

Report on the potential allergenicity of genetically modified organisms and their products

BSACI working party: G. Lack*, M. Chapman†, N. Kalsheker‡, V. King§, C. Robinson¶ and K. Venables**

*Department of Paediatric Allergy & Immunology, St Mary's Hospital, London, UK, †Health Sciences Centre, Charlottesville, Virginia, USA, ‡Department of Clinical Chemistry, University Hospital, Nottingham, UK, §Ministry for the Environment, Wellington, New Zealand, ¶Department of Pharmacology & Clinical Pharmacology, St George's Hospital Medical School, London, UK, and **Institute of Health Sciences, University of Oxford, Oxford, UK

Introduction

The application of genetic manipulation (genetic 'engineering') to crop breeding and food production is an extension of traditional agricultural goals. New varieties of crops are being sought that are easier to grow (by being harder and/or pest resistant), produce higher yields, and have improved nutritional, flavour or storage properties. Traditionally, realization of these objectives has relied upon hybridization of different crop varieties in which a large amount of genetic information is exchanged between the two varieties and the outcome of the hybridization is not predictable. However, analysis of plant genomes and gene cloning techniques advance the practice of crop breeding by permitting the incorporation of a desired trait through the insertion of a specific gene into a plant [1]. As the gene to be incorporated can in principle be derived from any source, even after 'engineering' it to modify some properties of the expressed protein, plant genetic engineering raises additional possibilities for creating 'designer' crops and producing non-food products in plants such as chemical intermediates, drugs and fuels [2–6].

As will be discussed later, the expression of plant genes can also be prevented by genetic manipulation, raising further possibilities for manipulating the attributes of 'engineered' crops. Although the outcome of plant genetic manipulation is more predictable than that of conventional breeding techniques, unexpected results can nevertheless arise from various technical obstacles in engineering the expression of a desired trait. Transgenes may lose their function [7]. Desired phenotypes may be expressed at only low levels because of position effects [8, 9] or inefficient incorporation of new genetic material into nuclear DNA [10].

The main crops under development for potential use in the UK include maize, oilseed rape, sugar beet, fodder beet and potatoes [11]. There are three GM food products that are currently sold for human consumption in the UK, including maize, soy and tomato paste. There is an enormous market for these products and 60% of processed foodstuffs contain soy produce [12]. In 1998 GM soy accounted for 30% of US soy production and it was predicted to increase to approximately 65% of

production in 1999 [13]. US soy is a major export product to EU countries, including the UK.

It should be noted that in addition to the important agricultural and socio-economic implications for the introduction of GM foods, there are potential applications in the domain of public health and medical treatment. For example, it is possible to increase synthesis of vitamin A precursors in rice [14]. This could reduce vitamin A deficiency, which occurs in parts of the world where the staple diet is lacking in vitamin A or its precursors. Additionally, there is the potential for the creation of hypoallergenic foods, which could improve quality of life for children and adults with persistent food allergies [15]. Furthermore, the use of recombinant food [16] and aeroallergens [17] that are modified to decrease allergenicity offer important therapeutic avenues in the immunomodulation of allergic diseases.

Despite such potential benefits there are concerns regarding possible adverse effects of GMOs on health and on the environment. In May 1999 the British Medical Association published a statement on GMO [12] addressing three areas of concern over potential health effects of GM foods. It focused on the transfer of antibiotic resistance, toxicity and allergenicity and highlighted the importance of doing further research on the potential allergenicity of GM products. This report will focus on the allergenic potential of GMOs and their natural or processed products, e.g. pollens, foods or other consumer products.

GM methodologies

There are four main approaches by which plants may be manipulated by genetic 'engineering'. These are: (i) gene insertion using a bacterial vector [18–20]; (ii) microballistic impregnation [21, 22]; (iii) poration [23–26]; and (iv) gene neutralization by means of antisense technology, homologous recombination (gene knock out) and gene replacement [27, 28]. The choice of technique depends upon the type of plant that is being engineered (not all approaches are suited to all plants) and whether the objective is incorporation of a new gene or 'neutralization' of an existing one. All of the procedures are usually conducted on plant cells in culture, from which mature, engineered plants are then grown.

In broad terms, gene transfer involving a bacterial vector relies upon the new gene (plant or otherwise) being inserted into a bacterium that can infect plant cells. Plant cells that

Correspondence: Gideon Lack, Department of Paediatric Allergy & Immunology, St Mary's Hospital, Praed Street, London W2 1NY, UK. E-mail: gideon.lack@st-marys.nhs.uk

successfully incorporate the new gene can then be selected and grown to maturity. The bacterium commonly used for this work is *Agrobacterium tumefaciens*, which occurs naturally in soil [18]. *A. tumefaciens* has the ability to infect many types of plant, and in so doing it alters their genetic information [18, 19]. It is this property that has made it valuable as a vector for the genetic manipulation of plants under controlled laboratory conditions. To achieve a genetic modification using this technique, a restriction enzyme is used to cut non-virulent plasmid DNA derived from *A. tumefaciens* and thus create an insertion point into which the gene can be ligated. The engineered plasmid is then put into a strain of *A. tumefaciens*, which contains a 'helper' plasmid, and plant cells are treated with the recombinant bacterium [18]. The helper plasmid assists the expression of the new gene in plant cells as these grow in culture [Fig. 1].

Even under carefully managed conditions, the efficiency of gene incorporation into the plant cells can be low. This makes it necessary to sort those plant cells that do not carry the new gene from those that do. A common way of achieving this is to insert into the plasmid vector a second gene that confers upon the plant cells an ability to grow in the presence of a particular antibiotic. The cells that grow under these conditions are those that have incorporated the genes from the bacterial plasmid. Because of concerns about the fate of the antibiotic-resistance genes, research is being conducted to assess their impact when encountered in genetically altered crops and foods [29]. Alternative types of marker are also under development. The utility of *A. tumefaciens*-mediated gene transfer is

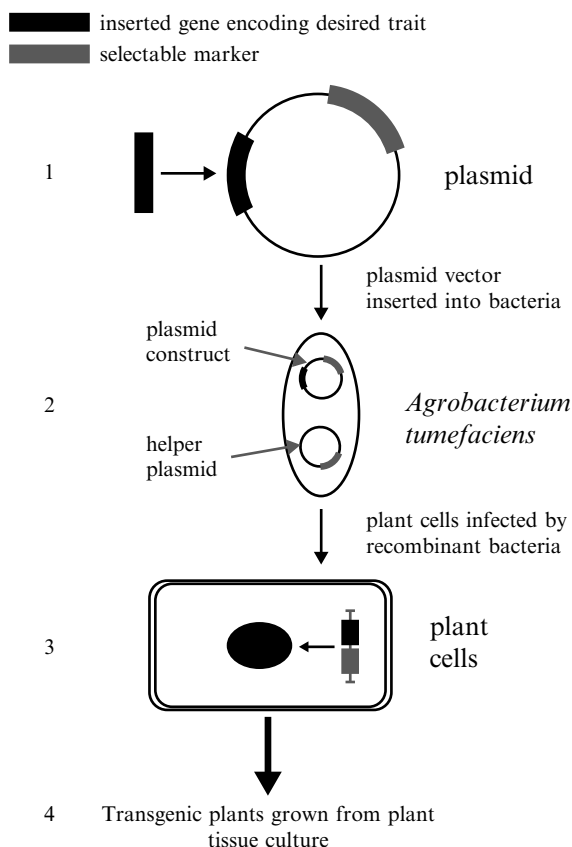


Fig. 1. Expression of new gene in plant cells grown in culture.

nicely illustrated by its use to develop potato plants that are resistant to Colorado beetle [30]. In this example the inserted gene *cryIIIa* is prokaryotic in origin and it was artificially modified to enhance its expression in the plant. The *cryIIIa* product encodes for an insecticidal protein from the soil bacterium *Bacillus thuringiensis*. When expressed in potato plants this protein is toxic to Colorado beetles by combining with a protein found within their digestive epithelium to form a membrane channel that results in cell lysis [30].

Agrobacterium-mediated gene transfer has been used for the genetic manipulation of many dicotyledonous plants, but it is less suitable for cereal crops. In cases such as these genes may be inserted by other techniques, although refinements in *Agrobacterium*-based transfer has allowed the production of genetically engineered rice [31, 32]. In microballistic impregnation the target gene (and any markers) is applied to minute particles of gold or tungsten, which are fired into plant tissue at high velocity. Cells that incorporate the target and marker genes can then be selected in culture and grown [21, 22]. Alternatively, poration by the application of a pulsed electric field or chemical treatment allows the transient formation of small pores in plant cells through which genes can be taken up and incorporated into the plant genome [23–26].

It is also possible to neutralize the expression of specific genes in the plant. The simplest genetic engineering approach is to employ an antisense sequence to the gene in question, but homologous recombination (gene knockout) and gene replacement provide alternatives. Antisense technology involves using the sequence of the non-coding strand of a particular gene to inhibit the expression of the desired gene. The application of antisense technology has resulted in a consumer food product, the *FlavrSvrTM* tomato, in which shelf-life is enhanced by suppression of the enzyme polygalacturonase [27, 28]. A similar increase in the shelf-life of tomatoes can also be produced using antisense technology against the enzyme 1-amino-1-cyclopropanecarboxylic acid synthase [28]. Antisense and gene knockout techniques also offer the potential to decrease the expression of known allergens.

IgE-mediated allergy

The past few decades have witnessed a significant increase in IgE-mediated allergic diseases. Several national and international studies have documented an increase in the prevalence of allergic rhinitis, asthma and eczema. Between 1% and 2% of the adult population suffers from IgE-mediated food allergies [33–35], and in children this number is estimated to be between 2% and 8% [36]. Peanut allergy alone is reported to occur in one in 200 individuals in the UK [37]. In order to understand the potential allergenicity of GMOs or indeed of any novel food or plant, it is necessary to provide a brief review of the pathophysiology of allergy and to address the specific issues that pertain to allergic sensitization and to food allergy. In doing so we highlight both areas of knowledge as well as areas of uncertainty.

Pathophysiology of allergy

IgE-mediated allergic reactions occur when allergens, by binding to and cross linking specific IgE antibodies on mast cells,

cause mast cell degranulation. The range of clinical manifestations includes rhinitis, conjunctivitis, urticaria, angioedema, bronchospasm, diarrhoea, vomiting and hypotension [38]. In severe cases, life-threatening anaphylaxis will ensue. Typically these symptoms are acute, occurring minutes after allergen exposure, but the release of chemokines and other inflammatory mediators can cause a subsequent influx of inflammatory cells into the target tissue, resulting in delayed symptoms that occur hours after exposure.

Allergic sensitization must be distinguished from an allergic reaction. Sensitization is the process whereby the immune system of an individual becomes reactive to an allergen. This requires exposure to an allergen over a period of time, resulting in allergen-specific CD4 T cell responses. These CD4 T cells exhibit a predominantly Th2 pattern of cytokine production. Initially, allergen-specific B lymphocytes elaborate IgM but following cognate interaction with CD4 T cells and under the influence of IL4 and IL13, they undergo isotype switching to produce IgE and IgG antibodies [39, 40]. This phenomenon of sensitization (induction of allergen-specific T and B cells and IgE production) is a prerequisite to the development of clinical allergy. The distinction between allergic sensitization and allergic reactions is critical and raises important considerations relevant to the potential allergenicity of GMOs. Thus, one must consider not only whether GM products can cause allergic reactions in sensitized individuals but whether a newly expressed transgene may lead to *de novo* allergic sensitization in the population [41–43].

Routes of allergen exposure

Allergens are compartmentalized according to the manner in which we encounter them. Thus we think of 'food allergens' as causing gastrointestinal and oral symptoms, following ingestion. We think of 'inhalant allergens' as causing the respiratory and ocular symptoms of hayfever and asthma. This division is somewhat artificial as in practice food allergens can behave as aeroallergens and conversely aeroallergens can behave as food allergens. Individuals can be exposed to any allergen by inhalation, by cutaneous contact, by ingestion or by injection.

Grass pollen allergy, for example, typically results in rhinitis and conjunctivitis. Although pollen grains are large and outside the respirable range, naturally occurring changes in weather conditions (rainfall, thunderstorms) cause pollen grains to break up into smaller particles, which can reach further down the respiratory tree and cause asthma. Cutaneous exposure to pollen when a child plays in the long grass may lead to urticaria, angioedema or worsening eczema. Food allergens can cause oral and gastrointestinal symptoms following ingestion, cutaneous symptoms following cutaneous exposure, and rhinitis and asthma following inhalation of aerosolized foods [44]. Even ingestion of food allergens as evidenced by double-blind placebo-controlled oral food challenges can lead to both upper and lower airway symptoms in addition to gastro-intestinal and cutaneous symptoms. Furthermore, there are important clinical cross-reactivities between aeroallergens and food allergens. Thus, individuals with birch-pollen allergies often suffer allergies to hazelnuts, apples, peas, potatoes, carrots, celery and kiwi fruit [45–47]. Individuals with grass pollen allergies are often allergic to peach, celery and tomato [46]. Ragweed pollen cross-reacts with watermelon, melon and banana [48, 49]. Different

routes of exposure, as well as the important cross-reactivity between food and aeroallergens, require careful consideration in the risk assessment of allergenicity of GM products.

Special considerations for food allergens

Food allergens, however, do present special problems that relate both to their physico-chemical characteristics and the way in which we use them. Common food allergens (peanut, egg, cow's milk, soy and wheat) are widely used in the food industry. Allergens such as peanut are very stable to high temperature. Consequently, peanut protein will retain its allergenicity even after roasting or sterilization processes used in the food industry [50]. Even a minimal amount of a food allergen present in a processed food product is sufficient to cause an allergic reaction and there are documented cases where less than 10 mg of ingested allergen has resulted in anaphylaxis [51]. These are important considerations in risk assessment of GM foods.

What is considered a non-allergenic food in one country may represent a significant cause of food allergies in other countries. Thus, in Europe and North America, rice is often used as part of a hypoallergenic diet, whereas in Japan it poses a problem [52]. Major allergens have been identified in rice that exacerbate eczema [53]. Although these common food allergens listed above would appear to account for the majority of world-wide food allergies, the list of foods that are potentially allergenic is extensive. To date, more than 160 food and food-related substances have been associated with presumed IgE-mediated allergy in individuals [54]. Food allergy is a major concern in public health and is affecting a growing proportion of the population.

It is important to note in the context of this report that the increase in prevalence in food allergies has been clinically apparent prior to the introduction of GM foods on to the market. The same possible causes that have been considered in the increase in atopic diseases have also been hypothesized to cause food allergies [55]. Thus, urban or industrialized lifestyle [56], exposure to cigarette smoke, lack of early exposure to viral and bacterial infections [57, 58], and changes in infant rearing and feeding practices [59], have all been proposed as causes. It is likely that socio-economic and cultural factors play an important role in the pathogenesis of food allergies. Generally, food allergies occur in societies where those foods are more commonly eaten. Thus, kiwi fruit allergy, which has become an increasing problem in European and North American countries during the past couple of decades, was very rarely seen prior to the introduction of kiwi fruit on to supermarket shelves [47]. Lentil allergy is commonly seen in Mediterranean countries where lentil is used as a weaning food [60].

It is likely that increased population exposure to foods leads to increased allergic sensitization. We know little about the route and timing and dose of allergen exposure that is required to cause allergic sensitization to foods. Sensitization to the food need not necessarily occur through ingestion but could occur through inhaled or cutaneous exposure. Intriguingly, the majority of peanut and egg allergic reactions in children occur upon first known exposure [61, 62]; therefore, low-dose exposure either *in utero* or in the neonatal period must have resulted in sensitization. There is *in vitro* evidence that low-dose antigen stimulation rather than high-dose exposure induces pro-allergic

cytokines and the production of IgE [63]. Some *in vivo* rodent models show that high-dose oral exposure leads to immunological tolerance [64–66].

There has indeed been considerable debate as to whether sensitization starts during pregnancy or in the neonatal period and whether exposure to food allergen either *in utero* or transmitted through breast milk may lead to sensitization [67]. Initially, the immune system of neonates shows a skewing towards Th2-like responses [68], which becomes more exaggerated during infancy only in atopic infants. There is therefore the concern that exposure to allergenic foods during this critical neonatal period may lead to allergic sensitization.

Community outbreaks of allergy and occupational exposure

Typically, allergies occur to a relatively small number of 'common' allergens in individuals who have an atopic family history. However, there are notable cases of community outbreaks of allergy and specific occupational exposures that have caused more unusual allergies. These have involved plant and other food products to which communities and workers have become sensitized and manifested allergic symptoms.

Thus, in Barcelona, epidemics of asthma followed release of aerosolized soy bean particles after unloading of soy bean cargo at the docks [69]. Those individuals who had epidemic asthma had specific IgE sensitization to soy bean and were shown to have experienced exposure to high levels of soy bean [70]. Similarly, exposure to castor bean has also been implicated in asthma epidemics [71].

Occupational allergies and asthma have been described in various settings, including plant and animal products [72]. For example, laboratory animal workers have been shown to develop symptoms and become sensitized to serum proteins in rat urine [73], to proteins from the urine and pelts of mice [74], to guinea-pig serum proteins [75] and rabbit salivary proteins [76]. Bakers' asthma is an important occupational allergic disease due to sensitization to airborne cereal dust. It occurs mainly in employees in bakeries and mills [77]. Importantly, in bakers' asthma, workers have been shown to be sensitized not only to cereal allergens, but also to baking additives, which are potent allergens [78]. Notably, the addition of mould enzymes such as α -amylase causes sensitization in 15% of bakers. There are several hundred other examples of occupational asthma and related allergies.

Consideration of these environmental and occupational causes of allergy and asthma is particularly relevant to the issues surrounding GM products as, indeed, for any new food product. First, it is clear that adults who are not normally at risk for food allergies can become sensitized to a variety of plant and animal allergens in the work environment. Sensitization to many of these foodstuffs and other allergens is occurring either by inhalation or through the skin. Secondly, some of these cases emphasize the risks associated with adding a protein derived from a known allergen into a foodstuff for economic reasons (e.g. α -amylase). Thirdly, these examples emphasize the fact that food allergens have important allergic implications, not only for individuals who consume these foods but also for individuals who are exposed to these foodstuffs: the farmers, the packers and the food processors. Finally, it will be particularly important to study changing patterns of allergic symptoms and immunoreactivity in such 'high risk' groups of people to

determine whether they show evidence of sensitization to new transgenic proteins. Indeed, such groups can be thought of as sentinel groups who deserve careful monitoring as part of our post-marketing surveillance of GM products.

Allergenic potential of GM crops

Statement of the problem

Genetic manipulation of crops creates several possible scenarios that are relevant to potential allergenic effects in humans. Some of the possible situations are: (i) unmodified plant gene from non-allergenic crop inserted into target crop; (ii) unmodified plant gene from crop known to cause allergy inserted into target crop; (iii) unmodified gene from non-plant source inserted into target crop; (iv) modified gene from non-plant source inserted into target crop; (v) existing gene from target crop modified and reinserted; (vi) gene expression down-regulated or up-regulated in non-allergenic or known allergenic crop.

Detection of allergens in GMOs

In 1996 a publication appeared in the *New England Journal of Medicine* describing how the 2S albumen protein from Brazil nut was transferred into soy bean [79]. The rationale behind this was to improve the content of soy bean with the sulphur containing amino acids cysteine and methionine as an improvement in cattle feed. As Brazil nuts are indeed known to cause allergic reactions in sensitive individuals, it was decided to determine the allergenicity of the transgenic soy bean. The results of detailed experiments showed that 2S albumen from Brazil nut was a major Brazil nut allergen (recognized by IgE from more than 50% of patients with Brazil nut allergy) and that the newly expressed protein in transgenic soy retained its allergenicity and therefore its potential ability to provoke clinical reactivity in patients with allergy to Brazil nut. In other words, patients allergic to Brazil nut and not to soy bean now showed an IgE-mediated immune response towards GM soy bean. On this basis, Hybrid International, the company that developed this, decided not to market the transgenic soy bean.

This particular example highlights the significant impact that GM foods could have on allergenicity in the public health area. There are two conflicting ways of interpreting this episode. Thus one interpretation is that GM foods could unleash a host of allergic public health problems, affecting an important percentage of the population and destroying consumer confidence in food safety. Soya protein is used extensively in a variety of foods in very small quantities and avoidance for soya allergy sufferers is extremely difficult. The transfer of a major nut allergen into soya would have severe consequences for nut allergy sufferers, who would now be exposed to an allergen in a wide range of foods that they would normally regard as safe. Indeed, a minority of these patients with severe reactivity to nuts would be at risk of life-threatening episodes. This problem could be compounded by the fact that there is important immunological cross-reactivity between major nut allergens: individuals allergic to one tree nut are frequently allergic to other tree nuts.

An alternative interpretation of this particular case is that it was possible to prevent the transfer of an allergen by using advanced immunological techniques together with clinical testing. In this case a solid phase immunoassay was used

together with immunoblotting to assess whether the protein transferred to soy bean from Brazil nut rendered the soy allergenic. Sera from eight individuals sensitized to Brazil nut produced a positive immunoassay, indicating that the transgene obtained from Brazil nut probably encoded like major Brazil nut allergen. It was thus possible to establish the allergenicity of the transgene product and prevent an unsafe food from entering the market.

Theoretical risks associated with GM proteins in foods

Further theoretical issues arise, where risk assessment is not possible. Recombinant proteins expressed in new plant hosts may be similar to natural proteins at the amino acid sequence level but may have reduced function compared with their natural counterparts if they do not fold correctly or undergo different post-translational modifications. The potential implications of this for allergenicity are unknown; the absence of some modifications may result in a lower allergenic threat, others may result in the formation of new immunological epitopes. These theoretical mechanisms are, however, unlikely to produce conformational changes or neo epitopes that have major immunological significance. While it is impossible to predict the risk of creating new conformational epitopes that have immunological significance, the experience with human recombinant factor VIII and insulin and other therapeutic recombinant proteins suggests that the creation of neo epitopes and immunogenicity are not problematic.

There is the potential for random integration of the novel gene in the host crop genome, resulting in unexpected expression of previously silent genes. This could potentially create new proteins to be expressed in the host species and therefore new potential allergens. The other possible consequence is that a protein expressed at low levels would be expressed at high levels. Although the possibility of this is unlikely, such new or increased protein expression could be recognized prior to a GM food becoming a crop. Thus techniques currently applied in the field of proteomics, such as high resolution two-dimensional gel electrophoresis, would allow identification of novel protein expression. If expressed at reasonable levels, mass spectrometry would allow sequencing of the new protein with the potential for comparisons to be made with known allergen sequences. Indeed, the technology to identify and sequence novel proteins in GM foods is considerably advanced and further technological advances in the near future are likely to improve the sensitivity and speed of these techniques.

GM foods in context: comparisons with traditional plant breeding and food processing methods

GM foods versus traditional plant breeding

An argument favouring the safety of GM foods is that this represents a far more precise and safe manner of genetic alteration in plants compared with traditional plant breeding methods (see Table 1). Traditional plant breeding, which has been practised for millennia, involves the transfer of thousands of genes and potentially new proteins to create a crop with certain desirable characteristics. Such large-scale genetic changes in the plants have significant implications for protein expression. Many changes in protein expression are likely to

Table 1. Comparison of GMO technology and traditional plant breeding

GM technology	Plant breeding
Transfer of single genes	Transfer of hundreds of genes
Gene sequence known	Genes are not known
Small change in protein expression	Large changes in protein expression
Between species	Within species

occur with these traditional methods, which we ignore. Conversely, GM foods allow the precise transfer of a single gene into a host species. There is precise information on the protein transferred and its sequence. We are therefore able to monitor its expression and to test for allergenicity in the new product.

The counter argument is that traditional plant breeding does not cross species barriers and in GM foods there is the potential to introduce a far greater diversity of genes into the host species. However, there are examples in traditional plant breeding where genetic material is exchanged between different species. Thus, 'wide' crosses have been made between barley or wheat species and related wild species that would not naturally cross [80]. Using this methodology, two species are crossed, resulting in a non-viable embryo that is then removed from the plant ('embryo-rescue') and allowed to develop further *in vitro*. The hybrid is then 'back crossed' several times with the wheat species, selecting for a particular phenotype of interest. However, a large amount of genetic material will inevitably be transferred from the alien species in addition to the selected gene; this could significantly alter protein expression in the plant. Wide crossing has been extensively used in wheat, particularly to confer resistance to fungal pathogens.

Another form of plant breeding practised, called 'mutation breeding', employs a chemical or physical stimulus to induce mutations in a crop species [81]. This induces a high number of random mutations in the plant that are unpredictable and very difficult to measure. Although a particular phenotype will then be selected, other unidentified mutations will inevitably be present in the new crop. This has been used, for example, to produce different phenotypes of barley, using gamma-irradiation. Thus a gamma-ray mutant barley was an important crop grown in the 1970s and 1980s for malting, brewing and distilling. Such methods of plant breeding have the potential to cause far greater changes in protein expression, protein conformation and allergenicity than with GM foods.

GM food compared with processed food

While we have detailed knowledge concerning the primary amino acid sequence of known allergens, three-dimensional knowledge is lacking for all but a few allergens. Importantly, the three-dimensional structure of protein allergens can be significantly modified by a number of processes used in the food industry (see Table 2).

Food processing has numerous purposes in the food industry, of which the most important are thermal denaturation, acidification and fermentation. Importantly, all these processes, especially thermal denaturation, cause significant conformational changes in protein structure that are far greater than those one would expect to find in GM foods [82–84]. Thus, heating generally reduces the allergenicity of a protein by alteration of conformational epitopes. Heating can create new epitopes,

Table 2. Comparison of allergenicity of GMO-derived foods and food processing methods in the industry

	GM food	Food processing
Change in plant genome	++	-
Change in protein composition	++	++
Modification of single protein	++	-
Modification of numerous proteins	-	++
Conformational change of protein	(+)	++
Neoallergens	(+)	+
New allergenic B cell epitopes	(+)	+
New sensitizing T cell epitopes	(+)	(+)

(-) Does not occur, (+) theoretically possible, + is known to occur occasionally, ++ occurs regularly.

potentially by unfolding the protein and exposing previously hidden linear epitopes. Alternatively, partial unfolding can create new conformational epitopes. However, heating can also increase allergenicity, depending on the protein involved and on the individual patient. Prausnitz and Kustner [85] reported the first example of novel heat-induced allergenic epitopes in fish. The patient was able to eat raw fish but had severe hypersensitivity symptoms after eating cooked fish. Similarly, Malanin *et al.* reported the creation of neo allergens in heated pecan nuts [86]. In this instance there was no hypersensitivity to untreated pecan nuts, whereas symptoms were induced by the formation of a neo allergen in roasted pecan nuts. Similarly, neo allergens have been described in heated soya bean husks [87]. Heat treatment of beta lactoglobulin causes at least one new conformational epitope on the molecule that is not found in the native state. There are data to suggest that the pasteurization of cow's milk may lead to increasing allergenicity [88].

Furthermore, it is important to note that food proteins are very rarely treated in isolation, but are normally found in an admixture of other food ingredients. This allows numerous potential interactions with other proteins and food components [83, 84]. Davis *et al.* point out that, 'The safety evaluation of novel food proteins must include heat denatured as well as "native" forms' [82].

Additionally, the unfolding caused by food processing could mean that linear T cell epitopes are seen by the immune system that would not normally be seen. This could result in enhanced immunogenicity of processed foods as compared with the native forms. Thus, conceivably, consumption of acidified dairy products or roasted nuts could increase sensitization following prolonged consumption of these foodstuffs. Despite these very significant changes in protein structure and conformation following food processing (Table 2), these issues have not been the subject of detailed scientific scrutiny.

Safety evaluation of GM foods

Food safety: the concept of substantial equivalence

Historically, allergy has not been a major safety concern in the food industry. Thus, the main food industry considerations in the past have focused on nutritional values of foods, issues of infection and toxicology. In more recent years, with the increasing prevalence of food allergy and growing reports of anaphylaxis, there has been increased consumer pressure on the food

industry for clear labelling of foods that may contain allergens and the need to identify very low levels of contaminant allergens in foods about to be released on the market. GMOs present a novel situation for the food industry and it is clear that new safety mechanisms will have to be put into place to prevent these problems from materializing.

Food safety as defined by the WHO consists of providing assurance that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use. The concept of substantial equivalence has been elaborated by the WHO and OECD [89–91]. It rests on the principle that if a novel food or food component is found to be substantially equivalent to an existing food or food component, it can be treated in the same manner with respect to safety. This determination needs to account for any processing that the food or food component may undergo, as well as its intended use. The comparison may be a lengthy process, involving several steps. Typically, this involves molecular characterization, phenotypic characteristics, and definition of nutrients and toxicants. This is practised in GM crops. Notably, glyphosphate-tolerant soy beans were generated by the addition of a glyphosphate tolerance gene to commercial soy beans [92]. Nutrients measured in soy bean seeds as well as anti-nutrients measured (trypsin inhibitor, lactins, isoflavones, bitate) were equivalent in both the glyphosphate-tolerant line and the parental soy bean [93]. Furthermore, in animal feeding studies there was no difference in the feeding value of such soy beans [94].

Extending the concept of substantial equivalence to allergenicity is, however, more difficult, but needs to be incorporated into the risk and safety assessment and practices that are required of the food industry. It has been possible, using SDS PAGE and immunoblotting, to show that glycoposphate tolerance does not cause quantitative or qualitative changes in the composition of endogenous soy bean allergenic proteins [95]. However, such experiments merely show that the allergen composition of a transgenic crop has not been altered and demonstrates allergenic similarity between the GM and wild-type crop. However, in the case of GM foods and transgenic crops, the real issue is 'substantial *inequivalence*'. We need to demonstrate that the expressed transgene is different to known existing allergens. This requires different chemical and immunological assays and a comprehensive data bank on allergen sequences. The second limitation of the concept of substantial equivalence is that it measures the 'here and now'. In other words, it looks at effects on the consumer at the time of consumption. Although the concept can be extended to demonstrate inequivalence of the transgenic product and new food to a multiplicity of known allergens, it is not possible to predict the immunogenicity of the transgenic product. In other words, we cannot say whether it will induce sensitization and cause *de novo* allergy in the population. This may only develop after months of exposure and might only be apparent in large epidemiological studies, years after exposure.

Predicting food allergy

Physico-chemical characteristics of food allergens There is no definite molecular weight above which proteins are allergenic and below which they are non-allergenic. Generally, there appears to be a lower limit for allergenicity for peptides with molecular weight at around 1200 to 1600 Da. As there

must be at least two IgE binding sites on a monomeric protein to cause mast cell degranulation, it is likely that a critical molecular weight value of about 3500 Da is likely to be applicable for IgE-mediated reactions. In practice, it is unusual for food allergens to have a molecular weight of less than 10 000 Da, though there are notable exceptions; most known food allergens have molecular weights between 10 000 and 70 000 Da [96, 97].

There are certain physical properties of food allergens that hold true for many food allergens. Thus, typically, food allergens are stable to changes in pH and heat treatment [86]. Research in both the food industry and in the fields of immunology and protein chemistry has been done with a view to elaborating the rules of allergenicity. Whereas certain properties have emerged that belong to a small number of well-known food allergens, there do not appear to be definite predictive 'rules of allergenicity' [98]. Certainly proteins can be suspected to be allergens on the basis of their characteristics. However, the absence of a certain characteristic in a protein does not allow us to definitively predict that it will not be allergenic. Stability to digestion, proteolysis or hydrolysis have also been noted [97, 99, 100]. Using a gastric model of digestion the stability to digestion of known food allergens (e.g. milk, egg, soy, mustard and peanut) was compared with that of common plant proteins. It emerged that the tested food allergens were all stable to digestion, unlike other plant proteins [97, 101–103].

However, not all allergens are stable and there are important exceptions. Tuck and Blumstein [104] reported patients who reacted to fresh melon, pear, peach, pineapple, grape and banana. In each case tinned fruit caused no reactions. Studies of fresh extracts of these fruits showed that when stored in a refrigerator, the extracts lost their ability to provoke a positive skin prick test after approximately 3 days. Indeed, changes in temperature do appear to decrease the allergenicity of a large number of fruits and vegetables. Thus, not all food allergens are stable and resistant to processing. Although these labile allergens are usually associated with the oral allergy syndrome, they can cause anaphylaxis. Recent studies have identified important allergens in vegetables that are easily digested using *in vitro* models and therefore differ from other food allergens that are resistant to digestion [105]. Furthermore, there are important exceptions amongst non-allergenic proteins. Thus gamma-thionin protein from wheat is very stable to digestion but is not an allergen.

Many food allergens are glycoproteins. Glycosylation occurs by covalent linkage of oligosaccharide chains (glycans) to the protein. This process is a post-translational modification of proteins. Furthermore, glycosylation can contribute towards the antigenicity of protein [97]. Carbohydrate moieties on a protein may indirectly contribute towards the antigenicity of a glycoprotein by increasing its solubility [98], or by increasing thermal stability and resistance to proteolysis.

While many of the above characteristics apply to food allergies, inhalant allergies do not have similar properties and are generally not heat stable and are not resistant to digestion. Therefore physico-chemical characteristics are of no known value in predicting whether a transgenic protein will cause inhalant allergies.

Structural comparison between proteins: predicting cross-reactivity Comparisons of the amino acid sequences of known allergens have not revealed a pattern that is characteristic of

allergenicity. Allergenic proteins do not have a unique primary structure that distinguishes them from non-allergens [103]. Comparing structural properties of a protein with those of known allergens can, however, be helpful in terms of predicting allergenicity and cross-reactivity. Antibodies bind to two sorts of epitopes: structural and conformational epitopes. Structural epitopes comprise a continuous amino acid sequence on the primary structure of the protein, whereas conformational epitopes are discontinued on the amino acid sequence of the folded protein [106]. It can be argued that allergenicity cannot be predicted by comparing amino acid sequence of the protein being tested and known allergens if the allergenic epitope is conformational. There is no reason to expect that IgE binding epitopes are similar in different food allergens.

Inspection of amino acid sequence similarity therefore has limitations. Thus, a 21 amino acid sequence from shrimp tropomyosin has a sequence identity of 87% with a 21 amino acid sequence of the fruit fly tropomyosin and of 60% with chicken tropomyosin. Whereas sequence identity is high between these different sequences from different species, their allergenicity in humans is very different [97, 107]. Shrimp tropomyosin is a clinically significant food allergen, with similar versions of this protein from beef or chicken very rarely causing allergic reactions [108]. There are, however, instances where conserved sequence identity is helpful in predicting cross-reactivity within certain families. There is substantial cross-reactivity amongst crustacea (with crab and crayfish sharing sequence identity for tropomyosin) [97]. Thus sequence homology has limited value in predicting cross-reactivity. Generally it is of greater value in assessing linear epitopes but is less reliable in making predictions about conformational epitopes. An absence of sequence homology between two proteins does not allow us to infer that these two proteins differ with respect to 3D structure and allergenicity.

The notion that there are three-dimensional (3D) structural determinants in a protein that govern its allergenicity is not supported by analysis of 3D structures of different allergens. Thus analysis of some 40 allergenic proteins (based on known or predicted configurations) reveals that allergens possess structurally diverse features and have no characteristic structural determinants [109].

The helper T cell epitope, however, is determined entirely by the primary amino acid sequence. While there are indeed a few cases of documented cases of cross-reactivity due to T cell epitope homology [110], our existing knowledge of the T cell epitope repertoire in allergens is limited. Expansion of existing allergen databases incorporating data on the amino acid sequences of major allergenic B cell and T cell epitopes, physico-chemical characterization of allergens and 3D determination of IgE-binding epitopes, may lead to improved predictive models for allergenicity. Importantly, internet sites on the world-wide web will allow such information to be rapidly updated and easily accessed [111–113].

Levels of allergen expression in foods Generally, proteins that are allergens are highly expressed in individual foods compared with other non-allergenic food proteins. This may be because a critical allergen load is required for allergic sensitization to occur. However, it should be pointed out that this is not a universal rule and *Gad c I*, which is the major allergen for cod fish, is expressed only at low levels [97].

Recent work on allergy to multiple fruits has shown that lipid transfer proteins are 'panallergens' that reside largely in the skin of fruits. These proteins are defensins involved in host immunity and their concentration may alter with ripening [114]. Maturation, ripening and host defence are all aspects that are targeted by genetic manipulation, and GMOs produced to this end could result in increased levels of these allergenic proteins.

Mouse models to predict allergenicity Animal models may be valuable in helping us understand the pathogenesis of allergies. However, their ability to predict allergenicity is not proven. Indeed, a murine model inaccurately determined that the 2S albumen protein from Brazil nut was not a major allergen because it failed to induce IgG1 production [115]. On this basis, the gene was transferred into soy.

One important criticism of mouse and rat models of allergy is that the intraperitoneal route of sensitization commonly used in animal models may not be relevant to human sensitization [116, 117]. While there are murine models for oral sensitization, an immunological adjuvant is usually required to achieve allergic sensitization [118, 119]. Although future studies may increase our understanding about the allergenicity of proteins, currently animal models do not provide reliable predictive information regarding allergenicity.

Existing approaches to evaluate safety of GM foods

In 1996 a Joint FAO/WHO Expert Consultation met to determine an approach to the evaluation of GMOs and the risk of allergenicity [120]. The consensus report recommended a stepwise assessment of foods, taking into account the source from which a gene was obtained, amino acid sequence comparisons with known allergens, immunological analyses (*in vitro* and *in vivo*) and physico-chemical properties of the gene product. The report stresses that there is no single diagnostic test to assess the probability that a GM food is allergenic, but that the totality of these assessments needs to be taken into account.

A decision tree is constructed, first determining the source of the introduced gene [121]. Significance is placed on whether the gene is from: (i) a commonly allergenic food; (ii) a less commonly allergenic food or other known allergen; or (iii) a source with no allergenic history. The report recommends that amino acid sequence of genes from all sources should be compared against a database of all known allergens, screening for immunologically significant sequence similarities. Based on a minimal peptide length for T cell reactivity, the report concludes that sequence identity comprising eight contiguous amino acids should be viewed as a possible risk for allergenicity and recommends that the gene product undergo further testing. The report includes a list of 198 sequences of food and non-food proteins reported to be allergens that are on available databases.

The stepwise approach varies, depending on the source of the gene.

(i) Commonly allergenic foods. The report recommends that if a food contains protein from a gene derived from a commonly allergenic food it be subjected to a series of solid phase immunoassays (RAST or RAST inhibition or ELISA). The suggestion is that each food should be tested against immune sera from at least 14 sensitized individuals with positive clinical histories. This should ensure a >99.9% probability of detecting the presence of a major food allergen and >95% probability of

detecting a minor allergen to which more than 20% of the population reacts. If the *in vitro* assay is positive, there is the potential for that protein to be allergenic and it is recommended that unless this possibility is dismissed by *in vivo* testing, food labelling on the GM product should include the source of the donor gene.

The report recommends in the event of negative or equivocal results with solid phase assays that skin prick testing should be undertaken using a minimum of 14 allergic individuals. A positive skin prick test would raise similar concerns as a positive *in vitro* reaction and it would require foods containing the newly introduced gene to be labelled. If the food is negative both with respect to immunoassays and skin prick tests, it is thought unlikely to contain allergen, but the report recommends that this be confirmed by negative double-blind placebo-controlled food challenges, again using 14 sensitive subjects. If the challenges are negative, there is no basis upon which to require labelling concerning the source of the gene.

(ii) Less commonly allergenic foods or other known allergens. In this situation the criteria recommended are less rigorous. It is recommended that gene products be subjected to solid phase assays using sera from 14 allergic individuals where feasible. Again, a positive result could be the basis for the requirement that the food be labelled with the source of the donor gene. It is pointed out that sera from individuals may be extremely difficult to obtain. If less than five sera are tested, this reduces the probability of detecting the presence of a major allergen to less than 95% and the report recommends that the gene product should be subject to physico-chemical evaluation. The protein products need to be evaluated for their stability *in vitro* to gastric digestion and food processing measures.

If it is found that the protein product is not resistant to digestion and/or processing, there is no basis for labelling the presence of gene products. For gene products resistant to digestion and/or processing, it is recommended that developers discuss the results with appropriate regulatory authorities.

(iii) Foods from sources with no allergenic history. In this situation, it is recommended that the starting point should be a comparison of the amino acid sequence (eight amino acids) against the database of known allergens, looking for sequence similarities. If a similar sequence to a known allergen is picked up, the assessment should be treated in the same manner as that for a food derived from a gene of less commonly allergenic foods or other known allergens. Products from sources with no allergenic history, even in the event of a negative sequence identity to known allergens, should still be subjected to physico-chemical evaluation to determine if the food is resistant to digestion and/or processing.

This scientific decision tree approach provides a way of assessing food allergies based on current knowledge about the biology and protein chemistry of food allergens and is indeed in use to ensure safety of GM food products from an allergenic standpoint. This approach to assessing allergenicity considers not only allergenicity based on B cell epitopes, but considers immunogenicity by stipulating recognition of potential T cell epitopes from food sources with no allergenic history. There are a number of shortcomings to such an approach. First, there are no definite rules of allergenicity, and not *all* allergens share the same typical physico-chemical characteristics. Secondly, these physico-chemical characteristics have been defined mainly for food allergens. GMO products as discussed earlier could act

as aeroallergens, which have different properties. Thirdly, this approach depends in part on identifying similarity in the amino-acid sequence of B cell and T cell epitopes. Given current knowledge it would not be possible to recognize conformational epitopes for the majority of allergens. Relatively few T cell epitopes have been defined for allergens. Fourthly, while this approach can reliably safeguard against the transfer of known allergenic genes, it does not provide a reliable way for predicting and preventing allergic sensitization to a protein derived from a source with no allergenic history.

Special considerations

Infants are at high risk for the development of food allergies in general. The concern here is that the introduction of a new protein in significant quantities during a critical window of opportunity in a high-risk individual will lead to sensitization to that particular protein. Indeed, because of such concerns, the Department of Health has issued recommendations that infants from atopic families not be fed peanuts during the first few years of life despite a lack of direct evidence [122]. Even though the transgene may be derived from a food that is not known to cause food allergies, there is the potential that high exposure in a high-risk individual at a critical time point will induce allergic sensitization. Such reasoning also provides the basis for the paediatric practice of the gradual introduction of food products in atopic children during the first year of life. However, there is considerable debate as to how sensitization to foods occurs in infants. Allergens can be transported transplacentally in the blood and can also be detected in breast milk. It therefore makes no sense to impose a restriction on GM products in infant formulae only, without restrictions on food consumed by pregnant or lactating women. If GMOs are able to cause new allergies, one would expect to observe this in infants. Careful surveillance for new allergies in infants is therefore required.

Pollination characteristics: special considerations

Genetic modification of crops clearly has the potential to affect pollination characteristics of plants. Any alteration that would lengthen the pollination period of a plant or, indeed, alter its dispersion characteristics, would have considerable potential to increase aeroallergen sensitization to that particular pollen. The results could translate into an increased prevalence of hayfever and asthma. However, the chance of this happening inadvertently in GM crops is no greater than in traditional plant breeding. Furthermore, other factors such as climate are likely to have greater effects on pollination. Indeed, traditional plant breeding now allows for winter cereals to be sown in November, in addition to spring cereals that are traditionally sown in March. Winter cereals allow germination over the winter and pollination will therefore start earlier. If a GM crop is produced where there is likely to be a change in pollination characteristics, aerobiological considerations must be taken into account before such crops are released. Other questions arise: is the transgenic protein expressed in pollen likely to cause a new form of pollen allergy? Could subtle changes in the composition of pollen grains allow the grains to

break up more easily into smaller, respirable particles and increase inhalant exposure to pollens? Another important question relates to whether a new transgenic protein is actually expressed in the pollen and whether this could lead to *de novo* inhalant allergy [123].

The engineering of hypoallergens

There is a possibility of using recombinant technology to change known allergens into hypoallergens. This has indeed been done for major allergens in rice [15] and soy [122] and such work is under way for peanut allergens [16].

While the idea of an allergen 'knock out model' is enticing, there are several potential problems. First, not all individuals will necessarily react to the same allergens. The concept of a major allergen is defined as that where more than 50% of the allergic population is sensitized. However, one person's minor allergen may represent another person's major allergen with respect to clinical reactivity. Even if only a few major allergens are identified for a particular food, the allergen may have several IgE binding epitopes and different individuals may have IgE directed against distinct epitopes. In order to create such hypoallergenic foods, care is needed to test these hypoallergens against large samples of sera and individuals. A situation where only 70% of allergic individuals tolerate a GM hypoallergen would be highly unsatisfactory and dangerous for the remaining 30%.

Hypoallergens have a potentially important role in allergic desensitization. Recently, single amino acid substitution in the major house dust mite allergen has been shown to reduce IgE binding by more than 10-fold [17]. Such an allergen could be used to desensitize allergic individuals without the worry of anaphylaxis. This approach could be particularly useful in peanut allergy. In peanut allergy, desensitization has been shown to have a partial effect. It is, however, impossible to achieve sufficiently high doses of maintenance injections because of IgE-mediated side-effects [125]. Indeed, the creation of recombinant peanut proteins that bind weakly to IgE may allow us to use desensitization more safely and effectively in the future. The application of recombinant food technology to immunotherapy thus has the potential to revolutionize the treatment of food allergies.

Labelling

This paper will not discuss the proposed legislations regarding the labelling of GM foods in various countries. We merely wish to raise special considerations in the labelling of GM foods that can affect the allergic patient. First, the labelling of conventional food allergens in food products is a major problem for patients with food allergies. Detailed labelling including information on known allergenic proteins should be required for all marketed foods.

A decision tree such as the one previously described to establish allergenicity in GM foods will provide protection for the consumer. It does, however, have the ability to create both anxiety and confusion in the allergic sufferer if due thought is not given to labelling details. Thus, a food labelled as having a protein expressed by a transgene derived from a peanut or tree nut, creates a dilemma for the consumer with peanut or tree nut

allergies even if the expressed protein is not a known allergen. The problem would be even more difficult if the transgenes were expressed in a variety of processed foods.

The creation of hypoallergens also has the potential to cause confusion for the allergic consumer. If both allergenic and non-allergenic peanut varieties exist in the market this could create serious labelling issues. The situation with respect to allergen avoidance is already difficult for individuals allergic to common allergens such as peanut. Allergic sufferers need to read every ingredient on the labels of packaged and processed foods in order to ensure that they do not consume hidden allergens. The life of allergic sufferers could become intolerably complicated and dangerous if they faced a choice of both allergenic and non-allergenic peanut products in the marketplace.

The argument that economic and medico-legal considerations would force supermarkets to purchase non-allergenic foods is compelling but there are no regulations to enforce this. If, indeed, hypoallergens are created, it will be important to institute appropriate legislation and regulatory procedures regarding distribution, sales and labelling to protect the allergic consumer.

Conclusions

The basic scientific techniques used for the production of GMOs do not pose any allergic threat *per se*. As a number of naturally occurring products are allergenic, there is a statistical probability that GM foods themselves could be allergenic. Taking proteins from a highly allergenic material and expressing them elsewhere makes this probability higher than the opposite extreme of making a transgenic crop from a hypoallergenic food protein. This has indeed been demonstrated by the Brazil nut/soy example, but the problem was detected prior to introducing the food on to the market [79].

Increasingly sophisticated genetic manipulations may increase the risk of inadvertent creation of neoallergens and some of these may evade detection, if the population is not yet sensitized to this new allergen. While no precise estimate of this potential risk can be made, there are no *a priori* reasons for GM technology to produce neoallergens to any greater extent than the introduction of other novel foods into the market. There is no evidence that the process of introducing a single transgene into the plant genome will create neoallergens to any greater extent than will the larger changes in composition that result from traditional and more advanced plant breeding methodologies. Thus far our experience with intravenously and subcutaneously administered recombinant/therapeutic proteins has not resulted in major allergic problems. The prevalence of food allergies, together with atopic diseases in general, has increased over the past two decades, well before the advent of GM technology. This increase has coincided with a period of significant change in dietary habits, the introduction of novel foods and changes in the food industry, but has not been linked to any changes in the genetic composition of foods that we consume.

It should be noted that no single food technology has ever been subjected to as much public and scientific scrutiny. Indeed, no previous food technology has emerged with as much detailed knowledge at the outset. GM technology allows precise identification and testing of a protein whose sequence and many physico-chemical characteristics are known. While our

knowledge of GMOs is clearly superior to that of protein changes brought about by traditional plant breeding and food processing, it would be unrealistic to assume complete knowledge. There is much information that remains to be acquired about allergens and the transgenic expression of proteins in GMOs. We need to improve our knowledge of the sequences of less well-characterized allergens and extend our databases, especially for T cell epitopes. Furthermore, the precise definition of the tertiary and quaternary structure of allergens and the effect on these of post-translational modification will provide important additions to our knowledge.

Post-marketing surveillance will provide an important way of monitoring the potential effects of GMOs on allergenicity and to that effect it will be important to identify and measure GM products in foods and correlate changes in exposure to epidemiological change in allergic symptoms in the population. The further scrutiny of sentinel groups, such as individuals in processing plants who handle GMOs and infants who are at risk for food allergies, may provide early warning signs to herald the emergence of new allergies to GMO proteins. The opportunity to develop successful vaccines against allergy and the possibility of developing hypoallergenic crops must be balanced against the smaller likelihood of new allergens developing. The expectation is that past research success will be overtaken by future advances so that any potential allergic risks will be reduced even further. In the light of the above, this paper recommends proceeding with caution, assessing individual GMOs on the basis of their individual merits and risks prior to introducing them into the market.

Acknowledgements

The authors would like to acknowledge Professor T. Lee and Professor P. Shewry for advice and discussion.

Glossary

Transgene: the gene transferred from a donor organism to the recipient organism.

Transgenic protein: the protein expressed by the transgene in the recipient organism.

Allergen: a protein capable of inducing a specific immune response in humans that results in disease when the person is subsequently re-exposed to that protein.

Hypoallergen: a protein that rarely or never causes allergic disease in human beings; or a protein that has been genetically modified to make it less allergenic.

Neoallergen: a novel protein, exposure to which increases the rate of allergic sensitization to that protein in the population.

Genetically modified organism: there is no internationally agreed definition of this term. The committee uses the term to refer to biological entities produced by targeted genetic modification using molecular biological techniques.

References

- 1 Fiske HJ, Dandekar AM. The introduction and expression of transgenes in plants. *Sci Hort* 1993; 55:5–36.

- 2 Cramer CL, Weissenborn DL, Oishi KK et al. Bioproduction of human enzymes in transgenic tobacco. *Ann N Y Acad Sci* 1996; 792:62–71.
- 3 Poirer Y, Nawrath C, Somerville C. Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers in bacteria and plants. *Bio Technol* 1995; 13:142–50.
- 4 Ma JK-C, Hein MB. Immunotherapeutic potential of antibodies produced in plants. *Trends Biotechnol* 1995; 13:522–7.
- 5 Keeler SJ, Maloney CL, Webber PY et al. Expression of de novo high-lysine alpha-helical coiled-coil proteins may significantly increase the accumulated levels of lysine in mature seeds of transgenic tobacco plants. *Plant Mol Biol* 1997; 34:15–29.
- 6 Beauregard M, Dupon C, Teather RM, Hefford MA. Design, expression and initial characterization of MB1, a *de novo* protein enriched in essential amino acids. *Bio Technology* 1995; 13:974–81.
- 7 Flavell RB. Inactivation of gene expression as a consequence of specific sequence duplication. *Proc Natl Acad Sci USA*, 1994; 91:3490–6.
- 8 Denis M, Delourme R, Gourret J-P, Mariani C, Renard M. Expression of engineered nuclear male sterility in *Brassica napus*. *Plant Physiol* 1993; 101:1295–304.
- 9 Nap JP, Keizer P, Jansen R. First-generation plants and statistics. *Plant Mol Biol Rep* 1993; 11:156–64.
- 10 Meier I, Groning B, Michalowski S, Spiker S. The tomato RBCS3A promoter requires integration into the chromatin for correct organ-specific regulation. *FEBS Lett* 1997; 415:91–5.
- 11 Department of the Environment. Transport and the Regions. Advisory Committee on Releases into the Environment. The Commercial Uses of Genetically Modified Crops in the United Kingdom: the Potential Wider Impact on Farmland Wildlife. London: Department of the Environment, 1999. www.environment.detr.gov.uk/acre/wildlife.
- 12 The British Medical Association Science Department. The Impact of Genetic Modification on Agriculture, Food and Health. An Interim Statement. London: BMA, 1999.
- 13 The Royal Society of Medicine. Statement on Genetically Modified Plants for Food Use. London: RSM, 1998.
- 14 Burkhardt PK, Beyer P, Wunn J et al. Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. *Plant J* 1997; 11 (5):1071–8.
- 15 Matsuda T, Alvarez AM, Tada Y. Gene engineering for hypoallergenic rice: repression of allergenic protein synthesis in seeds of transgenic rice plants by antisense RNA. In: Proceedings of the International Workshop on Life Science in Production and Food Consumption of Agricultural Products, Tsukuba, Japan, October 1993.
- 16 Bannon GA, Cockrell G, Connaughton C et al. Engineering, characterization and in vitro efficacy of the major peanut allergens for use in immunotherapy. *Int Arch Allergy Immunol* 2000; 124 (1–3):70–2.
- 17 Smith AM, Chapman MD. Reduction in IgE-binding to allergen variants generated by site-directed mutagenesis: contribution of disulphide bonds to the antigenic structure of the major house dust mite allergen Der p2. *Mol Immunol* 1996; 33:399–405.
- 18 Zambryski PC. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annu Rev Plant Physiol Plant Mol Biol* 1992; 43:465–90.
- 19 Van Larebeke N, Engler G, Holsters M et al. Large plasmid in *Agrobacterium tumefaciens* essential for crown gall inducing activity. *Nature* 1974; 252:169–70.
- 20 Sheng J, Citovsky V. *Agrobacterium*-plant cell DNA transport: have virulence proteins, will travel. *Plant Cell* 1996; 8:1699–710.
- 21 Baum K, Groning B, Meier I. Improved ballistic transient transformation conditions for tomato fruit allow identification of organ-specific contributions of I-box and G-box to the RBCS2 promoter activity. *Plant J* 1997; 12:463–9.
- 22 Christou P. Rice transformation: bombardment. *Plant Mol Biol* 1997; 35:197–203.
- 23 Bates GW. Electroporation of plant protoplasts and tissues. *Meth Cell Biol* 1995; 50:363–73.
- 24 Saunders JA, Lin CH, Hou BH et al. Rapid optimization of electroporation conditions for plant cells, protoplasts and pollen. *Mol Biotechnol* 1995; 3:181–90.
- 25 Chowira GM, Akella V, Fuerst PE, Lurquin PF. Transgenic grain legumes obtained by in planta electroporation-mediated gene transfer. *Mol Biotechnol* 1996; 5:85–96.
- 26 Terzaghi WB, Cashmore AR. Plant cell transfection by electroporation. *Meth Mol Biol* 1997; 62:453–62.
- 27 Kramer MG, Redenbaugh K. Commercialization of a tomato with an antisense polygalacturonase gene: the FlavrSavrTM tomato story. *Euphytica* 1994; 79:293–7.
- 28 Hamilton AJ, Fray RG, Grierson D. Sense and antisense inactivation of fruit ripening genes in tomato. *Curr Top Microbiol Immunol* 1995; 197:77–89.
- 29 World Health Organisation. Health Aspects of Market Genes in Genetically Modified Plants. Geneva: WHO Food Safety Unit, 1993.
- 30 Perlak FJ, Stone TB, Muskopf YM et al. Genetically improved potatoes: protection from damage by Colorado potato beetles. *Plant Mol Biol* 1993; 22:313–21.
- 31 Hiei Y, Komari T, Kubo T. Transformation of rice mediated by *Agrobacterium tumefaciens*. *Plant Mol Biol* 1997; 35:205–18.
- 32 Cheng X, Sardana R, Kaplan H, Altosaar I. *Agrobacterium*-transformed rice plants expressing synthetic *cryIA (b)* and *cryIA (c)* genes are highly toxic to striped stem borer and yellow stem borer. *Proc Natl Acad Sci USA* 1998; 95:2767–72.
- 33 Jansen JJ, Kardinaal AF, Huijbers GH, Vlieg-Boerstra BJ, Martens BP, Ockhuizen T. Prevalence of food allergy and intolerance in the adult Dutch population. *J Allergy Clin Immunol* 1994; 93:446–56.
- 34 Young E, Stoneham MD, Petrukevitch A, Barton J, Rona R. A population study of food intolerance. *Lancet* 1994; 343:1127–30.
- 35 Sampson HA. Food allergy. *JAMA* 1997; 278:1888–94.
- 36 Bock SA. Prospective appraisal of complaints of adverse reactions to foods in children during the first three years of life. *Paediatrics* 1987; 79:683–8.
- 37 Emmett SE, Angus FJ, Fry JS, Lee PN. Perceived prevalence of peanut allergy in Great Britain and its association with other atopic conditions and with peanut allergy in other household members. *Allergy* 1999; 54:380–5.
- 38 Sampson H. Food hypersensitivity: manifestations, diagnosis and natural history. *Food Technol* 1992; 46:141–4.
- 39 Pene J, Rousset F, Briere F et al. IgE production by normal human B cells induced by alloreactive T cell clones is mediated by IL-4 and suppressed by IFN- γ . *J Immunol* 1988; 141:1218–24.
- 40 Li JTC, Yunginger JW, Reed CE, Jaffe HS, Nelson DR, Gleich GJ. Lack of suppression of IgE production by recombinant interferon γ : a controlled trial in patients with allergic rhinitis. *J Allergy Clin Immunol* 1990; 85:934–40.
- 41 Lund JW, Nap J-P. Assessment of Allergenic Potential of Foods from Genetically Modified Plants. Coordination Committee Risk Assessment Research, 1997:1–40.
- 42 Tamarcaz P, Eigenmann PA. Risques allergiques des aliments génétiquement modifiés. *Mise au point* 2000; 2290:511–4.
- 43 King V. Genetic modification and allergenicity: the risks and the implications for policy and industry. MSc dissertation. London: University of London, 1978.
- 44 Crespo JF, Pascual C, Dominguez C, Ojeda I, Munoz FM, Esteban MM. Allergic reactions associated with airborne fish particles in IgE-mediated fish hypersensitive patients. *Allergy* 1995; 50:257–61.
- 45 Bircher AJ, VanMelle G, Haller E, Curty B, Frei PC. IgE to food allergens are highly prevalent in patients allergic to pollens, with and without symptoms of food allergy. *Clin Exp Allergy* 1994; 24:367–74.

- 46 Enberg RN. Food-induced oropharyngeal symptoms. *Immunol Allergy Clin N Am* 1991; 11:767.
- 47 Gall H, Kalveram KJ, Forck G, Sterry W. Kiwi fruit allergy: a new birch pollen-associated food allergy. *J Allergy Clin Immunol* 1994; 94:70–6.
- 48 Enberg RN, Leickly FE, McCullough J, Bailey J, Ownby DR. Watermelon and ragweed share allergens. *J Allergy Clin Immunol* 1987; 79:867–75.
- 49 Anderson LB, Dreyfuss EM, Logan J, Johnstone DE, Glaser J. Melon and banana sensitivity coincident with ragweed pollinosis. *J Allergy* 1970; 45:310–9.
- 50 Burks AW, Williams LW, Thresher W, Connaughton C, Cockrell G, Helm RM. Allergenicity of peanut and soybean extracts altered by chemical or thermal denaturation in patients with atopic dermatitis and positive food challenges. *J Allergy Clin Immunol* 1992; 90:889–97.
- 51 Yman IM, Eriksson A, Everitt G, Yman L, Karlsson T. Analysis of food proteins for verification of contamination or misbranding. *Food Agric Immunol* 1994; 6:167–72.
- 52 Khakhoo A, Roberts G, Lack G. The epidemiology of adverse food reactions. In: Dean T, ed. *Food Intolerance and the Food Industry*. Cambridge: Woodhead Publishing, 2000:163–219.
- 53 Matsuda T, Nakamura R. Molecular structure and immunological properties of food allergens. *Trends Food Sci Tech* 1993; 4:289–293.
- 54 Liebers V, Sander I, Van Kampen V, Raulf-Heimsoth M, Rozynek P, Baur X. Overview on denominated allergens. *Clin Exp Allergy* 1996; 26:494–516.
- 55 Von Mutius E. The environmental predictors of allergic disease (Review). *J Allergy Clin Immunol* 2000; 105 (1, Part 1):9–19.
- 56 Hirsch T, Weiland SK, Von Mutius E et al. Inner city air pollution and respiratory health and atopy in children. *Eur Respir J* 1999; 14 (3):669–77.
- 57 Von Ehrenstein OS, Von Mutius E, Illi S, Baumann L, Bohm O, von Kries R. Reduced risk of hay fever and asthma among children of farmers. Comment. *Clin Exp Allergy* 2000; 30 (2):187–93.
- 58 Martinez FD, Holt PG. Role of microbial burden in aetiology of allergy and asthma (Review). *Lancet* 1999; 354 (Suppl. 2):S112–5.
- 59 Kajosaari M. Atopy prophylaxis in infants. Prospective 5-year follow-up study of children with six months exclusive breast feeding and solid food elimination. *Adv Exp Medical Biol* 1991; 310:453–8.
- 60 Pascual CY, Fernandez-Crespo J, Sanchez-Pastor S et al. Allergy to lentils in Mediterranean paediatric patients. *J Allergy Clin Immunol* 1999; 103:154–8.
- 61 Hourihane JO'B, Kilburn SA, Dean P, Warner JO. Clinical characteristics of peanut allergy. *Clin Exp Allergy* 1997; 27 (6):634–9.
- 62 Ford RPK, Taylor B. Natural history of egg hypersensitivity. *Arch Dis Child* 1982; 57:649–52.
- 63 De Kruffy RH, Fang Y, Umetsu DT. IL-4 synthesis by in vivo primed keyhole limpet hemocyanin-specific CD4⁺ T cells. I. Influence of antigen concentration and antigen-presenting cell types. *J Immunol* 1992; 146:3468–76.
- 64 Stok W, van der Heijden PJ, Bianchi AT. Conversion of orally induced suppression of the mucosal immune response to ovalbumin into stimulation by conjugating ovalbumin to cholera toxin or its B subunit. *Vaccine* 1994; 12 (6):521–6.
- 65 Hoyne GF, Callow MG, Kuo MC, Thomas WR. Differences in epitopes recognized by T cells during oral tolerance and priming. *Immunology Cell Biology* 1994; 72 (1):29–33.
- 66 Ishii N, Moriguchi N, Sugita Y et al. Analysis of responsive cells in tolerance by the oral administration of ovalbumin. *Immunological Investigations* 1993; 22 (6–7):451–62.
- 67 Vadas P, Wai Y, Burks W, Perelman B. Detection of peanut allergens in breast milk of lactating women. *JAMA* 2001; 285 (13):1746–8.
- 68 Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Holt PG. Development of allergen-specific T cell memory in atopic and normal children. Comment. *Lancet* 1999; 353:196–200.
- 69 Antó JM, Sunyer J, Rodriguez-Roisin R et al. Community outbreaks of asthma associated with inhalation of soybean dust. Toxicoepidemiological Committee. *N Eng J Med* 1989; 320:1097–102.
- 70 González R, Polo F, Zapatero L et al. Purification and characterization of major inhalant allergens from soybean hulls. *Clin Exp Allergy* 1992; 22 (8):748–55.
- 71 Davison AG, Britton MG, Forrester JA, Davies RJ, Hughes DTD. Asthma in merchant seamen and laboratory workers caused by allergy to castor beans. analysis of allergens. *Clin Allergy* 1983; 13:553–61.
- 72 O'Neill CE, Lehrer SB. Occupational reactions to food allergens. In: Metcalfe DD, Sampson HA, Simon RA, eds. *Food Allergies – Adverse Reactions to Foods and Food Additives*. Boston: Blackwell, 1991:207.
- 73 Gordon S, Tee RD, Nieuwenhuijsen MJ, Lowson D, Harris J, Newman Taylor AJ. Measurement of airborne rat urinary allergen in an epidemiological study. *Clin Exp Allergy* 1994; 24 (11):1070–7.
- 74 Longbottom JL, Price JA. Allergy of laboratory animals; characterisation and source of major mouse allergens, Ag1 and Ag3. *International Arch Allergy Appl Immunol* 1987; 82:450–2.
- 75 Swanson MC, Agarwal MK, Yunginger JW, Reed CE. Guinea pig derived allergens. Clinicoimmunologic studies, characterisation, airborne quantitation, and size distribution. *Am Rev Respir Dis* 1984; 129:844–9.
- 76 Price JA, Longbottom JL. Allergy to rabbits. II. Identification and characterisation of a major rabbit allergen. *Allergy* 1988; 42:39–48.
- 77 Meredith SK, McDonald JC. Work-related respiratory disease in the United Kingdom 1989–92: report on the SWORD project. *Occup Medical (Lond)* 1994; 44:183–9.
- 78 Baur S, Sauer W, Weiss W. Baking additives as new allergens in baker's asthma. *Respiration* 1988; 54:70–2.
- 79 Nordlee JA, Taylor SL, Townsend JA, Thomas LA, Bush RK. 1996 Identification of a Brazil-nut allergen in transgenic soybeans. *New Eng J Med* 1996; 334:688–92.
- 80 Fedak G. Intergeneric hybrids with *Hordeum*. In: Shewry P.R., ed. *Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology*. Wallingford, UK: CAB International 1992:45–70.
- 81 Brock RD. Mutation plant breeding for seed protein improvement. In: *Seed Protein Improvement in Cereals and Grain Legumes*. Vienna: International Atomic Energy Agency, 1979:43–55.
- 82 Davis PJ, Williams SC. Protein modification by thermal processing. *Allergy* 1993; 53 (Suppl 46):102–5.
- 83 Boye JI, Mar C-Y, Harwalkar VR. 1997 Thermal denaturation and coagulation of proteins. *Food Sci Technol* 1997; 80:25–6.
- 84 Kilara A, Harwalkar VR. Denaturation. In: Nakai S, Modler HW, eds. *Food Proteins, Properties and Characterisation*. New York: VCH:71–165.
- 85 Prausnitz C, Kustner H. Studies on supersensitivity. *Centralbl f Bakteriell Abt Org* 1921; 86:160–9.
- 86 Malanin K, Lundberg M, Johansson SG. Anaphylactic reaction caused by neoallergens in heated pecan nut. *Allergy* 1995; 50:988–91.
- 87 Codina R, Oehling AG, Lockey RF. Neoallergens in heated soybean hull. *International Archives of Allergy and Immunology* 1998; 117:120–5.
- 88 Host A, Samuelsson EG. Allergic reactions to raw, pasteurised, and homogenized/pasteurised cow milk: a comparison. A double blind placebo-controlled study in milk allergic children. *Allergy* 1988; 43 (2):113–8.

- 89 Biotechnology and Food Safety. Report of a Joint FAO/WHO Consultation. Rome, Italy 30 September–4 October 1996. FAO: Rome.
- 90 World Health Organization. Application of the Principles of Substantial Equivalence to the Safety Evaluation of Foods or Food Components from Plants Derived by Modern Biotechnology. Report of a WHO Workshop. Geneva: World Health Organization, 1995.
- 91 Organisation for Economic Cooperation and Development. Safety Evaluation of Foods Produced by Modern Biotechnology: Concepts and Principles. Paris: OECD, 1993.
- 92 Hinchee MAW, Connor-Ward DV, Newell CA. Production of transgenic soybean plants using agrobacterium-mediated DNA transfer. *Bio Technology* 1988; 6:915–22.
- 93 Padgett SR, Biest Taylor N, Nida DL et al. The composition of glyphosate-tolerant soybean seeds is equivalent to that of conventional soybeans. *J Nutr* 1996; 126:702–16.
- 94 Hammond BG, Vicini JL, Hartnell GF et al. The feeding value of soybeans fed to rats, chickens, catfish and dairy cattle is not altered by genetic incorporation of glyphosate tolerance. *J Nutr* 1996; 126:717–27.
- 95 Lehrer SB, Reese G. Recombinant proteins in newly developed foods: identification of allergenic activity. *Int Arch Allergy Immunol* 1997; 113:122–4.
- 96 Taylor SL, Lemanske RF, Bush RK, Busse WW. Food allergens: structure and immunologic properties. *Ann Allergy* 1987; 59:93.
- 97 Taylor SL, Lehrer SB. Principles and characteristics of food allergens. *Critical Reviews in Food Science and Nutrition* 1996; 36 (Suppl.):S91–118.
- 98 Gilissen LJW, Nap J-P. Assessment of Allergenic Potential of Foods from Genetically Modified Plants. CCRO Project no. 1. The Netherlands: Ministry of Economic Affairs, 1997.
- 99 Bargman TJ, Taylor SL, Rupnow JH. Food allergies. In: Tu AT, ed. *Handbook of Natural Toxins*, Vol. 7, Food Poisoning. New York: Marcel Dekker Inc., 1992:337–70.
- 100 Taylor SL. Evaluation of the allergenicity of foods developed through biotechnology. In: *Proceedings of the 3rd International Symposium on Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms*. University of California, USA November 1994. 185–98.
- 101 Zikakis JP, Rzuclido SJ, Biasotto NO. Persistence of bovine milk xanthine oxidase activity after gastric digestion *in vivo* and *in vitro*. *J Dairy Sci* 1977; 60:533–41.
- 102 Astwood JD, Leach JN, Fuchs RL. Stability of food allergens to digestion *in vitro*. *Nature Biotechnol* 1996; 14:1269–73.
- 103 Astwood JD, Fuchs RL. Allergenicity assessment of food derived from genetically modified plants. *Food Technol* 1996; Feb:83–8.
- 104 Tuck L, Blumstein GI. Studies in food allergy. II. Sensitisation to fresh fruits: clinical and experimental observations. *J Allergy* 1942; 13:574–82.
- 105 Yagami T, Haishima Y, Namamura A, Osuna H, Ikezawa Z. Digestibility of allergens extracted from natural rubber latex and vegetable foods. *J Allergy Clin Immunol* 2000; 106:752–62.
- 106 Atassi MZ. Antigenic structures of proteins. Their determination has revealed important aspects of immune recognition and generated strategies for synthetic mimicking of protein binding sites. *Eur J Biochem* 1984; 145:1–20.
- 107 Daul CB, Slattery M, Reese G, Lehrer SB. Identification of the major brown shrimp (*Penaeus aztecus*) allergen as the muscle protein tropomyosin. *International Arch Allergy Immunol* 1994; 105:49–55.
- 108 Fox J. FDA attacks food allergens. *Bio Technol* 1994; 12:568–109.
- 109 Aalberse RC. Structural biology of allergens. *J Allergy Clin Immunol* 2000; 106 (2):228–38.
- 110 Romagnani S, Mohapatra SS. Human T-cell responses to grass pollen allergens. In: Mohapatra SS, Knox RB, eds. *Pollen Biotechnology. Gene Expression and Allergen Characterization*. New York: Chapman & Hall, 1996:164–75.
- 111 The Allergen Database. <http://www/csl.gov.uk/allergen> (as on 3 May 2001).
- 112 The Food Allergy and Anaphylaxis Network. <http://www/foodallergy.org> (as on 3 May 2001).
- 113 National Center for Biotechnology Information (National Institutes of Health). <http://www.ncbi.nlm.nih.gov> (as on 3 May 2001).
- 114 Sánchez-Monge R, Lombardero M, Garcia-Sellés FJ, Barber D, Salcedo G. Lipid-transfer proteins are relevant allergens in fruit allergy. *J Allergy Clin Immunol* 1999; 103:514–9.
- 115 Melo VMM, Xavier-Filho J, Lima MS, Prouvost-Dannon A. Allergenicity and tolerance to proteins from Brazil nut. *Food Agric Immunol* 1994; 66:185–95.
- 116 Atkinson HAC, Johnson IT, Gee JM, Grigoriadou F, Miller K. A Brown Norway model of food allergy: effect of plant components on the development of oral sensitisation. *Food Chem Toxicol* 1996; 34 (1):27–32.
- 117 Gee JM, Wall JM, Miller K et al. Effect of saponin on the transmucosal passage of β -lactoglobulin across the proximal small intestine of normal and β -lactoglobulin sensitised rats. *Toxicology* 1997; 117:219–28.
- 118 Li X-M, Serebrisky D, Lee S-Y et al. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol* 2000; 106:150–8.
- 119 Li X-M, Schofield BH, Huang C-K, Kleiner GI, Sampson HA. A murine model of IgE-mediated cow's milk hypersensitivity. *J Allergy Clin Immunol* 1999; 103:206–14.
- 120 Food and Agriculture Organisation at the United Nations. Report of the FAO Technical Consultation on Food Allergies, Rome, Italy, no. 13–14. FAO: Rome, 1995.
- 121 Metcalfe D, Astwood JD, Townsend R, Sampson HA, Taylor SL, Fuchs RL. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Review in Food Science & Nutrition* 1996; 36 (Suppl.):165–86.
- 122 Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment. *Peanut Allergy*. London: Department of Health, 1998.
- 123 Mohapatra SS, Knox RB. *Pollen Biotechnology, Gene Expression and Allergen Characterisation*. London: Chapman & Hall, 1996.
- 124 Samoto M, Fukuda Y, Takahashi K et al. Substantially complete removal of three major allergenic soybean proteins (*Gly m* Bd 30K, *Gly m* Bd 28K, and the α -subunit of conglycinin) from soy protein by using a mutant soybean, Tohoku 124. *Biosci Biotech Biochem* 1997; 61 (12):2148–50.
- 125 Nelson HS, Lahr J, Rule R, Bock A, Leung D. Treatment of anaphylactic sensitivity to peanuts by immunotherapy with injections of aqueous peanut extract. *J Allergy Clin Immunol* 1997; 99:744–51.