactivity between some parent and variant enzymes, there were clear differences with respect to allergenic activity measured in a guinea pig model with respect to allergic IgE antibody titers and dose-response analysis. Compared to the parent molecule, some of the variant enzymes tested provoked a more vigorous response at lower exposure concentrations. In the peanut allergen model, mutagenesis of the major peanut allergen epitopes to non-IgE binding peptides, produced both increased and decreased IgE binding activity as measured by immunoblot analysis with serum from peanut-sensitive individuals.

The ability of a protein (allergen) to induce an allergenic reaction can vary considerably. Thresholds of reactions differ both within and between individuals with time and level of exposure. Neonates, adolescents, and adults all have been shown to respond variably to exposure, dose and other unknown environmental factors (e.g., smoking) that may contribute to an altered immune response. As there is no unanimous agreement on the degree of hydrolysis or digestibility that will render a protein free of potential immunogenicity/allergenicity, except at the single amino acid level, only a causal relation between exposure (i.e. ingestion/inhalation/contact) of a particular protein and a subsequent obvious clinical reaction can be associated with diagnosis. Enzymatic digestion may expose epitopes that are shared by other non-related proteins or they may completely destroy epitopes. The question of the degree of protein digestibility can therefore stimulate or render the host tolerant to the peptides.

Thus, as with respect to immunological or non-immunological mediated adverse reactions, no single symptom is pathogonomic. Nor is any single laboratory test diagnostic for adverse reactions. Predictive approaches to determine aberrant potential of any protein must therefore be subject to a case-by-case critical appraisal, allied with implementation of monitoring of the potential postmarketing impact of the product on public health. Thus, a cooperative effort should develop between the registrant, Federal agencies such as EPA and the medical community, to monitor the extent of aberrant incidences upon exposure to the product. This recommendation aligns with previous recommendations by the Panel, specifically those addressing food allergenicity of Cry9C endotoxin and other non-digestible proteins.

With respect to the immune system and antibody production, the effect of the intact or fragmented protein on the immune response must also be taken into consideration with respect to the food source. For example, in the rat model, the immune system responds at a much lower concentration of the major milk allergen, beta-lactoglobulin, when seen in the context of whole milk compared to the protein alone. Thus, there appears to be a natural adjuvanticity of the food source that must be taken into consideration.

The Panel's response to this question also raised issues concerning protease inhibitors, -amylase inhibitors, and lectins. Comments by a Panel member concerning these issues are discussed in a later section of the report entitled "Additional Comments".

3. Assume that repeated dose (30 days) testing is indicated and that the test animal diet is appropriately adjusted to provide a healthy diet. Does the Panel agree that whole-food testing with plant-pesticide containing food products does not provide a means to apply an appropriate margin of safety in these studies? Would the plant-pesticide expressing food crop in question amended with pure protein plant-pesticide to yield a higher dose be an appropriate test substance? Or would the purified protein as test substance alone be more appropriate?

Taken together with knowledge of the amino acid sequence, its mechanism of action, and the fact that the pesticidal protein is readily degraded in gastric/intestinal fluids, this may be taken as a strong indication of the safety of the protein in the whole transgenic crop plant. However, 30-day feeding studies (OECD guideline 407) with both the pesticidal protein and transgenic crop plant and/or processed foodstuff can yield additional information (i.e., protein toxicity versus unintended effects) if realized that feeding studies with whole foods are difficult to carry out. One may have to deal with a number of experimental shortcomings such as limited dose ranges in order to prevent unbalanced diets, which may render the results difficult to interpret and of limited value.

The concentrations of heterologous proteins in plants are generally too small to be conveniently isolated and measured. As a consequence, it is also difficult to determine accurate measures of toxic doses, or to assign accurate margins of safety, for test animals when the toxin is administered as part of a balanced diet. One obvious solution is to add purified protein, typically expressed in bacteria, in varying amounts to the test food. This keeps the nutrients from the diet in balance and eliminates toxicity that might arise naturally from excessive feeding of certain classes of foods, such as tomatoes and potatoes. This treatment method will not generally help quantify other kinds of toxicity that may arise by chance from interactions of the heterologous protein with food material, nor will it increase the reliability of measures due to food gene disruption. In general there is merit in testing whole foods for toxicity, as they will be used by the consumer. However, proper tests of the toxicity of food products are difficult to devise, given the above mentioned limitations of diet.

Whole-feed testing with plant-pesticide containing food products does not provide a means to apply an appropriate margin of safety in the studies directed toward the assessment of the plant pesticidal protein. In principle, the protocol of such a whole food testing is primarily directed toward answering the question whether there are unintended effects (pleiotropic effects) due to genetic modification (OECD, 1993). Thus, additional testing should occur in the following scenarios:

(1) a completely new gene and/or transgenic crop plant;
(2) crop plant extensively changed as a result of biotechnology (metabolic pathway engineering);
(3) pesticidal proteins as antinutrients;
(4) pesticidal proteins without a clear threshold (i.e., bacterial toxins);
(5) crops/products with predicted high levels of intake of a toxic pesticidal protein (i.e., protease crop/product inhibitors);
(6) non-rapidly degradable proteins or crop plants with profoundly altered undesirable compositions.

More extensive testing with the whole product may be required for the assessment of pleiotropic effects due to genetic modification. But this will need careful judgement based on all the available background information on the food.

While whole food testing may not allow accurate assessment of safety margins, it might be necessary to dose food stuffs with exogenous toxins to determine accurate safety margins for that toxin. Pure toxin testing does not allow assessment of unexpected toxicities, or allergies, that might arise by interaction of the introduced protein with molecules normally present in the plant.

There may be several ways in which toxicity could arise from the expression of heterologous proteins in plants. In the simplest case, the heterologous protein may be directly toxic. It is possible that addition of an agent that is itself nontoxic, like an exogenous protein, may interact with elements of the food mixture to create an unexpected hazard. At this time, there is no clear example of such a phenomenon, but it is possible. It is also possible that insertion of a heterologous gene, as part of trait modification, could disrupt the expression, or regulation, of natural foodstuff genes. The resulting disruption may generate, or increase toxic or allergic agents independent of the introduced gene. Also, a heterologous protein that is not readily digestible by mammals might be able to elicit chronic effects. These situations may dictate a multidose testing regimen using whole foods that contain a heterologous gene (or induced trait).

The challenge to the scientific community is that protein pesticides introduced into genetically modified crop plants are often present at low levels. Proteins obtained from cultures of genetically modified bacteria are therefore employed in toxicity tests. This, however, carries the risk that toxic impurities may be present, which are uncommon for the crop plant, and that

protein processing, like glycosylation, may be different in plants and bacteria. To show that these proteins are equivalent, usually their molecular weights are compared. The current methods applied, however, do not allow the detection of the minor post- translational modifications, which might indeed be relevant (e.g., allergenicity, receptor binding, stability etc.).

Several Panel members recommended the following in vitro tests:

(1) identical behavior of the full length as well as the trypsinated forms on 2D-gel electrophoresis;
(2) identical immunoreactivity (i.e., binding) to poly- and/or monoclonal antibodies;
(3) identical patterns of post-translational modification (i.e. glycosylation). The production of large amounts of pure recombinant pesticidal proteins for use in toxicity trials and other experiments should be performed by over-expressing the encoding genes in, for instance, *Pichia pastoris*, an eukaryote that produces biologically active plant lectins such as snowdorp lectin (GNA) and *Phaseolus vulgaris* agglutinin E-form (PHA-E) [Gatehouse and Gatehouse, 1998]. In caseins the phosphorylated seryl residues appeared to be important immunoreactive regions within the molecule (Wal, 1998);
(4) sequence similarity of full length amino acid sequence (highly undesirable is the sequence analysis of 10-15 N and/or C-terminal amino acids and up to three short internal protein sequences). For example, two isoforms of bovine -lactoglobulin i.e., genetic variants which differed only by two point mutations on residues 64 and 118 (D and V versus G and A) showed modified allergenic properties [Wal, 1998];
(5) toxicity similarity to target herbivoral insect species (i.e. larvae).

However, other Panel members disagreed. The pesticidal protein should only have to be produced in the plant in which it will be used commercially. For example, if a Bt Cry protein is produced in *Pichia pastoris*, and is glycosylated and becomes immunogenic, this may not have any relationship to with the post-translational processing of the same protein in corn or other crops. If it is glycosylated in *P. pastoris* and becomes immunogenic, this could be very misleading and could lead to scientifically unsound conclusions with respect to its post-translational processing and immunogenicity in a wide range of other crops.

It is important to monitor for agronomic, phenotypic and biochemical changes during the development and breeding of the transgenic pest-resistant crop plant (Noteborn et al. 2000). The food product tested should be in a similar form to that which would be consumed by humans or animals. Many natural toxins, such as phytohaemagglutinins (lectins), trypsin inhibitors etc., are inactivated during cooking/processing while they are potentially toxic in the raw commodity. Based on these considerations, the following information should be provided relating to crop plant material:

(1) a detailed nutritional evaluation covering composition and potential bioavailability of macro- and micro-nutrients is essential before embarking on long-term toxicity studies in order to design the diets to be used and to avoid the complications of nutritional inadequacy of test diets;

(2) establishment of a database for natural plant compounds of potential dietary or other toxicological concern including variations in natural levels are urgently needed. On the other hand, monographs of major crops would help if their contents include information on the genetic background, natural levels of compounds including their natural variations and impact of environmental conditions;

(3) factors which should be considered before embarking on toxicity testing of transgenic crop plants are: identity, source (appropriate traditional food comparator), composition (macro and micro-nutrients, non-nutritive factors, natural toxicants in the traditional food used as comparator and in the transgenic crop plant, presence and nature of new chemical entities), potential intake (including specially vulnerable groups), and effects of processing/cooking.

It is generally considered that a longer-term study of 90-day duration according to OECD guideline 408 is the minimum requirement to demonstrate the safety of repeated consumption of a novel crop plant in the diet. This may need to be preceded by a pilot study of short duration (30-day) to establish a LOAEL/NOAEL of the pesticidal protein, and to ensure that the diet is palatable to the laboratory animals and that the levels of incorporation of the test article are appropriate e.g., that the semi-synthetic control human-type diet containing the comparator is not such as to produce effects from normal levels of nutrients and/or natural toxicants present in traditional foods accepted as safe. The highest dose level used in any animal feeding trials should be the maximal achievable without causing nutritional imbalance, while the lowest level used should be comparable to the anticipated human intake. Freeze-dried powders instead of fresh, raw materials including juices should be used as they also avoid some of the pitfalls which were encountered in early toxicity tests (e.g., gastric erosions) seen when attempts were made to administer tomato paste by gavage to rodents.

If required in exceptional cases, the 90-day study should be composed of at least the following test groups:
(1) pesticidal protein at LOAEL added to a semi-synthetic human-type diet;
(2) pesticidal protein at LOAEL added (spiked) to a semi-synthetic human-type diet containing the maximal achievable amount (MTD) of the transgenic crop plant;
(3) semi-synthetic human-type diet containing the maximal achievable amount (MTD) of the transgenic crop plant;
(4) semi-synthetic human-type diet.

The above described protocol illustrates the possibility of increasing the sensitivity of toxicity tests compared with that possible if the crops had been fed directly. However, this strategy is only applicable if preceded by detailed analysis of both the required biological endpoints (e.g., lectins and protease inhibitors) and the dietary composition to ensure that the expected changes in composition of the crop plant (i.e., insertion of a pesticidal protein) are the only ones occurring.

4. What is an accepted method of amino acid homology/similarity analysis that can be used to screen for a protein function like mammalian toxicity ? Are there any analyses that examine higher levels of protein organization (i.e., secondary, tertiary and quantriary) that could also be incorporated in these structural comparisons?

The Panel concluded that there is some evidence to indicate that secondary or tertiary structure may be important in plant allergens. This involves the profilins and other plant pathogenesis related proteins that have a considerable degree of barrel sheets in their structure. However, there is neither enough evidence nor sufficient numbers of plant proteins identified to establish this as a criterion. Plant protein structural integrity may play a significant role in the allergenicity of the protein, but data are not yet available to establish this as fact. Higher structure order, oligomer formation, may increase the resistance to the digestive enzymes of the gastric and mucosal systems prolonging the presence of the proteins in the GI tract and subsequently access to the immune system.

With respect to allergen epitopes, a minimal linear sequence of 8 amino acids appears to be critical. This does not however, take into consideration conformational epitopes. The degree of amino acid sequence homology in the active site of the toxin to known allergen epitopes or other toxic sequences should identify potential areas of concern. The available databases are sufficient for this purpose. However, it must be recognized that not all allergens have been characterized. Epitope, IgE binding, does not necessarily correlate with clinical disease. For example, there is considerable cross reaction (i.e., IgE binding to proteins found in peas, beans, soybean, and peanuts) among the legumes; however, upon challenge, it is unusual for an individual to respond clinically to more than a single legume food source. The binding site to tissues or other proteins that render a pesticide a viable candidate for use should also be a concern. For example, cholera toxin has two subunits, one that is immunogenic and one that has an adjuvant effect which increases mucosal inflammation. Both subunits have important implications to the immune response, such as direct immunoglobulin production and recruitment of cells of the immune system. Many pesticides have a mode of action (binding to specific amino acid sequences) that will allow access to immune cells. For example, many toxins have binding sequences to specific receptors on the target organism or tissue that will also bind to receptors on antigen-presenting cells (macrophages, epithelial cells) that could lead to direct or indirect peptide antigen recognition by the mucosal immune system. In the host-parasite or plant-pesticide interaction, there is "danger signal" recognition that may have relevance in the plant and/or bacterial recognition of toxin or proteins to the mammalian system as a "danger signal". This is based upon the immune system of mammals or recognition of danger to the plant or bacterial host that has developed and been refined during the course of evolution.

There are many computer-based amino acid search programs that can reveal similarities to toxins. Higher level searches of structure are not likely to be particularly fruitful at this time.

It is reasonable to compare the amino acid sequence and structure of a heterologous protein, expressed in a plant, with that of known toxins (or allergens). However, acute toxicity

studies need to be conducted regardless of the results of a protein homology search. In this sense, the search merely acts as an early alarm mechanism and can signal that very close scrutiny of a plant-expressed protein is warranted.

Apart from the 20 amino acids that are normally protein constituents, there are at least 25-30 amino acids that are known to be metabolic intermediates in the biosynthesis of amino acids or are found as secondary metabolites. As shown in the Table 1, some of these non-protein amino acids are potentially toxic or have been implicated in diseases of humans. Whether these compounds are simply by-products of metabolism or have been synthesized for a particular function, the fact remains that some of these amino acid homologues have been shown to have insecticidal activity. Because of their structural similarity to protein amino acids, they can act as metabolic antagonists which no doubt accounts for their protective effect, although in most cases they are not incorporated into newly synthesized proteins. The legumes, in particular, are rich in these compounds.

Unlike natural protein pesticides, these non-protein amino acids are not readily destroyed by processing or heat treatment. Their presence, however, can readily be detected and quantitated by conventional techniques used for analysis of amino acids. Rosenthal (1991) predicted that *the striking advances in molecular biology make possible production of transgenic plants possessing a newly acquired capacity to synthesize a given non-protein amino acid [with protective potential]*. It therefore becomes important to not only be aware of the presence of these novel amino acids in transgenic plants, but also to be able to evaluate whether they would in fact pose a risk to human health.

Similarity of amino acid sequence is often indicative of homologous structure and function and the higher the identity or similarity, the more likely the functional equivalence. If a protein to be expressed heterologously in plants (induced trait) has a very high similarity, say 80% amino acid sequence identity, to a protein known to be toxic to mammals, then there is reason to believe it too may be toxic. It is possible, however, for toxic homologs to have a much less obvious relationship. For example, plant toxins like ricin A chain and pokeweed antiviral protein show about 28% identity to one another and they show roughly 20% identity with the active enzyme from Shiga toxin. There are a number of protein databases, including SwissProt and GenBank. Some commonly used comparison programs are ALIGN, CLUSTAL, FASTA, MACAW, and BLAST that use differing alignment algorithms and report results in different ways. This makes it difficult to assign a numerical level that should trigger a more rigorous test regimen.

Table 1. Non-Protein Amino Acids

Plant Source	**Amino Acid Derivative**	**Toxic Effects**
Lathyrus odoratus	3-aminopropionitrile	Osteolathyrism
Lathyrus sativus *	3-N-oxalyl-2,3-diamino propionic acid	Neurolathyrism
Lathyrus latifolius	2,4-diaminobutyric acid	Neurotoxin
Vicia sativa *(vetch)	3-cyanoalanine	Neurotoxin
Cycas circinalis (cycad)	3-N-methylaminoalanine	ALS
Blighia sapida (ackee tree)	beta-(methylenecyclopropyl) alanine (hypoglycine A)	Hypoglycemia
Vicia faba * *Macuna* *	3,4-dihydroxyphenylalanine	Favism (?)
Astragalus	Selenomethionine	Selenosis
Canavalia ensiformis *	Canavanine	Lupus
Linum usitaissimum	Linatine (1-amino-D-proline)	Antimetabolite of pyridoxine
Lens esculenta	O-oxalyl homoserine	Antimetabolite of methionine

*Exhibits insecticidal activity

In addition to comparing the raw scores of sequence alignments, it may be useful to search protein sequences for signature sequences that are characteristic of families of enzymes; for example, there are signature sequences for chitinases and RIP toxins. These signatures are often important for the correct folding or mechanism of action of the protein and tend to be much more conservative than the overall sequence. It is also important to bear in mind that toxic functions may represent only a fraction, typically a self-folding domain, of a larger protein. A heterologous protein may exhibit only a low similarity, in total, to a known toxin, but could still contain a toxic domain that would give a stronger similarity signal when the sequence data are properly edited.

The tertiary structure of proteins is far more conservative than the amino acid sequence and gives a much better indication of a functional similarity. However, the structure of a protein is not generally known until it is well characterized biochemically. That is, if a heterologous protein has had its X-ray or NMR structure determined, its function is almost certain to be well established. In principle there is merit in attempting to assign a tertiary structure to the amino acid sequence of a candidate for heterologous expression, in order to assess more accurately its potential as a mammalian toxin. Computer programs can be used to put proteins of known structure into family classes, like the FSSP. Such information could suggest a correlation between proteins of differing biochemical function, but it is not likely that, say, a comparison of antifreeze proteins would reveal an unexpected relationship to a family of toxins. There are also a number of threading programs that attempt to fit a given amino acid sequence to an archetypal protein fold. Presently, these methods are unlikely to reveal significant information about toxicity because the amino acid sequence itself would have to suggest that the candidate resembled a known toxin. Programs that attempt to generate the fold of a protein de novo, say based on energy considerations, have not yet been shown to be robust.

If a sequence homology match to a known allergen is identified for the introduced protein (Gendel, 1998a,b), then this pesticidal protein should be assessed for immunoreactivity using sera from individuals sensitive to that particular allergen. The approach becomes similar to the one used when the genetic material is obtained from known allergenic sources (IFBC/ILSI decision-tree). If no sequence homology to known allergens is identified, then the stability of the protein to digestion and processing is assessed as part of the overall safety assessment.

The predictive value of the general biochemical characteristics of proteins with regard to folding and spatial configuration belonging to certain protein classes will be high, however, their predictive value in allergenicity assessments is rather limited. Research is needed to define the minimal food allergen epitopes, determine the effect of amino acid substitutions and clarify the role of the discontinuous epitopes. Even for the known allergens showing IgE binding sites, exceptions to postulated rules exist. Although it will be good to rate a given protein for all characteristics including toxicity, protein family, IgE responsiveness and protein modeling, it will not be possible to predict or exclude conclusively any potential allergenicity of the protein solely on the basis of these ratings. For example, there are reasons to expect that IgE binding

epitopes are not similar among food allergens. Post-marketing surveillance may be a useful tool to detect potential allergies in consumers to unsuspected newly introduced proteins.

Allergens with unknown structures, of course, cannot be considered for comparison. So far, from most other major food allergens, only information on their IgE binding sites is known, but not all IgE binding epitopes have been mapped (Hefle 1996b; Ruibal et al. 1997). If the newly introduced genetic material is derived from an allergenic source and the encoded proteinaceous plant pesticide is found allergenic, it is recommended not to consider it for market approval as a novel food/feed unless it can be properly labeled as allergenic [FAO, 1995].

Conformational structural analysis could be used to identify conformational characteristics on any protein with known three-dimensional structure to classify its possible biochemical properties (i.e. enzyme inhibitor, pore forming toxins, lectin-type protein etc.).

Specific aspects of the food allergens

Crop plants can contain both major and minor allergens (Bush and Hefle, 1996). Major allergens are defined as proteins for which 50% or more of allergic patients have specific IgE (King et al., 1994). The amino acid sequences of many food allergens are known [Metcalfe et al., 1996]. The amino acid sequences of the epitopes or IgE-binding regions of plant proteins are known in only some cases (Bush and Hefle, 1996). Many food allergens are present as major protein components, typically ranging between 1.0 and 80% of total protein (Metcalfe et al., 1996). Food allergens tend to be stable to digestion and (acid and heat-)processing (Taylor and Lehrer, 1996; Astwood et al., 1996). The ability of food allergens to reach and cross the intestinal mucosal barrier in immunologically intact form is likely a prerequisite to allergenicity [Sanderson and Walker, 1993].

In 1996, a decision tree was devised for this purpose by the International Food Biotechnology Council (IFBC) in conjunction with Allergy and Immunology Institute of the International Life Sciences Institute (ILSI) [Metcalfe et al., 1996]. The recommended strategy focused on: 1) the source of the gene, 2) the sequence homology of the newly introduced protein to known allergens, 3) the immunochemical reactivity of the newly introduced protein with IgE from the blood serum of individuals with known allergies to the source of the transferred genetic material, and 4) the physicochemcial properties of the newly introduced protein (Metcalfe et al., 1996). Moreover, three additional possibilities are considered: 1) the introduced gene is either derived from a common allergen (the 8 food groups generally mentioned in the literature, e.g. peanuts, soybeans, tree nuts, milk, eggs, fish, crustacea and wheat); 2) a less common allergen (the 160 foods identified by Hefle et al., 1996), or 3) is unknown as an allergen.

The most difficult assessment involves genetic material obtained from sources of unknown allergenic potential or toxicity. Indeed, there is no predictive allergenicity assessment protocol for plant pesticidal proteins from non-food sources. The relationship between protein function (i.e. allergenicity) and particularly primary structure is not well established. There is no

particular kind of biological function associated with food allergens and allergens in general. No specific biological function is connected with the ability of allergens to induce IgE production (Hefle, 1996a, Zhang and Mohapatra, 1996). For example, sequence comparison revealed putative functions, such as a thiol protease (Chua et al., 1988), a pathogenesis-related protein (Swoboda et al., 1994), an RNase (Bufe et al., 1995), a phospholipase (Hefle 1996a) and an exoglucanase (Zhang and Mohapatra, 1996). Helper T-cell epitopes and B-cell epitopes of various allergens have been identified (Kaminogawa, 1996, Hefle, 1996b). Unfortunately, comparisons of the amino acid sequences of these allergenic proteins has not yet yielded any unique or typical pattern specifically related to allergenicity. There does not appear to be a particular pattern of the (primary) structure of a protein denoting an allergen (Fuchs and Astwood 1996, Hefle 1996a). Some kind of predictive approach should be designed. Therefore, animal models, in vitro tests and protein structure should be taken into account.

The allergenic potential of an unknown protein can be estimated by comparison of the amino acid sequence to that of known allergens (King et al 1995; Metcalfe et al. 1996). Potential allergenicity of a given protein is supposed to require a sequence similarity with a known allergen of at least eight contiguous identical amino acids or chemically similar amino acid residues (Fuchs and Astwood 1996). Another criterion includes 30% amino acid identity of a given protein to a known allergen (Wal, 1998). It is important to realize, however, that such comparisons can only give an impression of the putative allergenicity of a given protein. Absence of any structural homology does not imply absence of allergenicity as it must be kept in mind that not much is known about the characteristics of food allergens.

The amino acid sequences of 198 major allergens including about 30 food allergens from plant origin are known (Metcalfe et al., 1996). In the IFBC/ILSI decision-tree strategy, it is assumed that antigenic sites are surface features of the proteins and should be related to regions of high hydrophilicity. The theoretical prediction of antigenic determinants would require a significant sequence similarity and would require a match of at least 8 contiguous identical amino acids (Metcalfe et al., 1996). The minimal peptide length for binding to helper T cells, which would be required for allergen sensitization, appears to be 8 or 9 amino acids (Roitt, 1994; Pernis, 1992; Rothbart and Gefter, 1991). Carbohydrates are not involved in this recognition. From only a few allergenic proteins some helper T -cell epitopes are currently known: ovalbumin [Shimojo et al., 1994), casein and lactoglobulin (Kaminogawa, 1996), bee venom (Fehlner et al., 1991a,b; Zhang and Mohapatra 1996) and several grass pollen allergens (Romagnani and Mohapatra, 1996). It is not yet clear whether these helper T -cell epitopes represent the complete spectrum of epitopes of each allergen for each individual.

The peptide length required for an IgE-binding epitope could be even longer (Rothbart and Gefter, 1991). B-cell epitopes are conformational, i.e., dependent on the tertiary structure of a protein. They may be composed of several adjacent amino acid sequences on the surface of the protein, but can also be determined by a carbohydrate moiety (Taylor and Lehrer, 1996). In the case of sequence recognition, it is generally assumed that B-cell epitopes consist of 15 to 22 amino acid residues, of which only a few (about 6) residues may be especially critical for binding to the

antibody (Kaminogawa, 1996; Wiedeman et al., 1996). There are virtually no consensus sequences among B-cell epitopes. Although polar or charged amino acids are often found, a single protein can generate different epitopes. The location of B-cell epitopes on the casein molecule was examined (Wal, 1998). Remarkably, up to five regions of the protein were recognized, but these regions differed between the mouse strains used. Two regions were recognized by antibodies of all mouse strains involved (Kaminogawa, 1996). There is an essential association between the potentially reactive fragment (epitope) of the protein, well defined and stable (i.e. caseins), and binding region of its corresponding IgE antibody, which is variable in affinity and specificity because of the genetic heterogeneity of the population. For example, caseine fragments are not always indistinguishly recognized by human IgG and IgE (Wal, 1998).

The accuracy of allergenicity assessment by amino acid homology analysis using alignment programs such as FASTA and the BLAST algorithm (Gendel, 1998a,b) and sequence homologies retrieved from several protein structure databases such as GenBank, TrEMBL, Swiss-Prot, PIR (e.g. Burks, 1999) is certainly questionable and does not give common and universal information with respect to the allergenic potency of a plant pesticidal protein. For example, using antisera of specific anti-alpha-lactalalbumin IgE from milk-allergic patients, Maynard et al. (1996) demonstrated that the allergenic sites in alpha-lactalalbumin may not always correspond with the antigenic determinants predicted by computer modeling, structure modeling or using sera of hyper immunized animals. Thus, there are reasons to expect that IgE binding epitopes are not similar among food allergens.

The database of allergenic sequences (but also for toxic fragments or domains) must be extensive enough to encompass all relevant sequence features related to allergenicity (but also to toxicity characteristics). An exact match based upon the identity of 8 contiguous, identical amino acids seems to be a conservative approach, but they occur infrequently by chance and thus trigger further testing. According to Gendel (1998a) it is likely that local alignments will be more useful in the case of allergenicity assessment (i.e., only regions with a high degree of similarity) between evolutionary unrelated proteins than using a global algorithm that optimize alignments/matches across the entire full-length of the protein as has been suggested by Taylor and Lehrer (1996) and Metcalf et al. (1996). Insufficient information is available on the amino acid sequences of allergen epitopes especially for food allergens. This lack of reliable criteria makes it difficult to assess the biological significance of the matches that are found (Gendel, 1998a). Concerning T-cells, the major birch pollen allergen had only a 4 amino acid sequence in common (Vieths, 1998). On the other hand, a comparison based on 4 instead of 8 identical amino acids might yield too many homologues sequences and thus too many 'false positives' for potential epitopes. Are exact matches really necessary? Can threonine and serine (OH-amino acids) substitute for one other? Moreover, the approach is also clearly limited in that it cannot identify discontinuous or conformational epitopes which depend upon the tertiary structure of the protein and, such an approach will likely yield some frivolous matches that have nothing to do with IgE binding.

Amino acid sequence homology analysis does not give much predictive information. For example, shrimp tropomyosin and chicken tropomyosin share a 60% amino acid sequence

homology, but only the shrimp protein is a potent allergen (Ruibal et al. 1997). In case of helper T-cell as well as on the B-cell level, there are examples of 4 contiguous amino acids which trigger the suspicion of (potential) allergenicity (Vieths, 1998; respectively, birch pollen and codfish). Discontinuous or conformational epitopes are not recognized as they cannot be described by one stretch of amino acids (i.e. depends on tertiary structure). Glycosylation patterns of proteins for recognition by B-cells are not part of the decision-tree. Thus, there is a risk of identification of identical homologous sequences in the amino acid sequence that are not correlated with potential allergenicity.

An additional issue is the relevance of glycosylation patterns of proteins for recognition by helper T and/or B-cells. Many food allergens are glycoproteins (Lehrer et al., 1996). Glycosylation, covalent linkage of oligosaccharide chains (glycans) to the protein backbone, is a major post-translational modification of proteins. In crop plants, two types of glycosylation occur, i.e., O-linked bound to hydroxyl groups of serine, threonine or hydroxyproline or N-linked bound to asparagine. The amino acid sequence of Asn-X-Thr (or Ser) is generally thought to be the N-glycosylation site of plant proteins (Faye et al. 1989). The involvement of O-linked glycans in allergenicity of plant glycoproteins remains unclear (Petersen et al. 1995, Garcia-Casada et al. 1996; Gupta et al. 1996). N-glycosylation of a given protein can be directly responsible for antigenicity in case the immune system reacts towards the likely immunogenic carbohydrate (N-glycan) epitope (Ohta et al. 1991; Tretter et al. 1993; Hijikata et al. 1994; Petersen et al. 1995; Zhang and Mohapatra 1996). Especially the -1,3 fucose and -1,2 xylose residue is suggested to be an antigenic epitope of plant glycoproteins [Faye et al. 1989; Bando et al. 1996; Batanero et al. 1996; Jenkins et al. 1996; Zeleny et al. 1999]. Xylosylation and fucosylation of N-linked glycans are suggested as the key features responsible for the high allergenicity and non-specific cross-reactivity of various pollen and vegetable proteins (Garcia-Casado et al. 1996). Alternatively, the protein glycosylation is indirectly responsible for the antigenic character of a glycoprotein. The presence of the carbohydrate groups on mature proteins affects several biochemical properties that increase the likelihood of allergenicity. A general character of glycoproteins is their highly increased solubility, proteolytic and thermal stability as compared to the unglycosylated counterpart (Faye et al. 1989; Jankiewicz et al. 1996; Faye et al. 1989).

3D-structural modeling may be very useful to reveal data on the structure-activity relationship of the major protein families (i.e. spatial/folding configuration comprises less than 6-7 families), such as storage proteins, calcium-binding proteins, enzymes, lipocalins, profilins, A-B toxins etc. For example, reviewing the structural properties of hemolytic delta-toxins.

The results of computer aided sequence alignments for linear epitopes with known allergens (SWISS-PROT: Bairoch and Apweiler, 1999; PIR: Barker et al., 1999) may vary depending on the method employed for comparing sequences (Gendel, 1998; http://www.sander.embl-heidelberg.de/course). In fact, as described before, epitopes may be sequential (linear sequences), or continuous, when they correspond to a fragment (peptide) of the primary structure, or they may be configurational, or discontinuous, when they correspond to distant fragments of the amino acid chain, brought close together by the folding and spatial configuration of the protein molecule. Their

sizes ranges from 4 to 6 (linear epitopes) and 15 to 22 (spatial epitopes) amino acid residues depending on the molecule. With the ILSI decision-tree linear approach, no three-dimensional epitopes can be compared with known allergens. This may change in the near future, however, as user-friendly protein modeling software became available recently, allowing for prediction of three-dimensional structures from linear sequences (for overview and progress see: http://www.embl-heidelberg.de/~rost/Papers/sisyphus.html).

For example, Kolaskar et al. (1999) predicted the 3D-structure of the envelope glycoprotein (Egp), a major structural antigen, of Japanese Encephalitis Virus (JEV) using the knowledge-based homology modeling approach and X-ray structure data of the Egp of tick-borne encephalitis virus as a template and mapped the conformational epitopes using the water-solvated structure.

Systems like the ExPAsy Molecular Biology Server including SWISS-MODEL compare a submitted protein sequence with a template sequence whose structure is already known (i.e. improvement of 3Dcrunch of Peitsch et al.) (http://www.expasy.ch/swissmod/SWISS-MODEL.html). As long as the template shares at least 25% sequence identity with the target sequence, SWISS-MODEL proceeds through a series of steps to generate a model structure based on the template. It may be possible to screen for discontinuous epitopes by using, for instance, also a sequence comparison based on 4 instead of 8 identical amino acids and using the proper algorithms (i.e. BLAST, VAST).

Entrez is an internet tool for retrieval of information on the structure and function of biological macromolecules [http://www4.ncbi.nlm.nih.gov/Entrez/]. It provides daily-updated databases of molecular sequences, 3D-structures, Medline citations pertaining to molecular genetics and information on molecular structure and function by a significant similarity score using the BLAST algorithm. Structural neighbor information in Entrez is based on a direct and quick comparison of 3D structure using the VAST (Vector Alignment Search Tool) algorithm by focusing on similarities that are surprising in the statistical sense rather than focusing on those occurring by chance in the protein structure [Bryant and Hogue, 1996; http://www.ncbi.nlm.nih.gov/structure/research/iucrabs.html].

Other useful databases are: 1) the PROSITE database (http://www.expasy.ch/sprot/prosite.html) consists of biologically significant patterns and profiles useful to determine to which known family of protein a new sequence belongs, or which known domain(s) it contains [Hofmann et al. 1999]; 2) the CATH database (http://www.biochem.ucl.ac.uk/bsm/cath) provides insights into protein structure/function relationships (Orengo et al., 1999); 3) the Dali databases organize protein space in the structurally known regions, and implies secondary and tertiary structures covering 36% of all sequences in SWISS-PROT (Holm and Sander, 1999).

5. Are peptide fragments that result from the breakdown of proteins more toxic than the intact proteins from which they originated? Are there examples of post-translationally modified proteins which have different toxicity compared to the non-post-translationally modified

proteins?

In general, peptide fragments that result from the breakdown of proteins are less toxic than the intact protein. Some toxins, like the maize-RIP, are expressed as pro-enzymes that must be activated by proteolysis. Proteins that are expressed in living system, including heterologously expressed protein pesticides, are ultimately degraded by an organism that ingests them. This process involves the action of a series of proteases that degrade the protein initially to peptides and eventually to amino acids that may be recycled or further metabolized. In general, proteins that are cleaved by proteases become inactive. Even a few breaks in the polypeptide chain can allow the protein to rearrange its structure, that is to partially denature, and to thereby lose native biological activity. There are clearly exceptions to this general rule. The human protein pro-opiomelanocortin (POMC) can be differentially cleaved to release eight peptide hormones. Some toxins are expressed as inactive precursors and are activated by specific proteolysis. An example of this is the ribosome inhibiting protein (RIP) from corn. The maize proRIP is cleaved in three places, removing short peptides from both termini and generating an active aggregate of two peptides of 17 and 8 kD. The famous insecticidal proteins from *Bacillus thuringiensis* are expressed as 130 kD protoxins that are activated by specific proteolysis in the alkaline environment of many insect guts.

However, the concern of this question appears to be the adventitious appearance of toxicity as a result of proteolytic digestion. Some small peptides arising from digestion could resemble toxic molecules. There are some small but potent toxins, like the 60 residue snake neurotoxins. However, these small proteins have evolved unusually stable folds, anchored by multiple disulfide bonds, and it is very unlikely that chance proteolytic fragments of heterologously expressed proteins would display this level of activity. There are even smaller peptide toxins like the 21 residue neurotoxic peptide, granulitoxin, from the sea anemone or the large family of conotoxins that are acetylcholine receptors. Again, these toxins have evolved under strong selective pressures and it is extremely unlikely that similar fragments would arise by chance from digestion of larger proteins. Finally, it is well known that mammals use a wide variety of very small peptides as powerful biological effectors including hormones. However, many of these peptides must be modified for activity and could not arise as active agents by chance digestion from other proteins; for example the tetrapeptide thyotropin release factor and the 11 residue pain mediator, substance P, are amidated at the C terminus.

In general, unmodified peptides arising in mammals from the digestion of plant material will have a very low probability of escaping the digestive and absorptive processes and reaching the blood or cytoplasm of cells in an intact and active state.

Proteins can cause a wide range of functional as well as adverse effects. As enzymes, they catalyze chemical reactions, certain proteins and peptides exhibit hormonal activity, and some act as antigens (i.e., allergens) or carrier molecules. On the other hand, peptide sequences from protein hydrolysates also can cause a wide range of functional as well as adverse effects (e.g. Meisel, 1998). Development in the field of functional foods will no doubt address the impact of, for instance, bioactive peptides on human health. For example, milk protein-derived bioactive peptides are

inactive within the sequence of the parent protein and can be released by enzymatic proteolysis during gut digestion or food processing (Meisel, 1998). Once they are liberated in the human body, bioactive peptides may act as regulatory compounds with hormone-like activity.

Examples of functional/hormone-like activities are peptide fragments containing one or two C-terminal proline residues showing Angiotensin I-coverting enzyme (ACE) inhibitory activities which are resistant toward digestion and active at low concentrations (50% inhibition: Val-Pro-Pro at 9 mol/L and Ile-Pro-Pro at 5 mol/L) (Takamo, 1998). Similar or homologous fragments/residues can be found in the Cry polypeptides such as in Cry1C: Glu-Pro-Pro-Pro (349-351), Ala-Pro-Pro (378-380) and Val-Pro-Pro (419-421) (US patent 6 033 874, Baum et al, par. 5.13) and in Cry9C: Arg-Pro-Pro (303-305), Asn-Pro-Pro (275-277/578-580). Whereas the amino acid sequences Tyr-Gly and Tyr-Gly-Gly possess strong immunomodulatory effects on human peripheral blood lymphocytes (PBL), respectively at 10^{-9} mol/L and 10^{-12} mol/L (Meisel, 1998).

In caseine, the phosphorylated seryl residues appeared to be important immunoreactive regions within the molecule (Wal, 1998). Related to this, it is well to remember the importance of careful scientific study and scrutiny of all aspects of the bioactive peptide arena (i.e., availability, concentration, structure-activity relationships etc.).

6. How does the breakdown of proteins differ in infants and individuals with digestive disorders compared to those with "normally" functioning digestive systems? Would risk from break-down products of an otherwise digestible plant-pesticide protein differ for these digestion impaired individuals compared to that posed by any other digestible proteins in their diet?

With respect to immunogenicity/allergenicity, a mature immune system and an intact gastrointestinal tract have been regarded to be prominent factors in the host's ability to respond to novel proteins. An immature gut or permeable mucosal epithelium is more likely to allow a higher degree of macromolecular transport and access to the immune system than the intact barrier of a normal mature gut. In association with a mature gut, the immune system must also be of sufficient maturity to respond to "danger signals". Both systems appear to be functioning optimally by age 3-5. The polarity of the Th1/Th2 cells in the mucosal membranes also plays a regulatory role in protective IgA versus aberrant IgE responses. Proteins causing an inflammatory response can trigger both protective and aberrant immunological responses as well as non-immunological responses in the host. Risk is relevant to the extent of hydrolysis or fragmented state of the protein and access to the immune system. Certainly, larger non-digestible proteins or protein fragments have a much more prolonged capacity to reach the immune system the longer they are in evidence in the intestinal tract. Recognition by antigen presenting cells, epithelial cells, and other immune associated cells, such as eosinophils and mast cells that have receptors to proteins and release immunomodulatory cytokines could play prominent roles in the host to plant-pesticide or other proteins.

Both immunologic and non-immunologic diseases are associated with impaired intestinal mucosal membranes. Celiac disease and wheat gluten is an example of a non-immunological

disease. However, inflammation of the gut has been associated with elevated IgE levels and may play an important role in the etiology of chronic urticaria. The intestinal mucosa of children with cows milk allergy shows an abnormal mucosa, which may be indistinguishable from that observed in the gluten-sensitive enteropathy, cited above. However, subjects with normal gut morphology occasionally have IgE plasma cells and often are only characterized by in increased intestinal permeability. Thus, it is clear that different pathological GI entities are multifactorial where different environmental factors and a complex genetic predisposition are in evidence. The extent and severity of the inflammatory response depends on the genetic diversity of bacteria or the amount of antigen on the one hand and the genetic constitution of the host on the other hand making predictions of allergenicity very difficult. What is known is that abnormal immune responses in the human gut are predominantly Th1-like inflammatory responses that can be stimulated by bacteria (normal or pathogenic) and peptides from other sources in a compromised intestinal tract. What determines the switch to IgE response and subsequent allergy is still unknown.

Different levels of stomach acidity can serve as potential causes for inducing not only different levels but also different types of toxicity. For example, the relatively nontoxic plant alkaloid cyclopamine is extremely embryotoxic and teratogenic; however, under acidic conditions of the mammalian stomach, much of this compound is converted by hydrolysis into a related alkaloid veratramine which is highly toxic rather than teratogenic.

7. Other than the predominant oral route of exposure, are there any additional routes of exposure of concern for the toxicity of plant pesticides? Are there any combinations of different routes of exposure that may result in an enhancement of potential adverse effects? If so, what tests should be considered to evaluate this combined effect?

There is some evidence that exposure to the skin and subsequent allergic responses other than contact dermatitis exist for exposure to plant pesticides. Contact of the skin with some proteins may cause sufficient arming of the immune response so that related incidences of protein sensitization can occur. There is marginal evidence of skin irritation leading to inhalation or ingestant allergy. For example, there is considerable evidence to suggest that contact exposure to latex may contribute to the latex-fruit allergy syndrome by cross reacting protein epitopes.

There is an emerging concept that in the healthy state, epithelial cells will induce anergy in CD4+ T cells and stimulate CD8+ T cells to inhibit local responses. However, in inflammation, the epithelial cells may provide additional stimulation to T cells that were either primed in another site or primed in situ. Additional studies are needed to appropriately address this issue of whether the epithelium at respective sites can initiate or perpetuate T cell activation at other sites.

The assessment of dermal irritation/toxicity (worker exposure) might be addressed through post-market surveillance reporting of any adverse reactions to (incidental) skin contact during product development or use. Descriptions of new causes of occupational IgE-mediated asthma include, for instance, the alkaline hydrolysis of wheat gluten derivative with resulting expression of new antigenic determinants (Newman et al. 1989).

Aero-allergen concentrations can be measured using an impaction filter device which traps inhalable particles; allergens extracted can be quantified by radioallergosorbent test (RAST) or ELISA etc.

8. Does the Panel believe there is any other area of toxicity that should be routinely examined for the safety of protein plant-pesticides? What new areas of inquiry should be considered for research into the safety of protein plant-pesticides?

As new and more complex transgenic pest-protected crop plants are designed, the kinds of information necessary to support risk assessments continue to evolve, creating the need for requirements on a case-by-case basis via general guidance documents.

Further research is needed on the issue of substantial equivalence. If bacteria are to be used as surrogate hosts for production of plant pesticidal proteins to be used in safety tests, a database should be developed demonstrating this approach is valid. A representative number of different plant pesticidal proteins should be produced in and purified from plants and be shown to be equivalent to those produced in bacteria by accepted biochemical tests (SDS-PAGE analysis for mass and Western Blot analysis prior to and after proteolytic cleavage for immunological characterization).

The potential complexity of crop plants produced by biotechnology conveying pest resistance via insertion of pesticidal genes, and possible undergoing subsequent processing/cooking means that a simple checklist approach to toxicity testing is not appropriate. A structured, case-by-case approach is needed, the details of which can be informed using a decision tree analysis, taking account of the nature of the pesticidal protein, its dietary role, consequent intake, and the target segment(s) of the population. Where possible, the safety assessment is facilitated by comparison with a traditional counterpart that provides a yardstick of safety.

Although past research has indicated little or no incidence of teratogenicity induced by plant proteins, break-down products of these proteins into small peptides of unusual structure should be monitored in order to ensure that compounds are not generated that bear a three-dimensional structural relationship to known teratogens.

To date, more than one hundred *Bt* gene sequences have been recorded (http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/toxins.html) and the following novel developments should be considered for research:

(1) coding sequences of the bacterial *cry* genes are often modified to enhance expression in plants;
(2) molecular modeling and mutagenesis are used to improve receptor binding, stability, and pore forming capacity of the Cry proteins;
(3) the pore forming or receptor binding domains are interchanged between Cry proteins in order to alter their specificity;
(4) expression of (part of a) Cry protein in various hosts may also alter its properties [Peferoen, 1997];

(5) transformation of the chloroplasts instead of the genome to reach higher levels of expression [Kota et al. [1999];
(6) increasing the structural stability of the Cry proteins, by means of protein engineering (e.g., Cry9c protein) [Atkinson et al., 1996; Atkinson and Meredith, 1998], and targeting the expression to plant tissues attacked by insects may even further increase the levels;
(7) stacking or pyramiding several insecticidal proteins into one crop plant, preferably with a different spectrum of activity. *Bt israelensis* produces a cytotoxic protein, CytA, which affects different cellular targets than observed in case of the Cry proteins [Wirth et al., 1997].

At present, only a limited number of crops have been developed that produce pesticidal proteins, and most of these produce insecticidal proteins derived from the bacterium, *B. thuringiensis*. These proteins have been tested extensively and have been shown to be non-toxic and non-allergenic to mammals under normal routes of entry, i.e., by ingestion. However, as new types of pesticidal proteins are introduced into plants for which no previous history of safety exists, these will have to be tested more extensively to ascertain their safety to allergenic individuals. For example, in the case of plants engineered to produce enzyme inhibitors, the following issues should be considered and addressed:

(1) target insects are capable to change their digestive enzyme cocktail in the gut [Estruch et al., 1997];
(2) long lasting resistance by inserting only one specific proteinase inhibitor gene may not be attainable;
(3) potato and tomato inhibitor levels of about 200 mg per kg of tissue of transgenic plants are said to be in the range of levels found in wounded tomato and potato plants. Similar inhibitor levels in unmodified and transformants are no guarantee for pest resistance (Gatehouse and Gatehouse, 1998);
(4) some of these inhibitors are known to have antinutritional and allergenic effects like the soybean Kunitz trypsin inhibitor (KTI subtypes) and the barley 15 kDa -amylase/trypsin inhibitor, which may constitute a health concern if incorporated in food crop plants (Gendel, 1998b; Franck, Oberaspach and Keller, 1997). However, not all of these inhibitors are detrimental to human and animal health as, for instance, cowpea trypsin inhibitor and a bean amylase inhibitor exerted no adverse effects in feeding trials with rodents (Gatehouse and Gatehouse, 1998; Pusztai et al., 1999). Thus, enzyme inhibitors (and lectins), which are part of a plant's defense mechanism against pests, have been identified as food allergens;
(5) substantial amounts of these proteins can be present in raw food, but are destroyed by food processing, like the trypsin inhibitor in soybeans (Oberaspach and Keller, 1997). Therefore, it is highly unacceptable to interchange pesticidal proteins among the various sources of origin (e.g., no soybean trypsin inhibitor inserted in tomato etc.);
(6) the content of similar defense related proteins has been increased in experimental plants by genetic engineering to create pest resistant varieties [Schuler et al., 1998].

In addition, there are specific aspects of transgenic plants expressing carbohydrate binding protein technology for consideration:

(1) combining proteinase inhibitors with lectins or with Cry proteins, either by cross breeding of primary transformants or by multiple gene insertion, is also contemplated in order to enhance pest resistance [Schuler et al., 1998];
(2) lectins bind to specific glycosylated receptors on the surface epithelium of the digestive tract of herbivores and lower plant pests. The presence of such lectins in edible crops may present health implications, and such proteins should be fully assessed with respect to their toxic potency for mammals;
(3) lectins differ in heat stability. For example, most legume lectins are inactivated by prolonged heat treatment, whereas non-legume lectins survive normal cooking processes;
(4) lectins are not degraded by gut proteases and most lectins are not metabolized either by gut bacteria (Gatehouse and Gatehouse, 1998; Peumans and Van Damme, 1996; Pusztai and Bardocz, 1996), pea lectin is relatively stable in the gastro-intestinal tract of the rat (Aubry and Boucrot, 1986).;
(5) the transfer of lectins derived from crops that are always cooked before consumption in order to destroy them, like beans, into crop plants that are consumed fresh, should be avoided;
(6) an increase in chronic (oral) exposure to the lectins due to genetic modification may also increase the chance for health damage: Peanut lectin (PNA) and Wheat germ agglutinin (WGA) are food allergens, and kidney bean Phaseolus Agglutinin (PHA) is an intestinal toxin [Oberaspach and Keller, 1997).

Additional Comments

Protease Inhibitors

As shown in Table 2, there are two main classes of protease inhibitors in plants: (1) proteins which inhibit the so-called serine proteases such as trypsin and (2) chymotrypsin as exemplified by the Kunitz inhibitor which is specific for trypsin and the Bowman- Birk inhibitor which inhibits both enzymes. There are also the cysteine protease inhibitors, so called because of their ability to inhibit enzymes which have cysteine at their active site. Both classes of inhibitors have been shown to have a growth retardant effect on insect pests and fungi which attack many of our important food crops. This protective effect is generally attributed to the ability of these inhibitors to inhibit the digestive enzymes present in the gut of these insects. Such findings have suggested the possibility that the genes responsible for the expression of these inhibitors could be transferred to plants as a defense against predators to which they are not normally resistant.

Based on a few examples, it would appear that plants genetically modified to exhibit protease inhibitor activity as a defense against predators holds great promise. This, of course raises the question as to whether these protease inhibitors present any health hazards to humans. It is well documented in the literature that protease inhibitors do in fact act as an antinutritional factor in most legumes, particularly the soybean which is quite rich in these inhibitors. Animal experiments have shown that these inhibitors depress growth by interfering with protein digestion, but, perhaps even more importantly, lead to hypertrophy and hyperplasia of the pancreas. The latter effect is due to a negative feedback mechanism wherein the inhibition of trypsin causes the pancreas to increase its size and secretory activity in order to keep up with the demand. This endogenous loss of nutrition via the pancreas creates a negative nitrogen balance with its attendant weight loss. The prolonged ingestion of the soybean trypsin inhibitors eventually leads to the formation of adenomatous nodules on the surface of the pancreas. Whether or not these effects would also be observed in humans cannot be determined at this time. However, the introduction of the Bowman-Birk inhibitor into duodenum of human subjects has caused a significant increase in the ability of the pancreas to secrete trypsin, chymotrypsin, and elastase. This would indicate that the human pancreas at least responds in a negative fashion to the effects of a protease inhibitor.

Since the trypsin inhibitors are destroyed by heat, their negative effect on the nutritional value of the protein can be minimized by the application of sufficient heat. However, because of the necessity of achieving a balance between the amount of heat necessary to destroy the trypsin inhibitors and that which might result in damage to the nutritional value or functional properties of the protein, most commercially available edible grade soybean products contain 5% to 20% of the trypsin inhibitor activity present in the raw soybeans from which they were prepared. That inadequately processed soybean products may find their way into the market place is illustrated by a report of an outbreak of gastrointestinal illness in individuals who had consumed underprocessed soy protein extender for a tuna fish salad.

Table 2. Protease Inhibitors as Pesticides

Conventional Plants

Type	Plant Source	Target Pest
Trypsin/Chymotrypsin	Soybeans	Bean weevils
		Corn borer
		Tomato fruit worm
		Beet army worm
		Fusarium
		Cricket
		Codling moth
	Cowpea	Cowpea weevil
		Lepidoptra species
		Coleoptera species
	Lathyrus sativus	Bruchid beetle
	Potato/Tomato	Fruit worm
		Fusarium
	Cabbage	Fungi
Cysteine Protease	Kidney bean	Bean weevil
	Soybean	Colorado potato weevil
		Corn root worm
		Coleoptera species
	Cowpea	Cowpea weevil
	Rice	Colorado potato weevil

Transgenic Plants

Type	Donor	Recipient	Target pest
Trypsin/chymotrypsin	Cowpea	Tobacco	Tobacco bud worm
	Potato	Tobacco	Tobacco horn worm
Cysteine protease	Rice	Tobacco	Coleoptera
	Rice	Tomato	Cyst nematode

Simple in vitro tests are available for measuring the trypsin inhibitor activity of plants and foods derived therefrom. The biological activity can be evaluated in rats in a number of different ways. In the short term (2 to 3 weeks), one can measure the level of trypsin activity that has been excreted into the feces as a result of an overactive pancreas or by direct measurement of the size of the pancreas. In the longer term, the size of the pancreas will increase with time and, after several months, the pancreas will progressively exhibit hypertrophy, hyperplasia, and ultimately after 60 or more weeks, will become cancerous. Like any other transgenic plant with pesticide properties, those containing trypsin inhibitor should be tested for their biological effects at dietary concentrations which, at minimum, are likely to be encountered in the transgenic plant itself. Such studies should also be accompanied by tests designed to evaluate how much heat treatment may be necessary to reduce the trypsin inhibitor activity to non-toxic levels.

amylase inhibitors

The common bean, *P. Vulgaris*, is particularly rich in a protein capable of inhibiting the starch degrading activity of - amylase in the gut of insects and thus confers resistance of this plant to cowpea and bruchid weevils (Table 3). This activity has been transferred to the green pea and the Azuki bean to provide these plants with resistance to insects to which they are normally vulnerable. There appears to be very little information available as to possible effects of the - amylase inhibitor in humans.

Lectins

Lectins are proteins found throughout the plant kingdom. These proteins have the unique ability to bind to carbohydrates or glycoconjugates in a very specific fashion. Numerous examples can be provided to show that these lectins can act as a defense against insects and fungi (Table 4). This protective effect is apparently due to the fact that the lectins bind to glycoprotein receptors on the surface of the basal membrane of the epithelial cells lining the intestinal wall of susceptible insects, thus interfering with the absorption of nutrients. Those lectins which display antifungal activity such as barley, rice, sting nettle, and the rubber tree apparently do so because of their ability to bind chitin and thus interfere with the formation of the cell walls of fungi. Advantage has been taken of the insecticidal and antimicrobial properties of lectins to produce transgenic plants which are resistant to these pests.

The lectins vary considerably in their toxicity to animals, ranging from the extreme toxicity of ricin and abrin to the relatively non-toxic lectin of the tomato seed. Among the edible legumes, the lectin from *P. vulgaris* has proved to be the most toxic. In fact, rats which are fed raw kidney beans, or the lectin derived therefrom die within a few days after consuming diets containing this lectin. The mode of action responsible for this toxic effect is similar to what was subsequently observed with the insects. There have been reports of severe gastrointestinal distress by individuals who have consumed kidney beans which have not been properly cooked.

The presence of lectins can be readily detected and quantitated by their ability to agglutinate red blood cells or by immunological techniques such as ELISA. Their biological effect is readily manifested in rats by growth depression and mucosal damage to the intestines, the severity of which will depend on the particular lectin being tested. Again, such plants should be incorporated into the diets fed to animals at a level which equals or exceeds the level necessary to act as an effective pesticide.

Table 3. -Amylase Inhibitor as a Pesticide

Conventional Plants

Plant source	Target pest
Phaseolus vulgaris	Cowpea weevil Bruchid weevil
Wheat germ	*Tenebrio molitor*

Transgenic Plants

Donor	Recipient	Target Pest
Phaseolus vulgaris	Green Pea	Bruchid beetle Mexican bean weevil Cowpea weevil
	Azuki bean	Azuki bean weevil

Table 4. Lectins as Pesticides

Conventional Plants

Plant Source	Target Pest
Phaseolus vulgaris	
Phytohemagglutinin	**Bruchid beetle**
Arcelin	**Bruchid beetle**
Soybean	**Manduca sexta** (leaf defoliating insect**)**
	Fungi
Wheat germ	**Cowpea weevil**
Barley	**Fungi**
Rice	**Rice plant hopper**
Potato	**Fungi**
Peanut	**Fungi**
Snow drop	**Wide range of insects**
	Peach-potato aphid
Sting nettle	**Fungi**
Rubber tree	
Amaranth	

Transgenic Plants

Lectin Donor	Plant Recipient	Pest Target
Arcelin from wild variant of *P. Vulgaris*	**Non-resistant bean**	**Bean weevil**
Soybean/Pea	**Tobacco**	**Tobacco budworm**
Snow drop	**Potato**	**Wide range of insects**
Hevein (rubber)	**Tomato**	**Fungi**

REFERNCES

Below are references to the scientific literature relevant to the Panel responses. The list includes citations not discussed by the Panel at the meeting but is provided as additional information relevant to the issues presented.

Astwood, J. D.; Leach, J. N.; Fuchs, R. L. (1996): Stability of food allergens to digestion in vitro. *Nature Biotechnology* **14**, 1269-1273.

Atkinson, H.A.C.. Johnson, I.T., Gee, J.M., Grigoriadou, F. & Miller, K., 1996. Brown Norway rat model of food allergy. Effect of plant components on the development of oral sensitation. Food Chem. Tox. **34**: 27 -32.

Atkinson, HAC, Meredith C Assessment of allergenic potential of novel proteins in food crops using the brown norway rat model. In: *Proceedings Third Karlsruhe Nutrition Symposium* (1998) Karlsruhe: Bundesforschungsanstalt für Ernährung. P. 70-75.

Aubry M, Boucrot P. Comparative study on the digestion of radiolabelled vicilin, legumin and lectin of Pisum sativum in the rat. *Ann Nutrition Metabolism* (1986) **30**: 175-182.

Bairoch A , Apweiler R. The SWISS-PROT protein sequence data bank and its supplement TrEMBL in 1999. *Nucleic Acid Research* (1999) **27**: 49-54.

Bando, N.. Tsuji, H., Yamanishi, R., Nio, N., Ogawa, T., 1996. Identification of the glycosylation site of a major soybean allergen, Gly m Bd 30K. Biosci. Biotech. Biochem**. 60**: 347- 348.

Barker et al. The PIR-International protein sequence database. *Nucleic Acid Research* (1999) **27**: 39-43.

Batanero, E., Villalba, M., Monsalve, R.I., Rodriguez, R., 1996. Cross-reactivity between the major allergen from olive pollen and unrelated glycoproteins: Evidence of an epitope in the glycan moiety ofthe allergen. J. Allergy Clin. Immunol. **97**: 1264 -1271.

Boulter, D. et. al. Genetic engineering of plants for insect resistance. Endeavor 14, 185-190, 1990.

Bowles, D.J. Defense-related proteins in higher plants. Annual Review Biochemistry 59, 873-907, 1990.

Bufe, A., Schramm, G., Keown, M.B., Schlaak, M., Becker, W.-M., 1995. Major allergen Phl p Vb in timothy grass is a novel pollen Rnase. FEBS Letters **363**: 6 -12.

Burks, C. (1999): Molecular biology database list. *Nucleic Acids Research* **27**, 1-9.

Bush, R. K.; Hefle, S. L. (1996): Food allergens. *Critical Reviews in Food Science and Nutrition* **36**, S119- S163.

Cheeke, P.R. and Shull, L.R. (eds.) Proteins and amino acids. In: Natural Toxicants in Feeds and Poisonous Plants. Avi Publishing Co., Westport CT., p. 235-291.

Christpeels, M.J. and Raikel, N.V. Lectins, lectin genes, and their role in plant defense. Plant Cell 3, 1-9, 1991.

Chua, K. Y.. Stewart, G.A., Thomas, W.R., Simpson, R.J., Dilworth, R.J., Plozza, T.M., Turner, K.J., 1988. Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. J. Exp. Med. **167:** 175- 182.

Commission of the European Communities (1997) Recommendations concerning the scientific aspects of the information necessary to support applications for the placing on the market of novel foods and novel food ingredients, in Off.J.Eur.Commun. No.L253/1-36, 16.9.97

Cornelissin, B.J.C. and Melchers, L.S. Strategies for control of fungal diseases with transgenic plants. Plant Physiology 10, 709, 1993.

Crickmore, N. Full list of Bacillus thuringiensis delta-endotoxin genes, 2000. http://epunix.biols .susx.ac.uk/H*ome/N*eil-Crickmore/Bt/toxins.html

Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., and Dean D. H. (1998) 'Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins,' *Microbiol. Mol. Biol. Rev.* **62**, 807-813.

Dufourcq J et al. In: The comprehensive sourcebook of bacterial toxin proteins (1999) p. 386-401.

Enneking, D. and Wink, M. Towards the elimination of anti- nutritional factors in grain legumes. In: Current Plant Science and Basic Biotechnology in Agriculture 14, 671-683, 1999.

Evans, C.S. Naturally occurring food toxicants. Toxin amino acids. In: Handbook of Naturally Occurring Food Toxicants. Ed. M. Recheigl, CRC Press, 1983, p. 3-1414.

Fowden, L., Lewis, D., and Tristram, H. Toxic amino acids: their action as metabolites. Advances in Enzymology 29, 89-163, 1967.

Ewen SWB, Pusztai A. Effects of diets containing genetically modified potatoes expressing Galanthus nivalis lectin on rat small intestine. *Lancet* (1999) **354**: 1353-1354.

Faye, L., Johnson, K.D., Sturm, A., Chrispeels, M.J., 1989. Structure, biosynthesis, and function of asparagine-Iinked glycans on plant glycoproteins. Physiol. Plant. **75**: 309- 314.

Ferry, G. (2000) Protein structure by numbers. *HMS Beagle. The BioMedNet Magazine.* (http://www.biomednet.com/hmsbeagle).

Fuchs, R.L., Astwood, J.D. 1996. Allergenicity assessment of foods derived from genetically modified plants. Food Technol. **50:** 83 -88.

FDA, *Foods derived from new plant varieties derived through recombinant DNA technology,* 1999, U.S. Food and Drug Administration. *http:llvm.cfsan*.fda.gov/-lrd/biocon.html.

Fehlner, P.F., Berg, R., Tam, J.P., King, T.P., 1991a. Murine T cell responses to melittin and its analogs. J. Immunol. **146**: 799- 805.

Fehlner, P.F., Kochoumian, L., King, T.P., 1991b. Murine IgE and IgG responses to melittin and its analogs. J. Immunol. **146**: 2664- 2670.

FAO (1996) Biotechnology and food safety. Report of a Joint FAO/WHO Consultation Rome 30 September-4 October 1996. FAO *Food and Nutrition Paper, 61*, Food and Agriculture Organisation of the United Nations, Rome.

FAO (1995): Report of the FAO Technical Committee on Food Allergies, Food and Agriculture Organization of the United Nations, Rome, Italy, November 13-14.

Franck Oberaspach, S.L., B. Keller, Consequences of classical and biotechnological resistance breeding for food toxicology and allergenicity. *Plant Breeding,* 1997. **116**(1): 1- 17.

Garcia-Casado, G., Sanchez-Monge, R., Chrispeels, M.J., Armentia, A., Salcedo, G., Gomez, L. 1996. Role of complex asparagine-Iinked glycans in the allergenicity of plant glycoproteins. Glycobiology **6**: 471- 477.

Gatehouse, A.M.R., J.A. Gatehouse. Identifying proteins with insecticidal activity: use of encoding genes to produce insect-resistant transgenic crops. *Pesticide Science*, 1998**. 52**(2): 165-175.

Gendel, S.M. (1998a): The use of amino acid sequence alignments to assess potential allergenicity of proteins used in genetically modified foods. *Advances in Food and nutrition research* **42**, 45-62.

Gendel, S.M. (1998b): Sequence databases for assessing the potential allergenicity of proteins used in transgenic foods. *Advances in Food and nutrition research* **42**, 63-92.

Gupta, N., Martin, B.M., Metcalfe, D.D., Subba Rao, P.V., 1996. Identification of a novel

hydroxyproline-rich glycoprotein as the major allergen in *Parthenium* pollen. J. Allergy Clin. Immunol. **98**: 902-912.

Hefle, S. L.; Nordlee, J. A.; Taylor, S. L. (1996): Allergenic foods. *Critical Reviews in Food Science and Nutrition* **36**, S69-S89.

Hefle, S.L., 1996a. The chemistry and biology of food allergens. Food Technol. **50**: 86 -92.

Hefle, S.L., 1996b. The molecular biology of food allergy. Immunol. Allergy Clinics N. Amer. **16**: 565 - 590.

Hilder, V.A. et. al. Genetic engineering of crops for insect resistance using genes of plant origin. In: Genetic Engineering of Plant Crops. eds. G. Lycett and D. Garrison. Butterworth, 1990, p. 55-66.

Hofmann, K., Bucher, P., Falquet, L., Bairoch, A. (1999) The PROSITE database, its status in 1999. *Nucleic Acids Research* **27**, 215-219.

Holm L, Sander C. Protein folds and families: sequence and structure alignments. *Nucleic Acids Research* (1999) **27**: 244-247.

ILSI (1996) The safety assessment of novel foods. Guidelines proposed by ILSI Europe Novel Food Task Force. International Life Sciences Institute, European Branch, Brussels.

Jankiewicz, A.. Aulepp, H., Baltes, W., Bogl, K.W., Dehne, L.I., Zuberbier, T., Vieths, S., 1996. Allergenic sensitization to native and heated celery root in pollen-sensitive patients investigated by skin test and IgE binding. Int. Arch. Allergy Immunol. **111**: 268 -278.

Janzen, D.H. Potentially defensive proteins in mature seeds of 59 species of tropical Leguminosae. Journal of Chemical Ecology 12, 1469- 1480, 1986.

Jenkins, N., Parekh, R.B., James, D.C., 1996. Getting the glycosylation right: Implications for the biotechnology industry .Nature Biotechnol. **14:** 975- 981.

Kaminogawa, S., 1996. Food allergy, oral tolerance and immunomodulation. Their molecular and cellular mechanisms. Biosci. Biotech. Biochem. **60:** 1749- 1756.

King, T. P.; Hoffman, D.; Lowenstein, H.; Marsh, D. G.; Platts-Mills, T. A.; Thomas, W. (1994): Allergen nomenclature. *International Archives of Allergy and Immunology* **10**, 224-233.

Kolaskar, AS, Kulkarni-Kale, U. Prediction of Three-Dimensional Structure and mapping of conformational epitopes of envelope glycoprotein of Japanese Encephalitis Virus. (1999) *Virology*

261, 31-42.

Kota, M., *et al.,* Overexpression of the Bacillus thuringiensis (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. *PNAS* (1999) **96**(5), 1840-1845.

Kuiper, H.A., Noteborn H.P.J.M. (1996) Food safety assessment of of transgenic, insect-resistant Bt-tomatoes in *Food Safety Evaluation* pp50-57. OECD, Paris.

Kuiper HA, Noteborn HPJM, Peijnenburg AACM Adequacy of methods for testing the safety of genetically modified foods. *Lancet* (1999) **354**: 1315-1316.

Liener, I.E. Toxic Constituents of plant foodstuffs, Academic Press, 1980.

Liener, I.E. Nutritional significance of lectins in the diet. In: The Lectins, ed. I.E. Liener, N. Sharon and I.J. Goldstein, Academic Press, 1986, p. 527-552.

Liener, I.E. Trypsin inhibitors: concern for human nutrition or not? Journal of Nutrition 116, 920-923, 1986.

Liener, I.E. Lectins. In: Herbivores. Their Interaction with Secondary Metabolites. Eds. G.A. Rosenthal and M.R. Berenbaum. Academic Press, New York, 1991, p. 1327-353.

Liener, I.E. Antinutritional factors related to proteins and amino acids. In: Foodborne Disease Handbook. Disease caused by hazardous substances. Eds. Y.H. Hui et. al., Marcel Dekker, Inc., New York, 1994, p. 261-309.

Meisel H. Overview on milk protein-derived peptides. *Int. Dairy J.* (1998) **8**: 363-373.

Metcalfe, D. D.; Astwood, J. D.; Townsend, R.; Sampson, H. A.; Taylor, S. L.; Fuchs, R. L. (1996): Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Reviews in Food Science and Nutrition* **36**, S165-S186.

Noteborn, H.P.J.M., Bienenmann-Ploum, M.E., van den Berg, J.H.J., Alink, G.M., Zolla, L., Reynaerts, A., Pensa, M., Kuiper, H.A. (1995) 'Safety asses-sment of the Bacillus thuringiensis insecticidal crystal protein CryIA(b) expressed in transgenic tomatoes', in Engel, K.-H., Takeoka, G.R., Teranishi, R. (eds.) ACS Symposium Series 605, Washington DC: ACS, pp. 134-147.

Noteborn, H.P.J.M., Lommen, A., van der Jagt, R.C.M., Weseman, J.M. (2000) 'Chemical fingerprinting for the evaluation of unintended secondary metabolic changes in transgenic food crops' J-Biotechnol. **77**(1): 103-14.

Noteborn, H.P.J.M., Lommen, A., Weseman, J.M., van der Jagt, R.C.M. and Groenendijk, F.P.F. (1998) 'Chemical fingerprinting and in vitro toxic-ological profiling for the safety evaluation of transgenic food crops' in Report of the Demonstration Programme on Food Safety Evaluation of Genetically Modified Foods as a Basis for Market Introduction, The Hague: Information & Newssupply department, Ministry of Economic Affairs, pp. 51-79.

OECD, 1993 Safety Evaluation of Foods Derived by Modem Biotechnology: Concepts and Principles, OECD, Paris, France

Ohta. M., Matsuura, F.. Kobayashi, Y., Shigeta, S., Ono, K., Oka, S.. 1991. Further characterization of allergenically active oligosaccharitols isolated from a sea squirt H-antigen. Arch. Biochem. Biophys. **290**: 474 -483.

Orengo et al. The CATH database provides insights into protein structure/function relationships. *Nucleic Acids Research* (1999) **27**: 275-279.

Peferoen, M. Progress and prospects for field use of Bt genes in crops. *Trends in Biotechnology*, 1997.**15**{5): 173-177.

Pernis, B. G. (1992): Antigen processing and antigen presentation. *Progress in Allergy and Clinica Immunology* **2**, 395-399.

Petersen, A.. Becker, W.-M., Moll, H.. Blumke, M., Schlaak, M.. 1995. Studies on the carbohydrate moieties of the timothy grass pollen allergen Phl p 1. Electrophoresis **16**: 869 -875.

Peumans, W.J., E.J.M. Van Damme, *Prevalence, biological activ;ty and genetjc manipulation of lectins in foods.* Trends in Food Science and Technology, 1996. **7**(4): 132-138.

Peumans, W.J. and Vandamme, J.M. The role of lectins in plant defense. Histochemical Journal 27, 253-271, 1995.

Pusztai et al. Expression of the insecticidal bean alpha-amylase inhibitor transgene has minimal detrimental effect on the nutritional value of peas fed to rats at 30% of the diet. *J Nutrition* (1999) **129**: 1597-1603.

Pusztai A, Bardocz S. Biological efects of plant lectins on the gastrointestinal tract: metabolic consequences and applications (minireview). *Trends in Glycoscience and Glycotechnology* (1996) **8**: 149-165.

Roitt, I.M., 1994. Essentiallmmunology. Blackwell Scientifjc Publications, London.

Romagnani S., Mohapatra SS 1996 Human T -cell responses to grass pollen allergens In:

Mohapatra, SS & Knox, RB (Eds) Pollen Biotechnology Gene Expression and Allergen Characterization Chapman & Hall, NewYork, pp 164-175

Reeck., Proteinase inhibitors and resistance to transgenic plants to insects. In: Insect control. The role of transgenic plants. Eds. N. Carozzi and M. Koziel, Taylor and Francis, 1997, p. 157-188.

Rosenthal, G.A. Nonprotein amino acids as protective allochemicals. In: Herbivores: their interaction with secondary plant metabolites. Eds. G.A. Rosenthal and M.R. Berenbaum, Academic Press, New York, p. 1-34.

Rothbart, J. B.; Gefter, M. L. (1991): Interactions between immunogenic peptides and MHC proteins. *Annual Reviews of Immunology* **9**, 527-565.

Rozan, P., Kuo, Y-H, and Lambein, F. Free amino acids present in commercially available seedlings sold for human consumption, a potential hazard for consumers. J Agr. Food Chem. 48, 716-273, 2000.

Ruibal NL et al. (1997) The potential allergenicity of novel foods. *J Sci Food Agri* **75**: 405-411.

Ryan, C.A. Proteinase inhibitor gene families: strategies for transformation to improve plant defenses against herbivores. BioEssays 10, 20-24, 1989.

SCF (1997) Opinions on the Assessment of Novel Foods expressed 7 June 1996, 12/13 December 1996. European Commission DG III/5915/97 Brussels.

Sears, C., Kaper, JB. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. *Microbiological Reviews* (1996) **60**: 167-215.

Swoboda, I, Scheiner, 0 Kraft D Breitenbach, M Heberle-Bors E., Vicente 0 1994 A birch gene family encoding pollen allergens and pathogenesis-related proteins Biochim Biophys Acta **1219:**457-464

Sanderson, I.R.; Walker, W.A. (1993): Uptake and transport of marcomoleculeds by the intestine: possible role in clinical disorders (an update). *Gastroenterology* **104**, 622-639.

Schuler, T.H. et al., Insect-resistant transgenic plants. *Trends in Biotechnology*, 1998. **16**{4):168-178.

Schroeder, H.E. et al. Gene technology for improved weed, insect and disease control and for seed protein quality. Current Plant Science and Biotechnology in Agriculture 34, 389 -396, 1999.

Stinzi, A. Plant pathogenesis-related proteins and their role in defense against pathogens. Biochimie 75, 686-706, 1993.

Takano T. Milk derived peptides and hypertension reduction. *Int. Dairy J.* (1998) **8**: 375-381

Taylor, S. L.; Lehrer, S. B. (1996): Principles and characteristics of food allergens. *Critical Reviews in Food Science and Nutrition* **36**, S91-S118.

Taylor, S. L.; Nordlee, J. A.; Rupnow, J. H. (1989): Food allergies and sensitivities. In: *Food Toxicology - A Perspective on the Relative Risks.* New York, USA; Marcel Dekker. pp. 255-295.

Tretter, V Altmann, F Kubelka V Marz L., Becker WM 1993 Fucose a1,3-linked to the core region of glycoprotein N-glycans creates an important epitope for IgE from honeybee venom allergic individuals Int Arch Allergy Immunol 102 259 -266

UK ACNFP (1995) Advisory Committee on Novel Foods and Processes Annual Report for 1994, Ministry of Agriculture, Fisheries & Food, London

Wal, J.M. Strategies for assessment and identification of allergenicity in (novel) foods. *Int. Dairy Journal* **8**, 413-423.

WHO (1995) Application of the principle of substantial equivalence to the safety evaluation of foods or food components from plants derived by modern biotechnology, Report of a WHO Workshop. World Health Organisation, Food Safety Unit, Geneva

Wirth, M.C., G.P. Georghiou, and B.A. Federici. CytA enables CrylV endotoxins of Bacillus thuringiensis to overcome high levels of CrylV resistance in the mosquito, Culex quinquefasciatus. *Proceedings of the National Academy of Sciences of the United States of America*, 1997. **94**(20): 10536-10540.

Wiedemann, P Giehl K, Almo SC Fedorov AA Girvin, M Steinberger P Rudiger, M Ortner M Sippl M Dolecek, C Kraft, D Jockusch B., Valenta R 1996 Molecular and structural analysis of a continuous birch profilin epitope defined by a monoclonal antibody J Biol Chem 271:29915-29921

Zeleny, R. Altmann, F. Praznik, W. (1999) 'Structural characterization of the N-linked oligosaccharides from tomato fruit'. Phytochemistry 51(2): 199-210.

Zhang, L, Mohapatra, SS 1996 Structure-function relationships of allergens In Mohapatra SS & Knox, RB (Eds) Pollen Biotechnology Gene Expression and Allergen Characteristics Chapman & Hall, New York pp 144- 163.