

Suggestions for the Assessment of the Allergenic Potential of Genetically Modified Organisms

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Abstract

The prevalence of allergic diseases has been increasing continuously and, accordingly, there is a great desire to evaluate the allergenic potential of components in our daily environment (e.g., food). Although there is almost no scientific evidence that genetically modified organisms (GMOs) exhibit increased allergenicity compared with the corresponding wild type significant concerns have been raised regarding this matter. In principle, it is possible that the allergenic potential of GMOs may be increased due to the introduction of potential foreign allergens, to potentially upregulated expression of allergenic components caused by the modification of the wild type organism or to different means of exposure. According to the current practice, the proteins to be introduced into a GMO are evaluated for their physiochem-

ical properties, sequence homology with known allergens and occasionally regarding their allergenic activity. We discuss why these current rules and procedures cannot predict or exclude the allergenicity of a given GMO with certainty. As an alternative we suggest to improve the current evaluation by an experimental comparison of the wild-type organism with the whole GMO regarding their potential to elicit reactions in allergic individuals and to induce de novo sensitizations. We also recommend that the suggested assessment procedures be equally applied to GMOs as well as to natural cultivars in order to establish effective measures for allergy prevention.

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Introduction

Genetically modified organisms (GMOs), in particular genetically modified plants (GMPs), have been developed in the last years for several reasons [1–3]. One reason for the development of GMOs is to enhance the food supply by increasing crop yields. Biotechnology companies have been developing plant crops with improved agronomic characteristics such as resistance to insects, herbicides, and diseases. For the future, traits resulting in climate tolerance and enhanced benefits for the consumer such as better taste, longer shelf life and increased nutritive value may be expected. GMOs are a result of genetic engineering which uses recombinant DNA technology to transfer genes from one organism to another [4]. The foreign DNA can be coated on particles, which are inserted directly into the cells of the plant using a particle gun. Another possibility is to insert the foreign DNA into vectors such as plasmids or plant viruses. These vectors serve as molecular carriers to infect or transform plant cells and insert the desired DNA into the plant genome. The bacterium commonly used is *Agrobacterium tumefaciens*, which has the ability to infect many types of plants.

Electroporation and modifications on the basis of antisense technology and gene replacement are other strategies for genetic engineering.

Since plants represent major components of our environment and nutrition, concerns have arisen that GMPs may act as more potent allergen sources compared to the wild-type plants [5–7]. These concerns gained increased support when it turned out that a Brazil nut allergen had been engineered into transgenic soybeans and other transgenic plants [8]. The latter event stimulated an awareness about the potentially increased allergenic risk of GMOs and the need to evaluate GMOs regarding their allergenicity [2, 9–13].

The Impact of GMOs on Allergic Diseases: Risk Factor or Potential Advantage?

The introduction of novel proteins into plants may bear an increased risk of eliciting allergic reactions in already sensitized allergic individuals or by inducing de novo allergic sensitizations. In fact, the allergenic potential of a GMO may be increased compared to the wild-type plant for several reasons. First, it is possible that the newly inserted protein either represents an allergen or may behave as an allergen due to the expression in a particular plant tissue. Second, expression of foreign proteins

may have unintended effects on the plant metabolism (e.g. pleiotropic effects) leading to the upregulated expression of allergens or proteins, which may become allergenic due to their increased amounts. Third, it has to be considered that transgenic plants may be used in industrial processing; hence other exposure routes and sensitization scenarios might become important. For example, manufacturing large amounts of transgenic soy containing a food allergen may induce respiratory sensitization due to the generation of allergen-containing dust [14, 15]. Finally, it is possible that foreign allergens that stem from transgenic plants appear in processed food without proper labeling and thus may cause unexpected severe allergic reactions in food-allergic patients.

On the other hand, biotechnology may contribute to reduce allergenic risks [10, 11]. For example, it has been demonstrated that the allergenic potential of plants may be reduced by targeted elimination of allergens using antisense technology [16, 17]. Furthermore, it has been suggested that potent plant allergens may be replaced by highly homologous proteins with similar biological function but reduced allergenic activity [18].

Currently there is limited evidence that GMPs present a higher allergenic risk compared to certain natural plant cultivars and natural plant products, which due to extensive global trading have been newly introduced into our diet (e.g. kiwi) [19–21], and into food additives used in our countries (e. g. sweet lupine to flour [22]). However, the possibility that GMPs may also have toxic or other as yet unknown hazardous effects on the consumer as well as on the environment has led to especially strict requests for their careful evaluation.

Currently Applied Guidelines for the Evaluation of the Allergenic Potential of a GMO

In 2001, a joint FAO/WHO expert consensus panel met in Rome to discuss foods derived from biotechnology and adapted a decision tree for the evaluation of the allergenicity of novel proteins introduced into food [23]. The resulting consensus recommendations by the expert panel are summarized in the form of the FAO/WHO 2001 decision tree (fig. 1). This decision tree considers two possible scenarios. In the first scenario, the introduced genes originate from a source known to be allergenic, whereas the second possibility is that the inserted gene is derived from a source not known to be allergenic.

If the inserted gene is derived from an allergenic source, sequence homologies of the novel protein to known allergens are investigated. It is suggested that a more than 35% amino acid sequence identity or the identity of six contiguous amino acids of the novel protein with a known allergen indicates that the protein may be allergenic. If no such amino acid sequence homologies can be identified, a serum screening of the novel protein is recommended. The serum screen is supposed to be done by applying an appropriate in vitro method and with sera from clinically well-characterized patients who are allergic to the allergen source from which the newly inserted protein is derived or who are allergic to allergen sources which are closely related to the source of the foreign protein. If a newly inserted protein reacts with serum IgE, it is defined 'as likely allergenic'. A negative result in the serum screening leads to further testing of the novel protein regarding its stability to pepsin resistance and its ability to sensitize animals after oral uptake or intraperitoneal administration. In addition to the serum screen, in vivo and/or in vitro testing (skin prick testing, basophil histamine release and oral challenge) in allergic patients may be appropriate, but this recommendation is not included in the decision tree.

If the expressed protein derives from a source not known to be allergenic, sequence homologies of the novel protein to known allergens will be investigated. Any sequence homology/identity as described above defines the novel protein as 'likely allergenic'. A negative result in the sequence comparison leads to a targeted serum screening where a panel of 50 well-characterized patients' sera should be tested with the new protein. In the case of a positive reaction, the protein may be evaluated by further in vivo and/or in vitro testing. In case the new protein would lack IgE reactivity, it would be subjected to testing for pepsin resistance and immunogenicity in animal models. It is claimed that the evaluation according to the FAO/WHO decision tree can predict high and low probability of allergenicity for a given protein.

Several other decision trees for evaluating the allergenicity of novel proteins have been suggested and are used. In the USA the IFBC (International Food Biotechnology Council) concept [24] is frequently used and a recently published minimonograph published by the US National Institute of Environmental Health Sciences provides an update on the current practice [25]. The IFBC concept served as a model for the FAO/WHO decision tree and consequently is very similar. It is still considered as a standard for placing novel foods on the market in the USA. The decision tree developed by Lehrer et al. [26] is

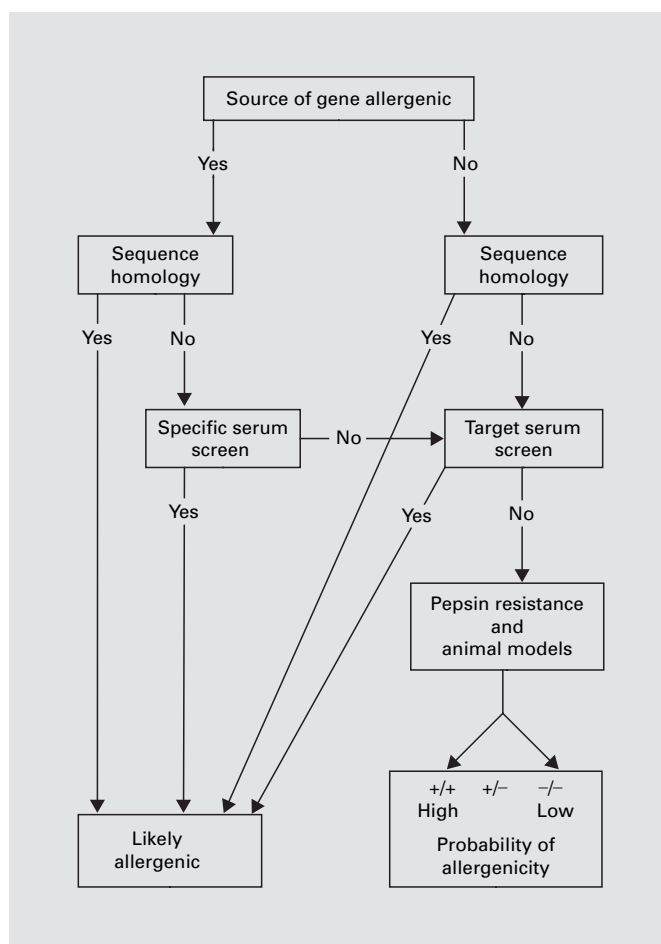


Fig. 1. The FAO/WHO 2001 decision tree for the evaluation of the allergenicity of novel proteins introduced into foods.

almost superimposable to the FAO/WHO decision tree. Jany and Greiner [27] developed a scheme on the basis of the European Union (EU) Recommendation 97/618/EC for the assessment of an allergenic risk of a novel protein, which similar to the previously mentioned decision trees, considers stability/instability of the novel protein during digestion in the gastrointestinal tract, glycosylation and isoelectric point, sequence homologies to known allergens, and homologies to 'safe' proteins as well as in vitro serology tests such as RAST and ELISA inhibition assays, and immunoblotting and in vivo tests such as skin prick tests for the evaluation of the allergenic activity [27].

Table 1. Frequently used arguments against an allergenic potential of GMOs

Source of the novel protein does not derive from a known allergen source
Lack of sequence homology to known allergens
The novel protein can be easily digested
The inserted gene is expressed at low levels
The novel protein is not glycosylated

A Review of Currently Applied Procedures for the Evaluation of the Allergenic Potential of GMOs to Be Released in Countries of the EU

In the EU, the assessment of the allergenic potential is required for market authorization of GM crops in Directive 2001/18/EC and in the preceding Directive 90/220/EEC. The allergenic potential of novel foods and food ingredients is mentioned in the EU Regulation 258/97 ('Novel Food Regulation' which for GMOs has been replaced by Regulation 1829/2003 in April 2004) and in the Recommendation 97/618/EC.

The Umweltbundesamt (Austrian Federal Environment Agency) and the Inter-University Research Centre for Technology, Work and Culture in Austria have recently conducted an analysis of eleven applications for market authorization according on the Directive 90/220/EEC of GMPs [28] in Europe regarding toxicity and allergenicity assessment. Shortly thereafter a second survey was carried out to investigate the practice in twelve 'novel food applications', which had been filed in the EU [29].

These applications contained basically no experimental work to assess the allergenic potential of the GMPs. Only one of the applications had studied the IgE reactivity of the foreign protein. All other applications contained only arguments as to why the GMP does not present an allergenic risk. These arguments are summarized in table 1. In most applications it was claimed that the protein was derived from a nonallergenic source, expressed at low levels in the GMP and may be easily digested in the gastrointestinal tract. Sequence comparisons were conducted to demonstrate that the new protein was not homologous to known allergens. Furthermore, the argument that a nonglycosylated protein is not allergenic was proposed.

In all applications only the newly inserted protein was discussed, whereas none of the applications considered

pleiotropic effects possibly caused by the insertion of the foreign protein, which may have effects on the expression of other allergenic proteins in the host organism. Mainly the oral route was considered as a possible route for allergic sensitization, whereas no consideration was given to other exposure or sensitization scenarios.

A Critical Assessment of the Current Guidelines for the Evaluation of the Allergenic Potential of GMPs

The evaluation of applications for placing GMPs on the European market has led to several interesting observations. First, it was noticed that many of the arguments forwarded in the applications to diminish the concerns regarding allergenic risk were either incorrect or insufficient to estimate the allergenic risk of a GMP with sufficient certainty (table 1). Below, we will critically discuss several of the most frequently used arguments in light of the currently available scientific data.

At present, there seem to be no clear guidelines for the experimental evaluation of the allergenic potential of GMPs. In this context we will discuss the decision tree of the FAO/WHO for evaluating the allergenic potential of GMPs and provide suggestions for improvement. In addition, we recommend that the whole GMP be evaluated rather than only the foreign protein introduced, in order to consider possible pleiotropic effects that may be caused by insertion of the foreign gene. Finally, it is felt that different exposure routes and circumstances of sensitization need to be considered. These may greatly vary depending on the intended use of the GMP.

Given the fact that there is almost no scientific evidence that GMPs present a higher allergenic risk than organisms without insertion of foreign genes we raise the question of whether different rules should be applied when evaluating the allergenic risk of GMPs and conventional crops and food. Numerous natural allergen sources (e.g., tropical fruits, fruits and vegetables with high allergen contents) have entered the market without having been subjected to any examination. A review of the literature regarding allergic reactions caused by food that has been newly introduced into our diet shows that there is a considerable number of reports about allergic reactions to newly introduced natural or processed food [21, 30–38] and food additives [22], whereas almost no data regarding increased allergenicity of GMOs are available [8]. It may, therefore, be pertinent to seek scientific methods that equally allow us to evaluate the allergenic risk of

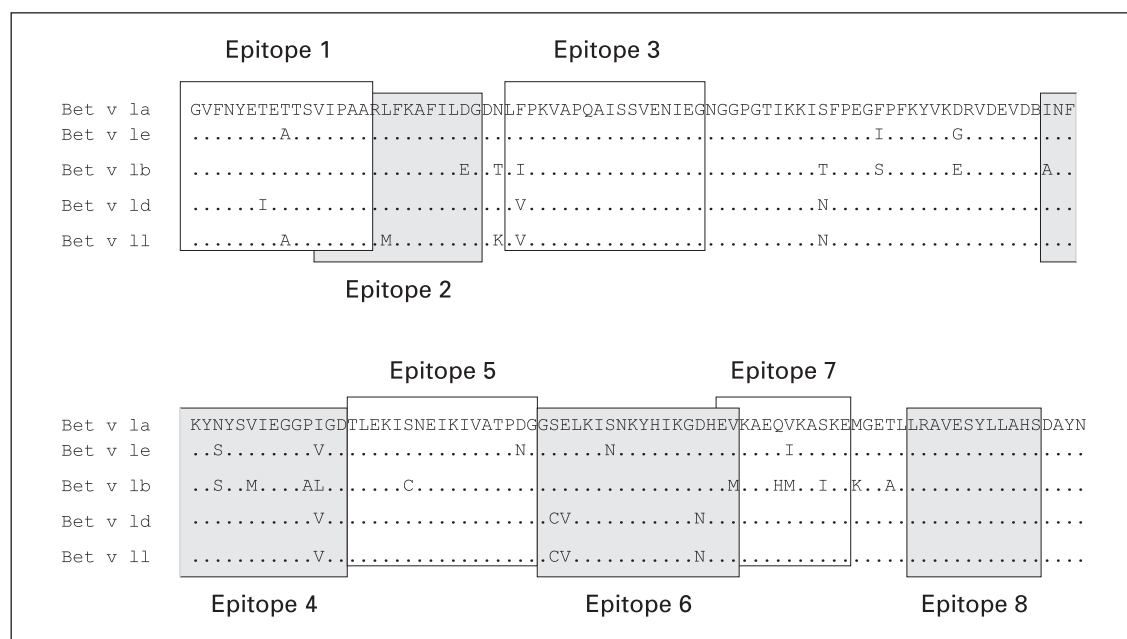


Fig. 2. Amino acid sequence comparison of five Bet v 1 isoforms. Boxes show T cell epitopes [with permission, 50].

conventional products, as well as GMPs. Suggestions for such methods will be given in one of the following sections.

Is it Important Whether a Newly Inserted Gene Is Derived from an Allergenic or Nonallergenic Source?

One argument frequently cited for the lack of allergenic potential of a particular GMP was that the newly inserted gene does not stem from a known allergen source. However, due to advances in research, it turns out that several antigen sources (e.g. maize, bacteria, yeast), which were thought not to play a role as allergen sources, have been discovered to contain allergenic molecules [39–41]. Moreover, if one follows the decision tree of the FAO/WHO [23], one will easily recognize that the origin of the newly inserted gene has no relevant implication for the evaluation of the allergenic potential of a GMO because the decision tree suggests that a sequence comparison has to be performed in any case. It should also be emphasized that even a protein which does not represent an allergen in its natural source may become a potent allergen through a new route of presentation. For example, it is possible that proteins may become highly allergenic in another

organism provided that they are expressed in a tissue which liberates the protein easily at the mucosa of individuals.

Furthermore, it is possible that the insertion of a non-allergenic protein into a host organism may unintentionally induce the activation of allergens and thus indirectly increase the allergenic potential of the GMP. In this context it should be mentioned that several potent plant allergens fall into the group of pathogenesis-related proteins, which become activated when the plant is infected or subjected to stressful conditions [42]. It is thus possible that the insertion of a nonallergenic protein may induce stressful conditions, which lead to upregulated allergen expression in the host organism.

The Questionable Value of Sequence Comparisons for Assessing the Allergenic Potential of a Protein

One of the most frequent instruments used for the assessment of the allergenic potential of a GMO is the sequence comparison of newly introduced proteins with allergen sequences submitted to various databases. There is no argument against performing a sequence homology search because it is very easy to perform. The questions

are what method of comparison should be selected and how should a meaningful sequence similarity be defined that will predict an allergenic risk. Meanwhile numerous methods of sequence comparison have been suggested for assessment of allergenicity. However, the routinely used sequence comparison technologies such as FASTA and BLAST [43–45] as well as all of the new methods developed more specifically for predicting the allergenic potential of a given protein [46–48] would provide the wrong results in many cases. For example, it is well known that nonallergenic isoforms of allergens exist which differ by only a few amino acids compared to their allergenic counterparts. Such nonallergenic isoforms have been described for many allergen sources (e.g. birch, hazel pollen) [49, 50]. Figure 2 shows an amino acid sequence comparison of five recombinant Bet v 1 isoforms which differ only in few amino acids. Isoform Bet v 1a shows a high IgE-binding activity, whereas isoforms Bet v 1d and Bet v 1l show a very low IgE-binding activity. Because of their low allergenic potential such isoforms have even been suggested as candidates for allergen-specific immunotherapy [50]. Likewise, proteins with significant sequence homology to major allergens but without any allergenic activity have been described [18]. For example, a cytokinin-inducible periwinkle protein (T1) was found to exhibit significant sequence homology with pathogenesis-related proteins and the Bet v 1 allergen family. The amino acid sequences of the periwinkle protein (T1) and the major birch pollen allergen showed 40.4% sequence identity, but in spite of the sequence homology the periwinkle protein showed immunologically distinct features from the Bet v 1 allergen family and exhibited no allergenic properties. These concrete examples show that sequence comparisons could identify proteins wrongly as allergens. On the other hand, we know that new allergens are discovered continuously and it is hence clear that the databases containing allergen sequences are incomplete at present. Accordingly, allergenic potential cannot be excluded with sufficient certainty when no sequence homology has been found. Another problem is that the sole analysis of the newly introduced protein does not take into account possible alterations in the host organism which may lead to its increased allergenic potential.

In conclusion, it can be stated that sequence comparisons can be easily performed and there is no reason to exclude these methods from allergenicity assessment. However, they can neither predict nor exclude with sufficient certainty allergenicity of a given protein nor do they provide any information on the allergenic potential of the GMP as a whole.

The Novel Protein Has to Be Tested with Sera of Allergic Patients: Differentiation between ‘Specific Serum Screen’ and ‘Targeted Serum Screen’?

According to the FAO/WHO decision tree of 2001 [23], a lack of sequence homology of the new protein with allergen sequences should lead to a serum screen. This represents, however, a rather recent change in the guidance for allergenicity assessment. Decision tree approaches that were used before 2001 required an *in vitro* digestibility study following a negative result of the homology comparison instead [23, overview provided in 27].

In case of an allergenic source organism a ‘specific serum screen’ and otherwise a ‘targeted serum screen’ should be conducted. Whereas the former means that sera from clinically well-characterized patients allergic to the source of the novel protein are used, the latter suggests the use of sera from patients allergic to related proteins of the newly inserted protein.

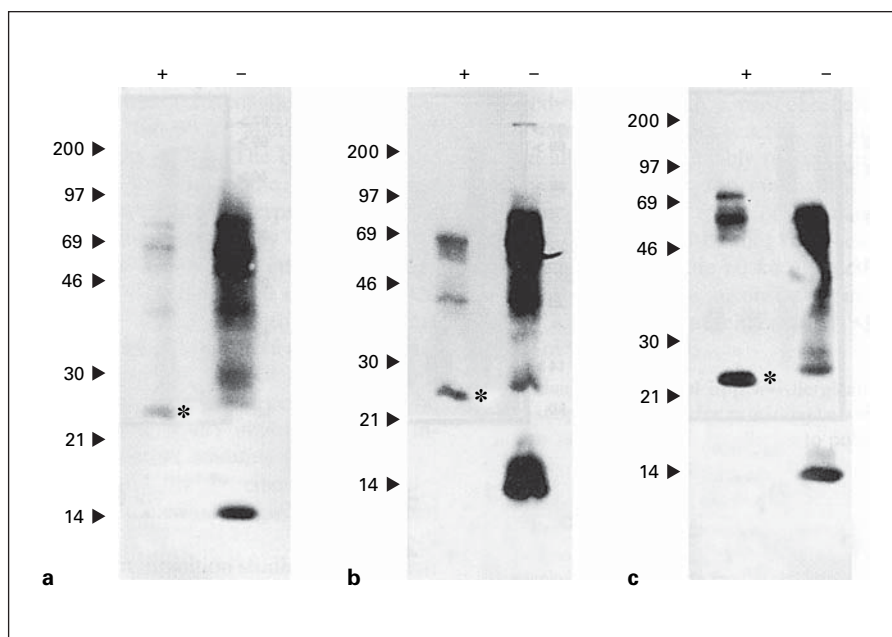
The review of applications for placing on the market as plants, seed and food conducted by the Austrian Umweltbundesamt showed that only one application had employed serological testing [28, 29]. However, we feel that the sole testing of the newly inserted protein is not sufficient. Instead, we suggest extending the serum screening from the target protein to the whole GMP in order to include effects that may indirectly increase the allergenicity of the GMP through pleiotropic effects. For example, it is possible that the insertion of a novel protein may lead to an upregulated expression of allergens or proteins with sensitization capacity. This concern is supported by the fact that many potent plant allergens fall into the group of pathogenesis-related proteins which are induced upon stress, pathogen attack and other stimuli [42].

To assess the allergenic risk caused by the GMP, it would be necessary to compare the GMP with the corresponding wild-type or parent organism as regards tissue. Only such a procedure would make it possible to judge whether the GMP bears a higher risk of allergenicity than the corresponding wild-type organism.

The Misconception that Allergens Are Always Characterized by Resistance to Proteolytic Digestion

The misconception that an allergen can be identified by its intrinsic stability against digestion, whereas nonallergenic proteins would be degraded goes back to studies

Fig. 3. a–c IgE reactivity of sera from mugwort pollen-allergic patients to nitrocellulose blotted untreated (–) and trypsinized (+) mugwort pollen extract. Stars indicate the position of trypsin [with permission, 54].



which were trying to identify general features of allergens [51]. Meanwhile several studies have clearly demonstrated that stability against digestion does not represent a feature which is strictly associated with allergenicity [52, 53]. A study by Heiss et al. [54] showed that trypsin digestion of mugwort pollen leads to digestion of the highly cross-reactive allergen profilin whereas another cross-reactive 60-kDa allergen turned out to be highly resistant against digestion (fig. 3). In light of recent scientific results it appears that digestibility studies cannot be considered suitable tools to address the allergenic potential of a protein or a GMP. The focus on resistance to digestion also may be overestimated considering studies which indicate that sensitization and boosting of the allergic immune response via the respiratory tract may be more important in allergic sensitization than mechanisms operating via the gastrointestinal tract [reviewed in 55–57].

Is Glycosylation a Feature of an Allergen?

According to current information about the nature of allergens the answer to the above question has to be a clear 'no'. Our knowledge regarding the molecular nature of allergens has increased tremendously during the last 15 years [reviewed in 58, 59; see allergen databases: www.allergenonline.com, <http://129.109.73.75/SDAP>]. Nevertheless, the analysis of applications for market

authorization of GMPs [28, 29] revealed that in almost all applications it was argued that the newly inserted protein is not glycosylated and hence will not exhibit allergenic activity. However, this assumption cannot be entertained further and a few examples are given below.

For example, profilins represent highly cross-reactive allergens but are not glycosylated [60]. Also another group of cross-reactive allergens belonging to calcium-binding proteins are not glycosylated [61]. Both families, profilins and calcium-binding proteins, represent potent well-characterized allergens, which have been tested for their allergenic activity in great detail. Although a few studies indicate that carbohydrate components might contribute to allergenic activity, there is now increasing evidence that carbohydrates are involved in IgE recognition but are poor elicitors of allergic reactions [62–65]. Only few reports are available describing carbohydrate components that bind IgE from sera of allergic patients and induce histamine release from blood cells [66, 67].

The Importance of the Expression Level and the Amount of a Protein in Allergenic Activity

Already the report of the Joint FAO/WHO expert consultation stated that it is not possible to draw conclusions regarding potential allergenicity of a given protein from its expression level [23]. Nevertheless, it turned out to be

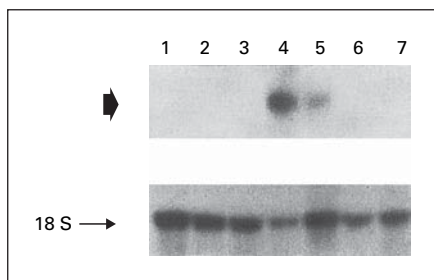


Fig. 4. Expression of the profilin transcript in different tobacco tissues and pollen. RNA from tobacco microspores (lane 1), mid bi-nucleate pollen (lane 2), embryogenic pollen (lane 3), mature pollen (lane 4), germinated pollen (lane 5), leaves (lane 6) and ovaries (lane 7) was separated by denaturing agarose gel electrophoresis, transferred to a nylon membrane and hybridized with the complete tobacco pollen profilin cDNA. Hybridization with the profilin transcript was found at 700 nucleotides in RNA of mature (lane 4) and germinated (lane 5) pollen (indicated by an arrow). Below, the same membrane was hybridized with an oligonucleotide complementary to 18S plant ribosomal RNAs to ensure that comparable amounts of intact RNA were transferred to the membrane [with permission, 68].

a common practice to argue against the allergenic potential of a protein by stating that the protein is expressed only at low levels in a GMO [28, 29].

Furthermore, it should be considered that the expression levels of certain allergens can vary depending on plant growth, developmental stages, tissue-specific expression and environmental stress. An example of the variability of allergen contents in different developmental stages and tissues is the allergen profilin. Profilins represent actin-binding proteins which have been described as cross-reactive plant allergens [60]. Profilin can be detected in different somatic plant tissues, but its expression and content were 50- to 100-fold higher in mature pollen than in seed or leaf. During early stages of pollen development no profilin could be detected by Northern blotting, whereas in mature pollen large amounts of profilin were found (fig. 4) [68].

Another study has investigated sixteen apple varieties regarding their contents of the major apple allergen, Mal d 1 [69]. The authors showed that certain apple strains contained larger amounts of Mal d 1 than others and concluded that the IgE-binding potency of different apple strains correlated with the amount of the allergen. Similar findings were made for the lipid transfer protein, Pru p 3, the major allergen of peach [70]. The concentration of lipid transfer protein Pru p 3 in peach peel extracts was approximately 7 times greater than in pulp extracts [70].

Thus it can be concluded that the expression level of a given protein cannot always be related to its potential allergenicity. Furthermore, expression levels of proven allergens may greatly vary in different strains, tissues and developmental stages, and can be influenced by a variety of factors.

Animal Models

At this point it may be useful to introduce a definition of terms which are frequently used in the context of evaluating the allergenic potential of a GMP. It is recommended that the term 'allergenic activity' be used to describe the propensity of a substance to induce allergic reactions in sensitized allergic patients. The allergenic activity of a certain component can be evaluated in allergic patients by provocation testing or by tests which closely mimic provocation testing. The goal is to find out whether the component in question can induce an allergic reaction in allergic individuals and to estimate the magnitude of this reaction. By contrast, the term 'allergenicity' describes the likelihood of inducing *de novo* sensitization in a nonallergic individual. Allergenicity cannot be determined by the above-mentioned methods but requires models of allergic sensitization which are mostly conducted in animals. There are several animals and strains described that can be used to investigate allergic sensitization and immune responses. They include rodent models as well as dog and primate models. Such models may be helpful to study the potential of a given protein to induce an allergic sensitization in a nonallergic individual. The current FAO/WHO 2001 decision tree recommends the use of animal models to study allergic sensitization and even suggests the use of different sensitization routes (oral sensitization, intraperitoneal administration) because different sensitization routes may induce different results [23]. It is also well established that different forms of allergic diseases may be provoked depending on the animal species/strain used and depending on the route of sensitization. Furthermore, there may be great differences regarding the MHC background of the animals used. Therefore, it will be quite difficult to make judgments and set cutoffs for determining whether a protein may be allergenic or not. However, it has been demonstrated that proteins can be compared regarding their ability to induce IgE responses in animals. It has been shown that immunization of Balb/c mice with purified natural and recombinant allergens induced IgE responses comparable to that seen in allergic patients [71]. Furthermore, it was

observed that potent and frequent allergens tended to induce higher IgE responses compared to less potent allergens. In this respect it appears that allergenicity is strongly related to the overall immunogenicity of an antigen.

At present, it seems impossible to develop animal models, which can predict or exclude with sufficient certainty whether a given protein can induce *de novo* allergic sensitization. It may, however, be possible to compare by simple allergic sensitization protocols new proteins with known allergens regarding the induction of IgE responses. Considering the possibility that the insertion of a foreign protein into an organism may induce an upregulation of allergen expression via indirect mechanisms, it may, however, be advisable to compare whole tissue extracts from the wild-type plant with tissue extracts from GMPs regarding their potential to induce IgE responses, rather than testing only the newly inserted protein. Using such a strategy it would be possible to define whether a particular GMP is more, equal or less potent in inducing an allergic immune response compared to the naturally occurring wild-type organism.

The Importance of Various Exposure Routes and Sensitization Scenarios

The analysis of applications for releasing GMPs revealed that the oral route of sensitization and eliciting allergic reactions was the primary consideration, whereas no attention was given to other exposure scenarios [28, 29]. The reason for this incomplete assessment may be that most of the GMPs were thought to be used for food production. However, it must be considered that GMPs may exhibit allergenic activity also via other routes, particularly when they are grown on a large scale and processed by different means. For example, pollens represent much more potent and frequent allergen sources than plant-derived food and it should, therefore, be considered that GMPs may also release allergens via pollen production and hence cause respiratory sensitization. Furthermore, processing of maize may lead to respiratory sensitization in bakers who are exposed to flour. In this context it has been reported that soybean dust caused severe outbreaks of asthma in Barcelona, Spain when the soybeans were unloaded in the city harbor [14, 15]. Many of the responsible soybean hull allergens were identified and characterized [14]. Another example for respiratory sensitization has been described in employees who are exposed to papain [72]. The cross-reactivity of IgE antibodies of papain with latex allergens has been reported using

sera of latex-exposed and papain-exposed people [73]. Hypersensitivity to papain was found in approximately 1% of an allergic population using skin prick tests and IgE measurements and was confirmed by oral challenge [74].

We, therefore, suggest that other exposure and sensitization scenarios be considered when assessing GMPs or other products rather than focusing on the gastrointestinal route only.

Guidelines for GMOs May Also Be Applicable for Natural Organisms and Processed Products

Allergic reactions to food represent an important health problem [75, 76]. In this context it may be useful to establish methods which allow detection, measurement and quantification of important allergens in natural and processed food. These methods are mainly based on the use of allergen-specific antibodies which allow the detection and quantification of allergens by immunological assays (e.g. sandwich ELISA protocols) [77, 78]. Similar methods have been developed for the measurement of numerous environmental allergens. Based on the detection of allergens in our environment and in our food, preventive strategies may be developed which may reduce the risk of allergen exposure. In fact, the above-mentioned methods for allergen detection and quantification can also be applied for GMPs, once they are released.

However, it has been required to assess GMPs regarding allergenicity prior to their release. This requirement is based on the assumption that GMPs might pose an unknown and perhaps higher allergenic potential compared to natural products. Concerns have been raised when it turned out that a brazil nut allergen was engineered into transgenic soybeans and other transgenic plants [8]. But, on the other hand, in numerous studies it was demonstrated that a variety of natural foods which have been newly introduced on the market have caused allergic reactions [30–38]. For example, kiwi fruits induce allergic reactions in a considerable number of patients as a consequence of their growing consumption in Europe. These patients exhibit IgE reactivity to the major kiwi fruit allergen which has been identified and characterized recently [21]. Another paper by Sten et al. [31] describes an edible nut (Nangai), which is cultivated on the islands in the Pacific, as a potent allergen source for patients suffering from allergy to grass, birch, and mugwort pollen, al-

though these patients were not exposed to Nangai nuts before. Furthermore, food additives such as sweet lupine and papain can represent allergen sources [22, 79]. Hefle et al. [22] describes that lupine-fortified pasta in a 5-year-old child with peanut sensitivity caused urticaria and angioedema after ingestion. Additionally, it is reported that some peanut-allergic patients show a cross-reactivity to sweet lupine. Taylor and Hefle [79] reported that ingredients such as papain, classified as rarely allergenic, should be declared in the ingredient listings on product labels.

In light of the increasing evidence of allergic reactions to newly introduced natural food it may also be worth considering the application of controlled allergenicity assessment for natural and perhaps even for processed products. In principle the Novel Food Regulation 258/97, which regulates novel food other than GM food, is a step in the right direction.

Hypersensitivities Other than IgE-Mediated Allergies Are Also Important for Risk Assessment

IgE-mediated allergies affect more than 25% of the population and belong to the most frequent immunologically mediated hypersensitivities. However, there are also several other types of hypersensitivity diseases such as celiac disease, cell-mediated reactions and immune complex diseases, which currently are not considered in the safety assessment of GMOs or other products [76]. The above-mentioned hypersensitivity diseases are less frequent than IgE-mediated allergies but may cause severe manifestations including gastroenteritis and lung diseases. Their immunopathogenic mechanisms are only poorly understood and assays for diagnosis are less well developed than for IgE-mediated allergies. Furthermore, there are no animal models reflecting human disease and it is, therefore, not possible to determine criteria whether GMOs, natural or processed products, may bear an increased risk of inducing these hypersensitivity diseases. At present, it is not possible to establish test systems that would allow a safety assessment regarding the non-IgE-mediated reactions and efforts will have to focus on the characterization of the antigens causing these diseases and on the investigation of the underlying pathomechanisms.

Suggestions for the Evaluation of the Allergenic Potential of GMOs

Based on the considerations made so far it is possible to develop a flow chart for the evaluation of the allergenic potential of GMPs. Figure 5 illustrates such a possible procedure for allergy risk assessment. Several aspects of this suggested assessment are different from the established procedures.

First, it is suggested that pleiotropic effects, which are caused by the insertion of a foreign gene, also be assessed, and that the effect of allergenic activity and allergenicity of the whole GMP is determined rather than exclusively focusing on the newly introduced protein. When the whole GMP is analyzed, it is mandatory to compare it with the wild-type organism with respect to allergenic activity and allergenicity. Assessing the whole GMP will focus not only on the allergenic potential of the foreign gene product, but also determine whether the insertion of the gene induces expression of other allergens. Such an evaluation could answer the question of whether the GMP has a greater, similar or perhaps reduced allergenic potential than the wild type.

Second, it is suggested that extracts from different tissues of the GMP and the wild type be tested, taking into consideration possible exposure scenarios. For example, large-scale farming of GMPs may lead to a considerable release of pollen whereas marketing of GMP seeds as food or industrial processing of other parts of the plant creates other exposure scenarios. Regarding the methods used for preparing tissue extracts, mechanical homogenization in combination with aqueous extraction similar to that used for the preparation of natural allergen extracts used for allergy diagnosis may be considered.

Third, it is recommended that the GMP be compared with the wild-type organism with respect to allergenic activity and allergenicity by experimental tests. In this context serological testing for IgE reactivity with a representative number of sera from allergic patients seems to be the only possibility because cellular (e.g. basophil histamine release tests) and *in vivo* tests (e.g. skin testing) cannot be performed with large numbers of patients for practical and ethical reasons. For the testing, sera should be selected that have a high likelihood of containing IgE against organisms which are related to the host organism or to the organism from where the foreign gene is derived. At least more than 100 sera from allergic patients of the population to be exposed to the GMP should be included in the analysis, but the selection of these sera will obviously be difficult to standardize. On the other hand, it

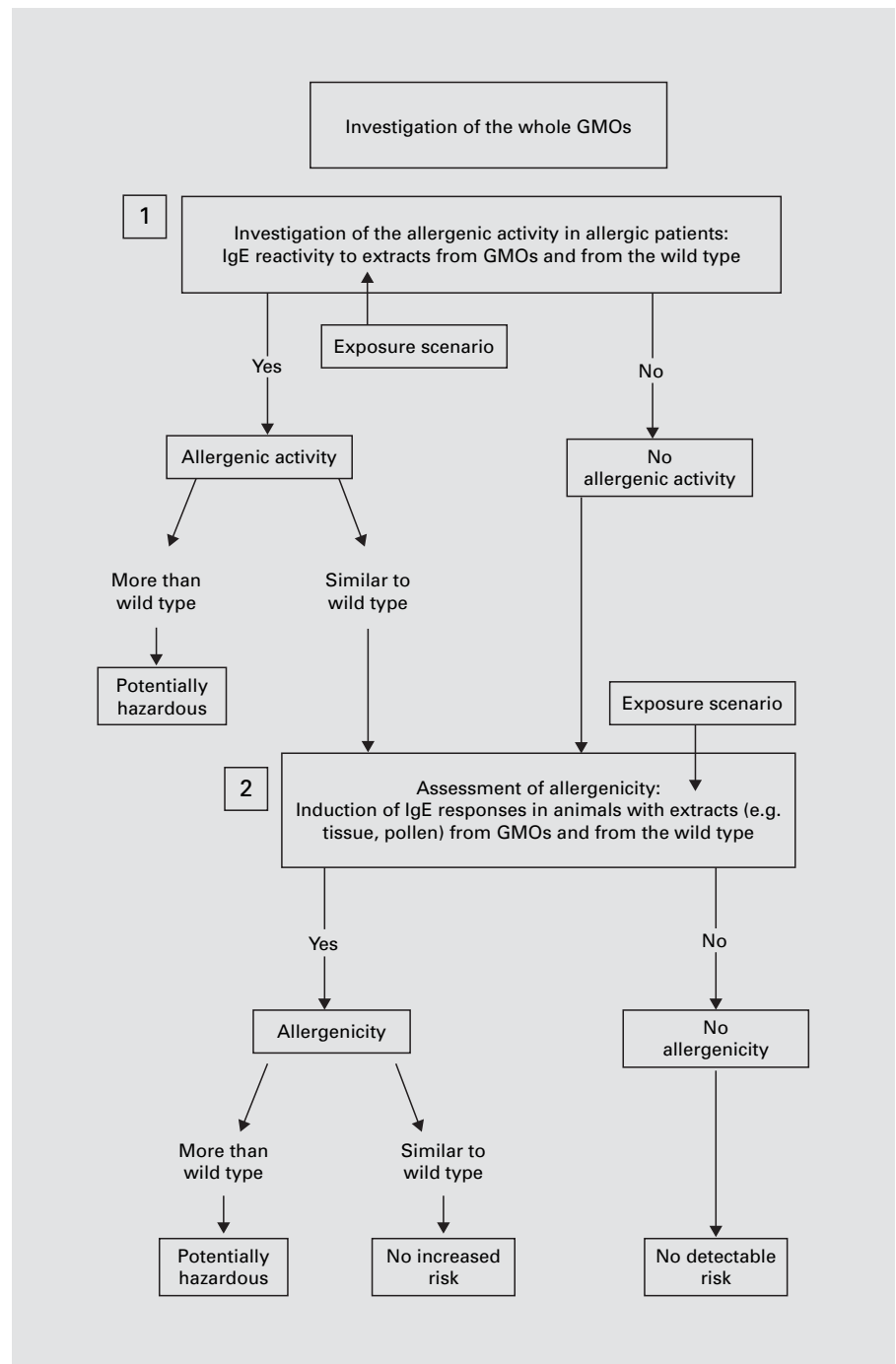


Fig. 5. Possible scheme for comparing the allergenic activity and allergenicity of a GMO with the corresponding wild type.

should be feasible to create representative serum banks for serological testing on a scientific basis because even companies developing diagnostic and therapeutic reagents already utilize this principle.

A more delicate issue to study is the question of whether a GMP may more easily induce an allergic immune

response than the corresponding wild type. To address this question one could use the well-established principle of injecting antigens with aluminium hydroxide into naïve mice and measuring the magnitude of IgE responses [reviewed in 80]. In fact it has turned out that potent and frequently recognized allergens induce higher IgE re-

sponses in mice than less potent allergens [71]. This assay is far from being perfect but is based on the reasonable assumption that antigens with low immunogenicity will less likely induce allergic immune responses. Again, this assay would involve a comparison of extracts from the GMP and the corresponding wild type to answer the question of whether the GMP bears a higher risk of allergenicity compared to the wild type. It should be considered that responses in mice can vary due to marked major histocompatibility complex gene effects showed for the major mite allergens Der p 1 and Der p 2 [81, 82]. Therefore, potential allergen sources should be analyzed using different strains of mice. Differences in the IgE responses of humans and animals to different house dust mite allergens are reported. For example, dogs allergic to house dust mite primarily produce IgE antibodies to the high-molecular-weight allergens and are lower responders to group 1 and 2 mite allergens, which are the major mite allergens associated with human allergy [83–86].

The ability of a protein when administered as a mixture of proteins to induce IgE antibodies in mice should be considered. A paper by Seitzer et al. [87] showed that mice immunized with timothy grass pollen extract produced IgE antibodies to most of the tested allergens, but did not give an IgE response to the major timothy grass pollen allergen Phl p 1. Furthermore, other immunization strategies such as the application of allergens by skin patch for induction of IgE responses should be considered

[88, 89]. Recently a paper was published that demonstrates that food allergy can be induced by allergen exposure through the skin [90].

The suggested experimental evaluations are certainly more meaningful than sequence comparisons, digestibility studies and guesses regarding allergenicity which are purely based on the physicochemical nature and characteristics of proteins. However, it must be clear that the suggested experimental criteria will not predict or exclude an increased allergenic potential of a GMP with absolute certainty. In this context it will be important to search for allergic reactions and sensitizations against GMPs after their release in the exposed population. Probably, this can be conducted in the course of postmarketing monitoring which is required by the new Regulation 1829/2003 for genetically modified food and feed and by Directive 2001/18/EC for placing on the market of GMOs as well.

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References

- Perr HA: Children and genetically engineered food: Potentials and problems. *J Pediatr Gastroenterol Nutr* 2002;35:475–486.
- Taylor S, Hefle SL: Will genetically modified foods be allergenic? *J Allergy Clin Immunol* 2001;107:765–771.
- Astwood JD, Bannon GA, Dobert RL, Fuchs RL: Food biotechnology and genetic engineering; in Metcalfe DD, Sampson HA, Simon RA (eds): *Food Allergy: Adverse Reactions to Foods and Food Additives*. Oxford, Blackwell Science, 2003, vol 3, pp 38–50.
- Newell CA: Plant transformation technology. Developments and applications. *Mol Biotechnol* 2000;16:53–65.
- Metcalfe DD, Astwood JD, Townsend R, Sampson HA, Taylor SL, Fuchs RL: Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit Rev Food Sci Nutr* 1996;36:S165–S186.
- Astwood JD, Fuchs RL: Allergenicity of foods derived from transgenic plants. *Monogr Allergy* 1996;32:105–120.
- Woolhiser MR, Metcalfe DD: Safety issues in food biotechnology; in Metcalfe DD, Sampson HA, Simon RA (eds): *Food Allergy: Adverse Reactions to Foods and Food Additives*. Oxford, Blackwell Science, 2003, vol 3, pp 38–50.
- Nordlee JA, Taylor SL, Townsend JA, Thomas AL, Bush RK: Identification of a brazil-nut allergen in transgenic soybeans. *N Engl J Med* 1996;334:688–692.
- Wuthrich B: Lebensmittelzusatzstoffe und 'Genfood' – eine Gefahr für Allergiker? *Schweiz Rundsch Med Prax* 1999;88:609–614, 616–618.
- Bucchini L: Allergenic risk from novel plants is very important and difficult to predict. *J Allergy Clin Immunol* 2001;108:654.
- Taylor SL, Hefle SL: Genetically engineered foods: Implications for food allergy. *Curr Opin Allergy Clin Immunol* 2002;2:249–252.
- Helm RM: Biotechnology and food allergy. *Curr Allergy Asthma Rep* 2002;2:55–62.
- Helm RM: Food biotechnology: Is this good or bad? Implications to allergic diseases. *Ann Allergy Asthma Immunol* 2003;90:90–98.
- Codina R, Lockey RF, Fernández-Caldas E, Rama R: Identification of the soybean hull allergens responsible for the Barcelona asthma outbreaks. *Int Arch Allergy Immunol* 1999;119:69–71.
- Anto JM, Sunyer J, Reed CE, Sabria J, Martinez F, Morell F, Codina R, Rodriguez-Roisin R, Rodrigo MJ, Roca J, Saez M: Preventing asthma epidemics due to soybeans by dust-control measures. *N Engl J Med* 1993;329:1760–1763.
- Bhalla PL, Swoboda I, Singh MB: Antisense-mediated silencing of a gene encoding a major ryegrass pollen allergen. *Proc Natl Acad Sci USA* 1999;96:11676–11680.
- Bhalla PL, Swoboda I, Singh MB: Reduction in allergenicity of grass pollen by genetic engineering. *Int Arch Allergy Immunol* 2001;124:51–54.

- 18 Laffer S, Hamdi S, Lupinek C, Sperr WR, Valent P, Verdino P, Keller W, Grote M, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Rideau M, Valenta R: Molecular characterization of recombinant T1, a non-allergenic periwinkle (*Catharanthus roseus*) protein, with sequence similarity to the Bet v 1 plant allergen family. *Biochem J* 2003;373:261–269.
- 19 Sutton SA, Assa'ad AH, Steinmetz C, Rothenberg ME: A negative, double-blind, placebo-controlled challenge to genetically modified corn. *J Allergy Clin Immunol* 2003;112:1011–1012.
- 20 Sten E, Skov PS, Andersen SB, Torp AM, Olesen A, Bindslev-Jensen U, Poulsen LK, Bindslev-Jensen C: A comparative study of the allergenic potency of wild-type and glyphosate-tolerant gene-modified soybean cultivars. *APMIS* 2004;112:21–28.
- 21 Pastorello EA, Conti A, Pravattoni V, Farioli L, Rivolta F, Ansaloni R, Spano M, Incorvaia C, Giuffrida MG, Ortolani C: Identification of actinidin as the major allergen of kiwi fruit. *J Allergy Clin Immunol* 1998;101:531–537.
- 22 Hefle SL, Lemanske RF Jr, Bush RK: Adverse reaction to lupine-fortified pasta. *J Allergy Clin Immunol* 1994;94:167–172.
- 23 FAO/WHO: Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods derived from Biotechnology. Food and Agriculture Organization of the United Nations (FAO), Rome, 2001.
- 24 Metcalfe DD, Astwood JD, Townsend R, Sampson HA, Taylor SL, Fuchs RL: Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit Rev Food Sci Nutr* 1996;36:S165–S186.
- 25 Metcalfe DD: Introduction: What are the issues in addressing the allergenic potential of genetically modified foods? *Environ Health Perspect* 2003;111:1110–1113.
- 26 Lehrer SB, Horner WE, Reese G: Why are some proteins allergenic? Implications for biotechnology. *Crit Rev Food Sci Nutr* 1996;36:553–564.
- 27 Spök A, Karner S, Stirn S, Gaugitsch H: Toxikologie und Allergologie von GVO-Produkten. Teil 2B. Untersuchung von Regelungen zur Sicherheitsbewertung von gentechnisch veränderten Lebensmitteln in der EU und den USA. Vienna, Umweltbundesamt, 2003, vol 164B.
- 28 Spök A, Hofer H, Valenta R, Kienzl-Plocherberger K, Lehner P, Gaugitsch H: Toxikologie und Allergologie von GVO-Produkten. Empfehlungen zur Standardisierung der Sicherheitsbewertung von gentechnisch veränderten Pflanzen auf Basis der Richtlinie 90/220/EWG (2001/18/EG). Vienna, Umweltbundesamt, 2002, vol 109.
- 29 Spök A, Hofer H, Valenta R, Kienzl-Plocherberger K, Lehner P, Stirn S, Gaugitsch H: Toxikologie und Allergologie von GVO-Produkten. Teil 2A. Untersuchung zur Praxis und Empfehlung zur Standardisierung der Sicherheitsbewertung von gentechnisch veränderten Lebensmitteln. Vienna, Umweltbundesamt, 2003, vol 164A.
- 30 Pastorello EA, Pravattoni V, Calamari AM, Banfi E, Robino AM: New plant-origin food allergens. *Allergy* 2002;57:106–110.
- 31 Sten E, Stahl Skov P, Andersen SB, Torp AM, Olesen A, Bindslev-Jensen U, Poulsen LK, Bindslev-Jensen C: Allergenic components of a novel food, Micronesian nut Nangai (*Canarium indicum*), shows IgE cross-reactivity in pollen allergic patients. *Allergy* 2002;57:398–404.
- 32 Fah J, Wuthrich B, Vieths S: Anaphylactic reaction to lychee fruit: Evidence for sensitization to profilin. *Clin Exp Allergy* 1995;25:1018–1023.
- 33 Wuthrich B, Borga A, Yman L: Oral allergy syndrome to a jackfruit (*Artocarpus integrifolia*). *Allergy* 1997;52:428–431.
- 34 Kelso JM, Jones RT, Yunginger JW: Anaphylaxis after initial ingestion of rambutan, a tropical fruit. *J Allergy Clin Immunol* 1998;102:145–146.
- 35 Blanco C, Ortega N, Castillo R, Alvarez M, Dumpierrez AG, Carrillo T: *Carica papaya* pollen allergy. *Ann Allergy Asthma Immunol* 1998;81:171–175.
- 36 De Greef JM, Lieutier-Colas F, Bessot JC, Verot A, Gallerand AM, Pauli G, de Blay F: Urticaria and rhinitis to shrubs of *Ficus benjamina* and breadfruit in a banana-allergic road worker: Evidence for a cross-sensitization between Moracea, banana and latex. *Int Arch Allergy Immunol* 2001;125:182–184.
- 37 Niggemann B, Reibel S, Hipler C, Wahn U: Anaphylactic reaction to lychee in a 12-year-old girl: Cross-reactivity to latex? *Pediatr Allergy Immunol* 2002;13:64–67.
- 38 Focke M, Hemmer W, Wohrl S, Gotz M, Jarisch R: Cross-reactivity between *Ficus benjamina* latex and fig fruit in patients with clinical fig allergy. *Clin Exp Allergy* 2003;33:971–977.
- 39 Pastorello EA, Farioli L, Pravattoni V, Spano M, Scibola E, Trambaioli C, Giuffrida MG, Ansaloni R, Godovac-Zimmermann J, Conti A, Fortunato D, Ortolani C: The maize major allergen, which is responsible for food-induced allergic reactions, is a lipid transfer protein. *J Allergy Clin Immunol* 2000;106:744–751.
- 40 Bunikowski R, Mielke M, Skarabis H, Herz U, Bergmann RL, Wahn U, Renz H: Prevalence and role of serum IgE antibodies to the *Staphylococcus aureus*-derived superantigens SEA and SEB in children with atopic dermatitis. *J Allergy Clin Immunol* 1999;103:119–124.
- 41 Lindborg M, Magnusson CG, Zargari A, Schmidt M, Scheynius A, Cramer R, Whitley P: Selective cloning of allergens from the skin colonizing yeast *Malassezia furfur* by phage surface display technology. *J Invest Dermatol* 1999;113:156–161.
- 42 Hoffmann-Sommergruber K: Plant allergens and pathogenesis-related proteins. What do they have in common? *Int Arch Allergy Immunol* 2000;122:155–166.
- 43 Pearson WR: Flexible sequence similarity searching with the FASTA3 program package. *Methods Mol Biol* 2000;132:185–219.
- 44 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: Basic local alignment search tool. *J Mol Biol* 1990;215:403–410.
- 45 Altschul SF, Lipman DJ: Protein database searches for multiple alignments. *Proc Natl Acad Sci USA* 1990;87:5509–5513.
- 46 Hileman RE, Silvanovich A, Goodman RE, Rice EA, Holleschak G, Astwood JD, Hefle SL: Bioinformatic methods for allergenicity assessment using a comprehensive allergen database. *Int Arch Allergy Immunol* 2002;128:280–291.
- 47 Stadler MB, Stadler BM: Allergenicity prediction by protein sequence. *FASEB J* 2003;17:1141–1143.
- 48 Soeria-Atmadja D, Zorzet A, Gustafsson MG, Hammerling U: Statistical evaluation of local alignment features predicting allergenicity using supervised classification algorithms. *Int Arch Allergy Immunol* 2004;133:101–112.
- 49 Breiteneder H, Ferreira F, Hoffmann-Sommergruber K, Ebner C, Breitenbach M, Rumpold H, Kraft D, Scheiner O: Four recombinant isoforms of Cor a I, the major allergen of hazel pollen, show different IgE-binding properties. *Eur J Biochem* 1993;212:355–362.
- 50 Ferreira F, Hirtlenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, Grimm R, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breitenbach M, Rheinberger HJ, Ebner C: Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: Potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med* 1996;183:599–609.
- 51 Astwood JD, Leach JN, Fuchs RL: Stability of food allergens to digestion in vitro. *Nat Biotechnol* 1996;14:1269–1273.
- 52 Yagami T, Haishima Y, Nakamura A, Osuna H, Ikezawa Z: Digestibility of allergens extracted from natural rubber latex and vegetable foods. *J Allergy Clin Immunol* 2000;106:752–762.
- 53 Fu TJ, Abbott UR, Hatzos C: Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid – A comparative study. *J Agric Food Chem* 2002;50:7154–7160.
- 54 Heiss S, Fischer S, Müller WD, Weber B, Hirschwehr R, Spitzauer S, Kraft D, Valenta R: Identification of a 60 kd cross-reactive allergen in pollen and plant-derived food. *J Allergy Clin Immunol* 1996;98:938–947.
- 55 Bohle B, Swoboda I, Spitzauer S, Breiteneder H, Hoffmann-Sommergruber K, Scheiner O, Ebner C, Kraft D, Valenta R: Food antigens: Structure and function; in Metcalfe DD, Sampson HA, Simon RA (eds): Food Allergy: Adverse Reactions to Foods and Food Additives. Oxford, Blackwell Science, 2003, vol 3, pp 38–50.

- 56 Valenta R: The future of antigen-specific immunotherapy of allergy. *Nat Rev Immunol* 2002;2:446–453.
- 57 Valenta R, Ball T, Focke M, Linhart B, Mothes N, Niederberger V, Spitzauer S, Swoboda I, Vrtala S, Westritsching K, Kraft D: Immunotherapy of allergic disease. *Adv Immunol* 2004; 82:105–153.
- 58 Valenta R, Kraft D: Recombinant allergen molecules: Tools to study effector cell activation. *Immunol Rev* 2001;179:119–127.
- 59 Valenta R, Kraft D: From allergen structure to new forms of allergen-specific immunotherapy. *Curr Opin Immunol* 2002;14:718–727.
- 60 Valenta R, Duchêne M, Pottenburger K, Sillaber C, Valent P, Bettelheim P, Breitenbach M, Rumpold H, Kraft D, Scheiner O: Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 1991;253:557–560.
- 61 Niederberger V, Hayek B, Vrtala S, Laffer S, Twardosz A, Vangelista L, Sperr WR, Valent P, Rumpold H, Kraft D, Ehrenberger K, Valenta R, Spitzauer S: Calcium-dependent immunoglobulin E recognition of the apo- and calcium-bound form of a cross-reactive two EF-hand timothy grass pollen allergen, Phl p 7. *FASEB J* 1999;13:843–856.
- 62 van der Veen MJ, van Ree R, Aalberse RC, Akkerdaas J, Koppelman SJ, Jansen HM, van der Zee JS: Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. *J Allergy Clin Immunol* 1997; 100:327–334.
- 63 Mari A, Iacovacci P, Afferni C, Barletta B, Tinghino R, Di Felice G, Pini C: Specific IgE to cross-reactive carbohydrate determinants strongly affect the in vitro diagnosis of allergic diseases. *J Allergy Clin Immunol* 1999;103: 1005–1011.
- 64 Mari A: IgE to cross-reactive carbohydrate determinants: Analysis of the distribution and appraisal of the in vivo and in vitro reactivity. *Int Arch Allergy Immunol* 2002;129:286–295.
- 65 van Ree R: Carbohydrate epitopes and their relevance for the diagnosis and treatment of allergic diseases. *Int Arch Allergy Immunol* 2002;129:189–197.
- 66 Batanero E, Crespo JF, Monsalve RI, Martin-Esteban M, Villalba M, Rodriguez R: IgE-binding and histamine-release capabilities of the main carbohydrate component isolated from the major allergen of olive tree pollen, Ole e 1. *J Allergy Clin Immunol* 1999;103:147–153.
- 67 Bublin M, Radauer C, Wilson IB, Kraft D, Scheiner O, Breiteneder H, Hoffmann-Sommergruber K: Cross-reactive N-glycans of Api g 5, a high molecular weight glycoprotein allergen from celery, are required for immunoglobulin E binding and activation of effector cells from allergic patients. *FASEB J* 2003;17: 1697–1699.
- 68 Mittermann I, Swoboda I, Pierson E, Eller N, Kraft D, Valenta R, Heberle-Bors E: Molecular cloning and characterization of profilin from tobacco (*Nicotiana tabacum*): Increased profilin expression during pollen maturation. *Plant Mol Biol* 1995;27:137–146.
- 69 Vieths S, Jankiewicz A, Schöning B, Aulepp H: Apple allergy: The IgE-binding potency of apple strains is related to the occurrence of the 18-kDa allergen. *Allergy* 1994;49:262–271.
- 70 Carnés J, Fernández-Caldas E, Gallego MT, Ferrer A, Cuesta-Herranz J: Pru p 3 (LTP) content in peach extracts. *Allergy* 2002;57:1071–1075.
- 71 Vrtala S, Ball T, Spitzauer S, Pandjaitan B, Suphioglu C, Knox B, Sperr WR, Valent P, Kraft D, Valenta R: Immunization with purified natural and recombinant allergens induces mouse IgG1 antibodies that recognize similar epitopes as human IgE and inhibit the human IgE-allergen interaction and allergen-induced basophil degranulation. *J Immunol* 1998;160: 6137–6144.
- 72 Novey HS, Keenan WJ, Fairshir RD, Wells ID, Wilson AF, Culver BD: Pulmonary disease in workers exposed to papain: Clinico-physiological and immunological studies. *Clin Allergy* 1980;10:721–731.
- 73 Baur X, Chen Z, Rozynek P, Duser M, Raulf-Heimsoth M: Cross-reacting IgE antibodies recognizing latex allergens, including Hev b 1, as well as papain. *Allergy* 1995;50:604–609.
- 74 Mansfield LE, Ting S, Haverly RW, Yoo TJ: The incidence and clinical implications of hypersensitivity to papain in an allergic population, confirmed by blinded oral challenge. *Ann Allergy* 1985;55:541–543.
- 75 Sampson HA: Food allergy. *J Allergy Clin Immunol* 2003;111:S540–S547.
- 76 Metcalfe DD, Sampson HA, Simon RA: Food Allergy: Adverse Reactions to Foods and Food Additives, ed 3. Oxford, Blackwell Science, 2003.
- 77 van Ree R: Value of monoclonal antibody-based assays: Advantages and drawbacks. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankfurt a M* 1994;87:127–139.
- 78 Aalberse RC, van Ree R: Monoclonal antibody assays for allergens: Pick your antibodies with care! *J Allergy Clin Immunol* 2000;106:625–626.
- 79 Taylor SL, Hefle SL: Ingredient and labeling issues associated with allergenic foods. *Allergy* 2001;56(suppl 67):64–69.
- 80 Epstein MM: Do mouse models of allergic asthma mimic clinical disease? *Int Arch Allergy Immunol* 2004;133:84–100.
- 81 Hoyne GF, Callow MG, Kuo MC, Thomas WR: Inhibition of T-cell responses by feeding peptides containing major and cryptic epitopes: Studies with the Der p I allergen. *Immunology* 1994;83:190–195.
- 82 Hoyne GF, Callow MG, Kuo MC, Thomas WR: Characterization of T-cell responses to the house dust mite allergen Der p II in mice. Evidence for major and cryptic epitopes. *Immunology* 1993;78:65–73.
- 83 Nuttal TJ, Pemberton AD, Lamb JR, Hill PB: Peripheral blood mononuclear cell responses to major and minor Dermatophagoides allergens in canine atopic dermatitis. *Vet Immunol Immunopathol* 2002;84:143–150.
- 84 Masuda K, Tsujimoto H, Fujiwara S, Kurata K, Hasegawa A, Yasueda H, Yamashita K, DeBoer DJ, de Weck AL, Sakaguchi M: IgE sensitivity and cross-reactivity to crude and purified mite allergens (Der f 1, Der f 2, Der p 1, Der p 2) in atopic dogs sensitive to Dermatophagoides mite allergens. *Vet Immunol Immunopathol* 1999;72:303–313.
- 85 McCall C, Hunter S, Stedman K, Weber E, Hillier A, Bozic C, Rivoire B, Olivry T: Characterization and cloning of a major high molecular weight house dust mite allergen (Der f 15) for dogs. *Vet Immunol Immunopathol* 2001;78:231–247.
- 86 Weber E, Hunter S, Stedman K, Dreitz S, Olivry T, Hillier A, McCall C: Identification, characterization, and cloning of a complementary DNA encoding a 60-kd house dust mite allergen (Der f 18) for human beings and dogs. *J Allergy Clin Immunol* 2003;112:79–86.
- 87 Seitzer U, Bussler H, Kullmann B, Petersen A, Becker WM, Ahmed J: Characterization of immunoglobulin E responses in Balb/c mice against the major allergens of timothy grass (*Phleum pratense*) pollen. *Clin Exp Allergy* 2003;33:669–675.
- 88 Spergel JM, Mizoguchi E, Brewer JP, Martin TR, Bhan AK, Geha RS: Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *J Clin Invest* 1998;101: 1614–1622.
- 89 Huang CH, Kuo IC, Xu H, Lee YS, Chua KY: Mite allergen induces allergic dermatitis with concomitant neurogenic inflammation in mouse. *J Invest Dermatol* 2002;121:289–293.
- 90 Hsieh KY, Tsai CC, Wu CH, Lin RH: Epicutaneous exposure to protein antigen and food allergy. *Clin Exp Allergy* 2003;33:1067–1075.